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Sterol 24-Methyltransferase as a Drug Target in Parasitic Protozoa

A thesis submitted to the University of Wales for the Degree of

Doctor of Philosophy

By

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A ma mère, avec ton mon amour,

A mon père, à jamais dans mon coeur.

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Sapientia est potentia

ABSTRACT

Amongst parasitic diseases, leishmaniasis, South American Trypanosomiasis (Chagas' disease) and Human African Trypanosomiasis (HAT or sleeping sickness) are some of the most important. The causative organisms for these diseases are *Leishmania spp*, *Trypanosoma cruzi* and *Trypanosoma brucei* species respectively. The current drugs available to treat these diseases have many disadvantages such as parenteral mode of administration, long duration of therapy, toxic side effects and increasing cases of resistance. In addition there have been a number of cases of *leishmania*/HIV co-infection, which are not very responsive to current drug treatment. The diseases are discussed in Chapter I.

Sterol biosynthesis is an important drug target in parasitic protozoa. While ergosterol is the major sterol present in fungi and some protozoa cells, cholesterol is the major sterol in mammalian cells. This is discussed in detail in Chapter II. In particular, the enzyme sterol 24-methyltransferase (24-SMT) is described in detail. This enzyme is involved in the biosynthesis of ergosterol, but is not found in the biosynthetic pathway of cholesterol.

This enzyme uses zymosterol as a substrate and *S*-adenosyl-L-methionine (SAM) as co-factor. Azasterols (Figure A-1) have been reported[1] to interfere with sterol biosynthesis, as they are able to mimic a high energy intermediate.

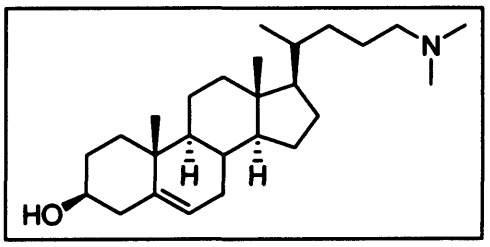


Figure A-1: 25-azasterol

[1] Rahier, A.; Taton, M.; Bouviernave, P.; Schmitt, P.; Benveniste, P.; Schuber, F.; Narula, A. S.; Cattel, L.; Anding, C.; Place, P. Design of High-Energy Intermediate Analogs to Study Sterol Biosynthesis in Higher-Plants. *Lipids* **1986**, *21*, 52-62.

In this project, the synthesis of new analogues of the carbocation intermediate, have been achieved. Two types of azasterols were prepared containing a sterol nucleus and SAM moieties as side chains. The role of the position 3 was also investigated in order to assess a possible structure activity relationship, and synthetic results are presented Chapters IV and V.

In vitro screening of the compounds against *Leishmania donovani*, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi* and *Plasmodium falciparum* was carried out with some promising data in the nanomolar range (Chapter VI). Enzyme assays have also been carried out, but these compounds were not found active against both *Leishmania major* and *Trypanosoma brucei brucei* 24-SMT enzymes.

In order to investigate further the mode of action of these compounds, synthesis of proteomic probes was attempted. Synthetic design and attempts are presented Chapter VII.

To establish whether there was 24-SMT present in the blood stream form of *Trypanosoma brucei brucei*, a Northern blot was carried out. This confirmed transcription of the enzyme which was then cloned, over expressed and purified (Chapter VIII). Enzyme assays were carried out against the recombinant enzyme.

Part of this thesis is in press for publication in "Bioorganic & Medicinal Chemistry" journal, and referred in the Appendix 2 as:

Preparation of Transition-State Analogues of Sterol 24-Methyl Transferase as Potential Anti-Parasitics

ABBREVIATIONS

Ac	Acetyl
Ado	Adenosine
Amp	Ampicillin
Bn	Benzyl
Boc	1,1-Dimethylethoxycarbonyl (*tert*-butoxycarbonyl)
BSA	Bovine serum albumin
bsf	Blood stream form
cat.	Catalytic
CNS	Central nervous system
CL	Cutaneous Leishmaniasis
d	Doublet
Da	Dalton
dATP	Deoxyadenosine triphosphate
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
dCTP	Deoxycytidine triphosphate
DFMO	Difluoromethylornithine
dGTP	Deoxyguanosine triphosphate
DIPC	Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DIPU	Diisipropyl urea
DMAP	Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ED_{50}	Effective dose for 50% reduction of organisms
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

EDTA	Ethyl diamino tetra acetic acid
eq.	Equivalent
ES	Electrospray ionisation
Et	Ethyl
FBS	Fetal bovine serum
h	Hour
HOBt	1-Hydroxybenzotriazole
HRMS	High resolution mass spectrometry
Hz	Hertz
IC_{50}	Concentration required for 50% inhibition
IPTG	Isopropyl-β-D-thio-galactopyranoside
IR	Infrared spectroscopy
iPr	Isopropyl
J	Coupling constant
Kan	Kanamycin
Kb	Kilobase
kDa	Kilodalton
Ki	Inhibition constant
kV	Kilovolt
LB	Luria Bertani
LRMS	Low resolution mass spectrometry
m	Multiplet
M	Molar concentration
MCL	Mucocutaneous Leishmaniasis
Me	Methyl
MED	Minimal effective dose
min.	Minute
Mp	Melting point
MS	Mass spectroscopy
mRNA	Messenger ribonucleic acid
ms	Milli-second
MS	Mass spectrometry

MST	Mean survival time
MW	Molecular weight
µCi	Microcurie
NMR	Nuclear magnetic resonance spectroscopy
ODC	Ornithine decarboxylase
PBS	Saline phosphate buffer
PCR	Polymerisation chain reaction
PG	Protecting group
Ph	Phenyl
PKDL	Post Kala-azar dermal Leishmaniasis
pmol	Picomol
ppm	Part per million
Pr	Propyl
Pyr	Pyridine
q	Quadruplet
quint	Quintuplet
R	Substituent
R_f	Retention factor
RNA	Ribonucleic acid
r.t.	Room temperature
s	Singlet
SAM	*S*-adenosyl-$_L$-methionine
sat.	Saturated
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	Starting material
24-SMT	Sterol 24-methyltransferase enzyme
SOD	Superoxide dismutase
t	Triplet
TBAF	Tetrabutylammonium fluoride
tBu	*tert*-Butyl
TBDMS	*tert*-Butyldimethylsilyl

TBTU	2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEA	Triethylamine
TFA	Trifluoroacetic acid
TEMED	N,N,N',N'-Tetramethyl diaminoethane
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Ts	p-Toluensulfonyl
UV	Ultraviolet spectrometry
VL	Visceral Leishmaniasis
WHO	World health organisation
Xgal	5-Bromo-4-chloro-3-indoyl-β-D-galactosidase

GUIDELINES TO NOMENCLATURE

G.1 Steroid nomenclature[2]

G.1.1 Definition of steroids and sterols

Steroids are compounds possessing the skeleton of cyclopenta[*a*]phenantrene and derive from one or more bond scissions or ring expansions or contractions. Methyl groups are normally present at C-10 and C-17 positions. An alkyl chain may also be present at C-17. Sterols are steroids carrying a hydroxyl group at C-3 and most of the skeleton of cholestane. Additional carbon atoms may be present in the side chain.

[2] IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). The Nomenclature of Steroids. *Eur. J. Biochem.* **1989**, *186*, 429-458.

G.1.2 Numbering and ring letters

The standard numbering system for steroids and lettering system for the rings of the tetra cycles is shown in the Figure G.1[3]. If one of the two-methyl groups attached to C-25 is substituted, it is assigned the lower number (26). If both are substituted, the lower number is assigned to the carbon carrying the substitute first cited in alphabetical order.

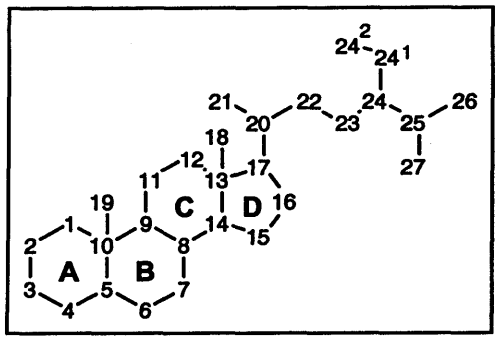


Figure G.1: Numbering of steroids

G.1.3 Absolute configuration

When the rings of the steroid are denoted as projections on to the plane of the paper, the formula should be oriented as in the Figure G.2. Atoms or groups above the plane of the ring system are designated β (beta), and those below the plane α (alpha). Solid lines are used to depict bonds to β atoms or groups, and dashed lines for bonds to α atoms or groups. Bonds to atoms or groups, whose configuration is not known or is unspecified, are denoted by wavy lines[2].

[3] IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) and IUPAC-IUB Commission on Biochemical Nomenclature (CBN). The Nomenclature of Steroids. *Eur. J. Biochem.* **1969**, *10*, 1-19.

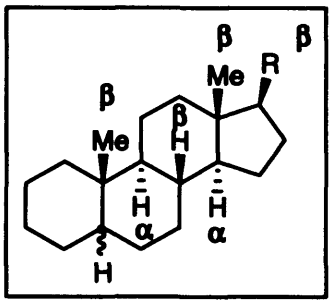


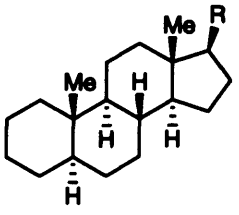
Figure G.2: Stereochemistry of steroids

G.1.4 Ring junctions and side attachments

Unless implied or stated to the contrary[2], use of steroid name implies that atom groups attached at the bridgehead position 8, 9, 10, 13 and 14 are oriented as shown in the Figure G.2, and side chain attached at position 17 is assumed to be β-oriented. The configuration of hydrogen (or substitute) at the bridgehead 5 is always designed by adding α, β or ξ after the numeral 5, this numeral and letter placed immediately before the stem name.

G.1.5 Nomenclature of steroids

According to the IUPAC system of nomenclature, steroids are derivatives of cyclopentano-perhydrophenanthrene. They are alicyclic and mostly saturated (non-aromatic) structures. Instead of applying the systematic IUPAC nomenclature, steroids are named by a modified system based on stem names. A list of stem names approved by the IUPAC commission is depicted in Figure G.3[2]. It is important to note that stereochemistry is implied in the stem names as explained before.



R =		
	H	5α-androstane
	CH_2CH_3	5α-pregnane
	$CH(CH_3)CH_2CH_2CH_3$[a]	5α-cholane
	$CH(CH_3)CH_2CH_2CH_2CH(CH_3)_2$[a]	5α-cholestane
	$CH(CH_3)CH_2CH_2CH(CH_3)CH(CH_3)_2$[ab]	5α-ergostane

[a] 20-R configuration
[b] 24-S configuration

Figure G.3: Stem names of steroids

The following expressions were introduced to define skeletal modifications in regards to the stem names[2]:

- X-homo: stepwise expansion by one methylene unit at carbon X;
- X-nor: stepwise contraction by one methylene unit at carbon X;
- ξ(X): undefined stereochemistry at carbon X;
- ΔX (Y): unsaturation between carbon X and carbon Y, indicated by changing –ane to –ene, -adiene, -yne, etc... or an to –en-, -adien-, -yn-, etc...;
- Esters of steroids: they are named by the appropriate steroid substitute group followed by that of the acyloxy group in its anionic form, replacing the terminal –e of the hydrocarbon name by –yl, -diyl, etc. and insert a Greek letter to designate the position and configuration in the molecule.

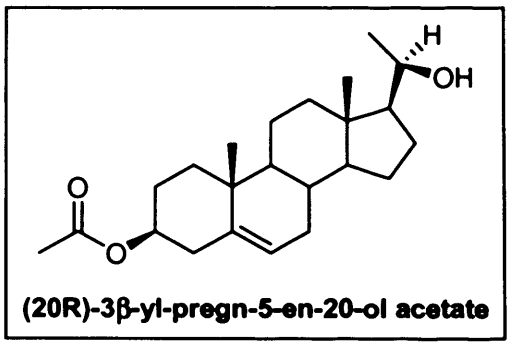


Figure G.4: Example of the use of stem name

G.1.6 Trivial names

However, some steroids and sterols are referred by trivial names commonly used in the literature. For example, the IUPAC name of the compound Figure G.5 is 4,4,14-trimethyl-5α-cholest-8-en-3β-ol. Its trivial name is 24,25-dihydrolanosterol.

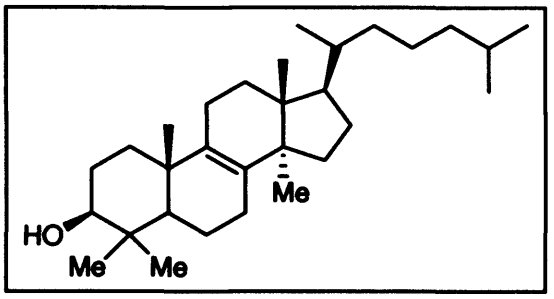


Figure G.5: 24,25-Dihydrolanosterol

In this report, the steroids were named according to the specialised IUPAC nomenclature aforementioned. All compounds were considered as derivatives of cholenic acid. The numbering system at C-3 position was denoted by using numbers followed by (').

G.2 Nomenclature of α-amino acids[4]

In non-cyclic amino acids, the number 1 is reserved for the carboxylic acid, whereas the methylene groups are numbered 2, 3, 4... Amino acids have a centre of chirality at C-2. The naturally occurring α-amino acids have an S configuration. Trivial names are commonly used, and are generally replaced by three letters codes that identify the structure and the stereochemistry at C-2 (Figure G.6).

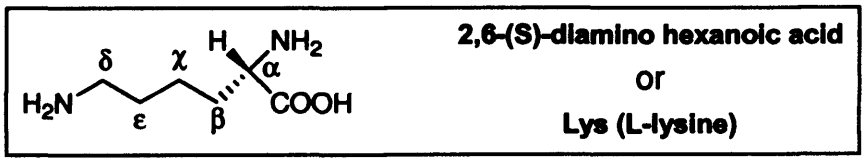


Figure G.6: Numbering and stereochemistry of α-amino acids

[4] IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature and Symbolism for Amino Acids and Peptides. *Eur. J. Biochem.* **1984**, *138*, 9-37.

CONTENTS

CHAPTER I

LEISHMANIASIS AND TRYPANOSOMIASIS

Diseases caused by parasitic protozoa affect many people in large areas of the world[5,6,7,8]. The cure of these diseases depends mainly on chemotherapy[6]. Several antiparasitic drugs are available, but are often associated with toxic side effects and development of drug resistance. Therefore new effective drugs have to be found and this is the subject of this thesis.

This work concerns the synthesis of new drugs against Leishmaniases and Trypanosomiases. Therefore, a description of their origin, geographic distribution, life cycle and current treatment is in Chapter I.

The aim of the research is to synthesise specific sterol 24-methyltransferase (24-SMT) enzyme inhibitors. This enzyme, involved in the sterol biosynthetic pathway, does not have a counterpart in the mammalian pathway, and this is described in detail in Chapter II.

I.1. Leishmaniasis and Trypanosomiasis main features

Leishmaniases and Trypanosomiases are important diseases caused by parasitic protozoa of the family Trypanosomatidae and are (Table 1.1)[9]:

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[5] Van den Bossche, H. Chemotherapy of Parasitic Infections. *Nature* **1978**, *273*, 626-630.

[6] Hide, G.; Mottram, J. C.; Holmes, P. H.; Coomb, G. H. Preface: Current Research into the Biology and Control of Trypanosomiasis and Leishmaniasis. *In: Trypanosomiasis and Leishmaniasis. Biology and Control.CAB International, Wallingford, UK* **1997**, xiii.

[7] Croft, S. L. The Current Status of Antiparasitic Chemotherapy. *Parasitology* **1997**, *114*, S3-15.

[8] http://www.WHO.int.

[9] Molyneux, D. H. Vectorborne Parasitic Diseases - An Overview of Recent Changes. *Int. J. Parasit.* **1998**, *28*, 927-934.

- Leishmaniasis, caused by species and subspecies of *Leishmania*;
- South American Trypanosomiasis or Chagas' disease, caused by *Trypanosoma cruzi*;
- Human African Trypanosomiasis (HAT) or sleeping sickness, caused by subspecies of *Trypanosoma brucei*.

Disease	Species	Vector	Geographical Distribution
Leishmaniasis (cutaneous, visceral, mucocutaneous, diffuse cutaneous)	*Leishmania donovani* *Leishmania infantum* *Leishmania major* *Leishmania tropica* *Leishmania* spp.	Sand flies	India, Africa (Sudan), Southern Europe, Middle East, Asia, Africa, Central and South America
South American Trypanosomiases (Chagas'disease)	*Trypanosoma cruzi*	Reduviid bug (Kissing bug)	Brazil, Chile, Argentina, Bolivia, Paraguay, Uruguay, Venezuela, Central America
Human African Trypanosomiasis (Sleeping sickness)	*T. brucei gambiense* *T. brucei rhodesiense*	Tsetse fly (Glossina sp.)	East and Southern Africa, West and Central Africa

Table I.1: Human pathogenic *Trypanosoma* and *Leishmania* spp.

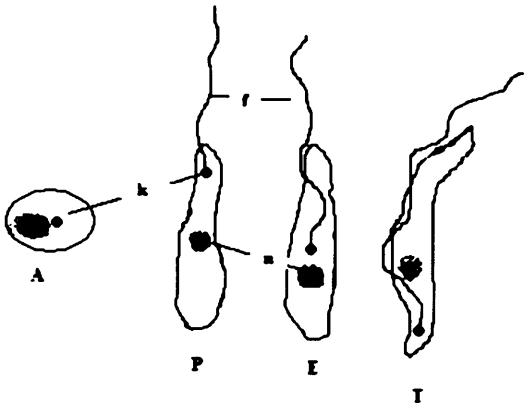
These organisms have a number of unique features, which differentiate them from other organisms, including the kinetoplast (a network of interconnected mini and maxi circles of DNA which is within the mitochondrion) and glycosomes (Figure I.1)[10].

Trypanosomatids cause disease in man and vertebrate animals. These parasites live within the tissues of their hosts, such as skin, gut lumen, tissue cells (usually intracellular) or tissue fluids (plasma or lymph). They are essentially parasites of the blood stream, although some adapt to an intracellular existence. They have different morphological forms which change

[10] James, D. H.; Gilles, H. M. Human Antiparasitic Drugs: Pharmacology and Usage. *John Wiley & Sons, UK* **1985**.

during the life cycle of the parasite. The more generally accepted terminology is based on the position of the flagellum. Five forms are described:

- **Amastigotes**: rounded form devoid of an external flagellum with the kinetoplast placed near the nucleus. This is the intracellular form in the mammalian host (*Leishmania and T. cruzi*);
- **Promastigotes**: elongated form with the kinetoplast anterior to the nucleus; flagellum with no undulating membrane; form found in the vector (*Leishmania*);
- **Epimastigotes**: elongated form with the kinetoplast anterior to the nucleus but with the flagellum emerging to join a short undulating membrane. Stage found in the vector (*T. cruzi, T. brucei*);
- **Trypomastigotes**: elongated form with the kinetoplast posterior to the nucleus; the flagellum joining a long undulating membrane that extends almost the complete length of the parasite. This is the blood-stream form (*T. cruzi, T. brucei*);
- **Metacyclic Trypomastigotes**: similar to trypomastigotes. Form found in both the vector and the blood stream (*T. cruzi*).



A: Amastigote, P: Promastigote, E: Epimastigote, T: Trypomastigote, k: kinetoplast, n: nucleus, f: flagellum

Figure I.1: Morphological forms of kinetoplastid flagellate[10]

I.2 Leishmaniases

Figure I.2: *Leishmania* promastigote[8].

I.2.1 Transmission and infection

Leishmaniasis is a parasitic infection transmitted by the bite of a female sand fly. At least 14 species and subspecies of the genus *Leishmania* are known to be responsible for different forms of the disease. The diseases (Visceral (VL), Cutaneous (CL) and Mucocutaneous (MCL) leishmaniasis) are usually categorised broadly according to the long-established clinical classification (Table I.2).

Species or Complex	Subspecies	Diseases
Leishmania donovani	*L.d. donovani*	VL
	L.d. infantum	VL
	L.d. chagasi	VL
Leishmania tropica	*L.t. tropica*	CL
	L.t. minor	CL
	L.t. major	CL
	L.t. aethiopica	CL
Leishmania mexicana	*L.m. mexicana*	CL
	L.m. amazonensis	CL
	L.m. pifanoi	CL
Leishmania braziliensis	*L.b. braziliensis*	CL / MCL
	L.b. guyanensis	CL
	L.b. panamensis	CL
	L.b. peruviana	CL

Table I.2: Species and subspecies of leishmaniasis

The disease has four main clinical syndromes, which depend on the parasite species and the cellular immune system of the patient:

- **Visceral leishmaniasis (VL) - also known as kala-azar**: this is a systemic infection caused by the subspecies *L. donovani* complex. It involves the reticuloendothelial system (RES) where the parasites multiply within macrophages causing an intermittent fever and dysfunction of the liver, spleen, bone marrow and lymph nodes. It is usually fatal if left untreated. Response to the available drugs is relatively good, but relapses often occur as the dermal form (Post Kala-azar Dermal Leishmaniasis: PKDL). Resistance to the drugs is widespread;
- **Cutaneous Leishmaniasis (CL)**: this is the most common form of the disease complex. Patients can have more than one primary skin lesion with satellite lesions or pain, and bacterial infection, which cause serious disability and leave the patient with atrophic scars. Cutaneous infection can remain sub clinical or become manifest apparent after a variable incubation period that averages several weeks. A dermal lesion may occur also as an outcome of visceral leishmaniasis when therapy has been incomplete or unsuccessful, and this must be distinguished from exclusively dermal types, referred to PKDL;
- **Mucocutaneous Leishmaniasis (MCL)**: this is caused by the subspecies *L. braziliensis braziliensis*. It initially causes skin lesions, on the face arms and legs, which after a variable period will spread to the cartilage and other connective tissue of the nasopharynx by the transfer of infected macrophages. Disfiguration is the fatal consequence in the more serious cases and reconstructive surgery is an important part of the therapy;
- **Diffuse Cutaneous Leishmaniasis**: difficult to treat due to disseminated lesions that resemble leprosy and do not heal spontaneously. This form especially is related to a defective immune system and it is often characterised by relapses after treatment.

I.2.2 Geographic distribution

Leishmaniasis is endemic in 88 countries: 16 are developed countries, 59 are developing countries and 13 of them are among the least developed. 350 million people are estimated at risk on the five continents, Africa, Asia, Europe, North America and South America. It is believed that worldwide 12 million people are affected by leishmaniasis. More than 90% of the world's cases of visceral leishmaniasis are in Bangladesh, India, Sudan and Brazil. The Figure I.3 shows the distribution of the disease. The areas in light blue are endemic for leishmaniasis and the areas in dark blue are endemic for leishmaniasis with reported cases of HIV co-infection[8]. Recent data in southern Europe[11] indicated that 3 to 7% of people infected with HIV-1 developed visceral leishmaniasis, and more than 50% of visceral leishmaniasis in adults were related to infection with HIV. This suggests that Leishmania/HIV co-infection is becoming more important where both species are endemic.

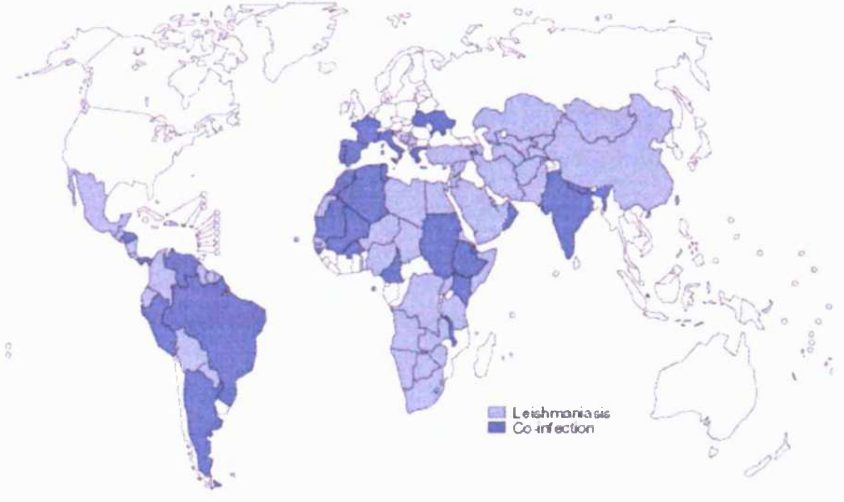
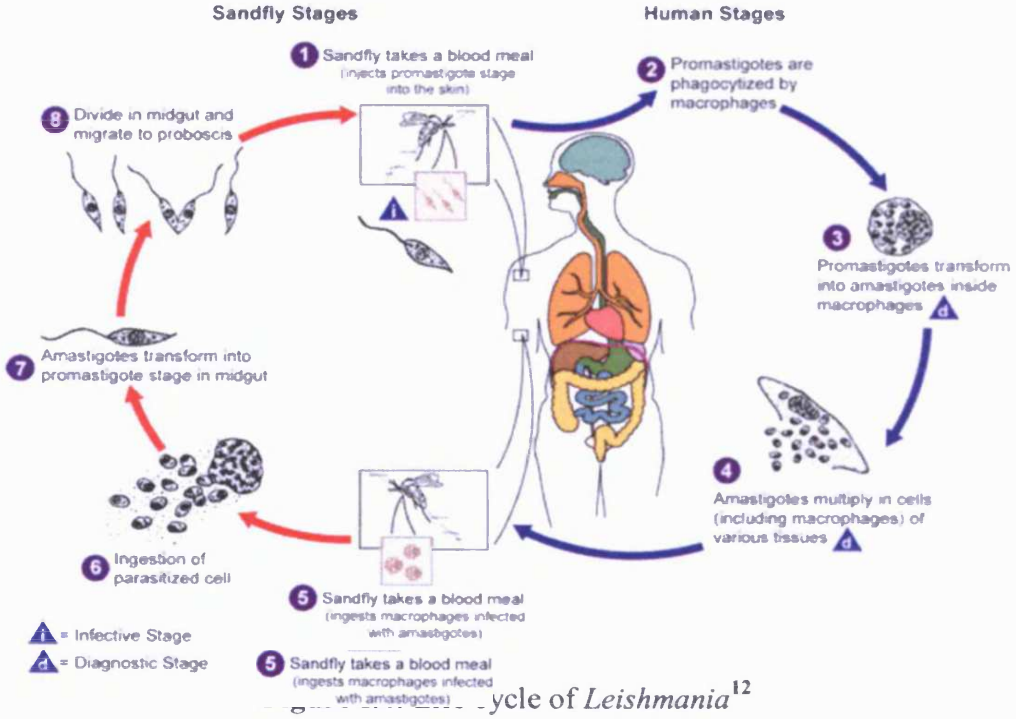


Figure I.3: Geographical distribution of Leishmaniasis[8].

[11] Tremblay, M.; Olivier, M.; Bernier, R. Leishmania and the Pathogenesis of HIV Infection. *Parasitol. Today* **1996**, *12*, 257-261.

I.2.3 Life cycle

The female sandfly first infects itself with the *Leishmania* parasites contained in the blood it sucks from its human or mammalian host (Figure I.4). During a period of 4 to 25 days, the parasite continues its development first in the intestine and after in the salivary glands inside the sand fly where it undergoes major transformation. This transformation takes place in the insect's midgut. The amastigote form changes to the flagellated promastigote, which divides by repeated binary longitudinal fission before migrating to the vector's proboscis ready for inoculating a fresh vertebrate host. Once in the host, the promastigotes are rapidly phagocytised by macrophages in which they transform to amastigotes to divide repeatedly causing the rupture of the macrophage. The released amastigotes infect other macrophages in which division is repeated while some remain available for vector uptake and transmission.



ycle of *Leishmania*[12]

[12] http://www.dpd.cdc.gov.

I.2.4 Drug treatment

I.2.4.1 Antimonials

Vianna who employed the toxic antimony compound, potassium antimonyl tartrate in cases of mucocutaneous leishmaniasis first successfully treated leishmaniasis in 1912 in Brazil[10]. Since the 1940s, the pentavalent antimony compounds sodium stibogluconate (Pentostam®, GlaxoSmithKline, UK, Figure I.5) and meglumine antimonate (Glucantime®, Novartis, France, Figure I.5) have been the mainstays of antileishmanial therapy[13,14].

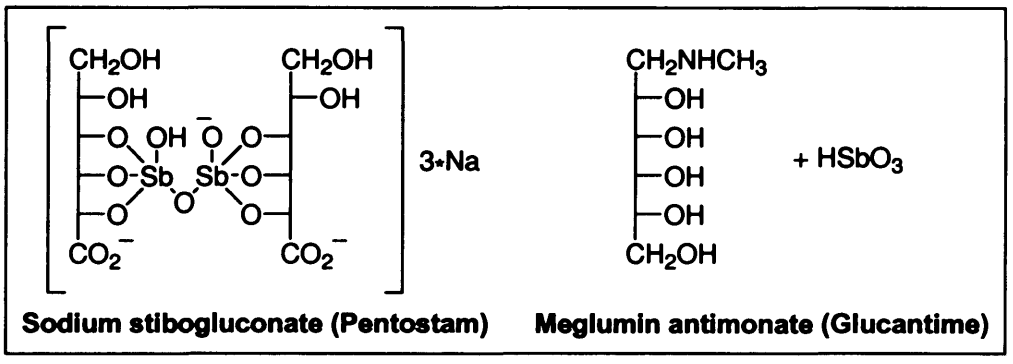


Figure I.5: Antimonial drugs

The exact chemical composition of these compounds is not known and therefore nor is the mechanism[15,16]. Nevertheless, the trivalent antimonial preparation was shown to be more potent than the pentavalent preparation against both promastigotes and amastigotes[17]. The activity was proposed to be

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[13] Herwaldt, B. L.; Berman, J. D. Recommendations for Treating Leishmaniasis with Sodium Stibogluconate (Pentostam) and Review of Pertinent Clinical-Studies. *Am. J. Trop. Med. Hyg.* **1992**, *46*, 296-306.

[14] Herwaldt, B. L. Leishmaniasis. *Lancet* **1999**, *354*, 1191-1199.

[15] Mottram, J. C.; Coombs, G. H. Leishmania-Mexicana - Enzyme-Activities of Amastigotes and Promastigotes and their Inhibition by Antimonials and Arsenicals. *Exp. Parasitol.* **1985**, *59*, 151-160.

[16] Opperdoes, F. R. The Glycosome of Leishmania as a Possible Target for Chemotherapeutic Attack. Leishmaniasis Current Status and New Strategy for Control. Edited by Hart DT, Published by Plenium Press, New York **1989**, 859-864.

[17] Roberts, W. L.; Berman, J. D.; Rainey, P. M. In-Vitro Antileishmanial Properties of Trivalent and Pentavalent Antimonial Preparations. *Antimicrob. Agents Chemother.* **1995**, *39*, 1234-1239.

mainly due to inhibition of glycosome structure or function[18]. Although these drugs are usually highly effective, they have disadvantages such as parenteral mode of administration, long duration of therapy (several weeks) and toxic side effects (e.g. fatigue, body aches, electro-cardio graphic abnormalities).

Development of drug resistance is a major problem in some parts of the world with conventional antimonial therapy[19,20,21,22,23] (occurring in 5 to 70% of patients in some endemic areas). In addition, some *Leishmania* species have greater susceptibility to antimonial compounds than others[24].

I.2.4.2 Diamidines

Diamidines were successfully developed as trypanocidal agents. Pentamidine (Figure I.6) has become the chosen member of this group for clinical use for visceral leishmaniasis (*L. donovani*) resistant to antimonials. A 95% cure rate was observed with cutaneous leishmaniasis in Columbia[25]. The side effects such as vomiting, abdominal pain, and hypertension are common as pentamidine is probably more toxic than antimonial compounds.

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[18] Berman, J. D.; Waddell, D.; Hanson, B. D. Biochemical-Mechanisms of the Antileishmanial Activity of Sodium Stibogluconate. *Antimicrob. Agents Chemother.* **1985**, *27*, 916-920.

[19] Gutteridge, W. E.; Coombs, G. H. Biochemistry of Parasitic Protozoa. *The MacMillan Press LTD, London, UK* **1977**.

[20] Ouellette, M.; Papadopoulou, B. Mechanisms of Drug-Resistance in Leishmania. *Parasitol. Today* **1993**, *9*, 150-153.

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[22] Sundar, S.; More, D. K.; Singh, M. K.; Singh, V. P.; Sharma, S.; Makharia, A.; Kumar, P. C. K.; Murray, H. W. Failure of Pentavalent Antimony in Visceral Leishmaniasis in India: Report from the Center of the Indian Epidemic. *Clin. Infect. Dis.* **2000**, *31*, 1104-1107.

[23] Rijal, S.; Chappuis, F.; Singh, R.; Bovier, P. A.; Acharya, P.; Karki, B. M. S.; Das, M. L.; Desjeux, P.; Loutan, L.; Koirala, S. Treatment of Visceral Leishmaniasis in South-Eastern Nepal: Decreasing Efficacy of Sodium Stibogluconate and Need for a Policy to Limit further Decline. *Trans. Roy. Soc. Trop. Med. Hyg.* **2003**, *97*, 350-354.

[24] Allen, S.; Neal, R. A. The *in vitro* Subsceptibility of Macrophages Infected with Amastigotes of Leishmania spp. to Pentavalent Antimonial Drugs and Others Compounds with Special Relevance to Contaneous Isolates. *Leishmaniasis Current Status and New Strategy for Control. Edited by Hart DT, Published by Plenium Press, New York* **1989**, 711-720.

[25] Balana-Fouce, R.; Reguera, R. M.; Cubria, J. C.; Ordonez, D. The Pharmacology of Leishmaniasis. *General Pharmacology* **1998**, *30*, 435-443.

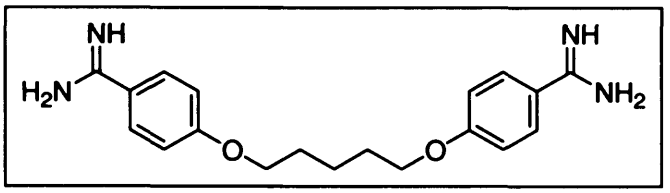

Figure I.6: Pentamidine

I.2.4.3 Amphotericin B

A more recent group of antileishmanial drugs are the polyene macrolide antibiotics of which Amphotericin B is the clinically used derivative. They are highly active against *Leishmania* but systematically toxic. Their use is limited to the cases completely resistant to the other drugs, especially for advanced cases of mucocutaneous leishmaniasis. Amphotericin B consists of a large glycosidic molecule containing a large lactone ring linked to an amino sugar, mycosamine (Figure I.7).

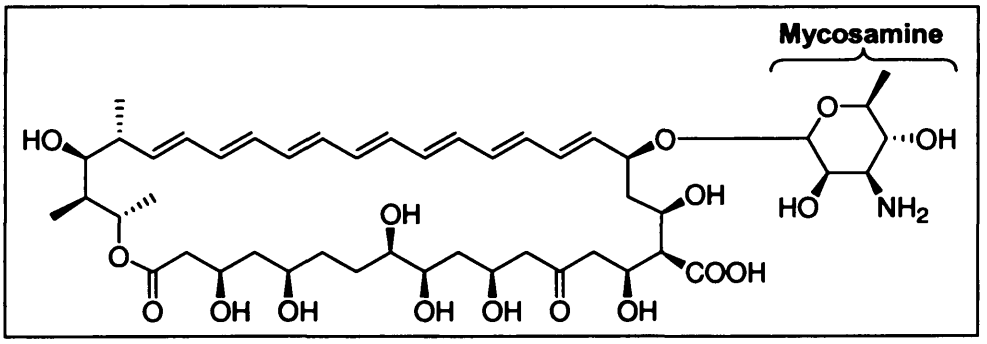

Figure I.7: Amphotericin B

Amphotericin B is thought to bind to the cellular membrane, in particular to the sterols, and generate pores through which ions can pass, destroying the osmolytic balance of the host[10]. New lipid formulations[26] are really effective with less adverse effects against visceral leishmaniasis: over

[26] Yardley, V.; Croft, S. L. A Comparison of the Activities of Three Amphotericin B Lipid Formulations Against Experimental Visceral and Cutaneous Leishmaniasis. *Int. J. Antimicrob. Agents* **2000**, *13*, 243-248.

90% of patients were cured 6 month after treatment with only one injection in India[27] and two injections in Mediterranean areas[28] resulting in a lower cost.

I.2.4.4 Miltefosine

Miltefosine (Figure I.8) is an alkylphospholipid, which was first administered as an anti-cancer drug. Results from studies in India[29,30] (Phase II) showed that orally-administered miltefosine was well tolerated, and is now registered for drug treatment. Studies against visceral leishmanisis demonstrated that patients were apparently cured on day 21, and a long-term therapy proved to be effective for 90% of cases. The mode of action of miltefosine has been investigated but is still not known[31,32]. It has also been shown that *L. donovani* was more sensitive to this drug than the other strains, limiting the use of this drug in particular areas[33]. The disadvantage of miltefosine is its teratogenic properties which narrow the therapeutic window.

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[27] Sundar, S.; Jha, T. K.; Thakur, C. P.; Mishra, M.; Singh, V. P.; Buffels, R. Single-Dose Liposomal Amphotericin B in the Treatment of Visceral Leishmaniasis in India: A Multicenter Study. *Clin. Infect. Dis.* **2003**, *37*, 800-804.

[28] Syriopoulou, V.; Daikos, G. L.; Theodoridou, M.; Pavlopoulou, I.; Manolaki, A. G.; Sereti, E.; Karamboula, A.; Papathanasiou, D.; Krikos, X.; Saroglou, G. Two Doses of a Lipid Formulation of Amphotericin B for the Treatment of Mediterranean Visceral Leishmaniasis. *Clin. Infect. Dis.* **2003**, *36*, 560-566.

[29] Sundar, S.; Rosenkaimer, F.; Makharia, M. K.; Goyal, A. K.; Mandal, A. K.; Voss, A.; Hilgard, P.; Murray, H. W. Trial of Oral Miltefosine for Visceral Leishmaniasis. *Lancet* **1998**, *352*, 1821-1823.

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[32] Verma, N. K.; Dey, C. S. Possible Mechanism of Miltefosine-Mediated Death of Leishmania donovani. *Antimicrob. Agents Chemother.* **2004**, *48*, 3010-3015.

[33] Escobar, P.; Matu, S.; Marques, C.; Croft, S. L. Sensitivities of Leishmania Species to Hexadecylphosphocholine (Miltefosine), ET-18-OCH3 (Edelfosine) and Amphotericin B. *Acta Trop.* **2002**, *81*, 151-157.

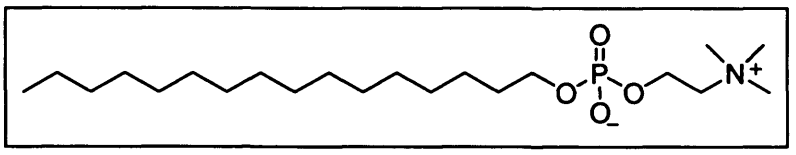

Figure I.8: Miltefosine

I.2.4.5 Paromomycin

Paromomycin (Figure I.9) is an aminoglycoside used for all forms of leishmaniasis and effective against antimony-resistant leishmaniasis, but administrated parentally. This drug showed a 90% cure rate in India[34] and in Guatemala[35]. A lipid formulation[36] has also been tested, and gave a higher activity than with antimony treatment, giving significant size reduction of the lesions. The mode of action of paromomycin is related to the inhibition of RNA synthesis and modification of membrane lipids and membrane fluidity, leading to altered membrane permeability[37].

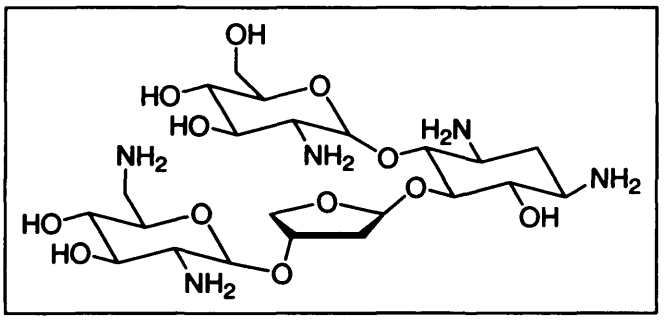

Figure I.9: Paromomycin

[34] Thakur, C. P.; Kanyok, T. P.; Pandey, A. K.; Sinha, G. P.; Messick, C.; Olliaro, P. Treatment of Visceral Leishmaniasis with Injectable Paromomycin (Aminosidine). An Open-Label Randomized Phase-II Clinical Study. *Trans. Roy. Soc. Trop. Med. Hyg.* **2000**, *94*, 432-433.

[35] Arana, B. A.; Mendoza, C. E.; Rizzo, N. R.; Kroeger, A. Randomized, Controlled, Double-Blind Trial of Topical Treatment of Cutaneous Leishmaniasis with Paromomycin plus Methylbenzethonium Chloride Ointment in Guatemala. *Am. J. Trop. Med. Hyg.* **2001**, *65*, 466-470.

[36] Goncalves, G. S.; Fernandes, A. P.; Souza, R. C. C.; Cardoso, J. E.; de Oliveira-Silva, F.; Maciel, F. C.; Rabello, A.; Ferreira, L. A. M. Activity of a Paromomycin Hydrophilic Formulation for Topical Treatment of Infections by *Leishmania (Leishmania) amazonensis and Leishmania (Viannia) braziliensis*. *Acta Trop.* **2005**, *93*, 161-167.

[37] Maarouf, M.; Lawrence, F.; Brown, S.; RobertGero, M. Biochemical Alterations in Paromomycin-Treated *Leishmania donovani* Promastigotes. *Parasitol. Res.* **1997**, *83*, 198-202.

I.3 South American Trypanosomiases - Chagas' disease

South American Trypanosomiasis or Chagas' disease is a serious health problem affecting millions of people in Latin America where it is endemic. 20 million people are infected and 100 million are estimated at risk[8]. It was first described in 1909 by the Brazilian physician Carlos Chagas who also discovered its agent and mode of transmission[38].

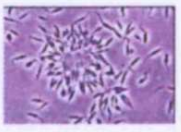

Figure I.10: *Trypanosoma cruzi* trypomastigote[8]

I.3.1 Transmission and infection

Chagas' disease is caused by the parasite *T. cruzi* (Figure I.10) and is usually found in small domestic animals, especially cats, dogs, rabbits, guinea-pigs as well as rodents and small feral species, which act as reservoirs of infection. The parasite is transmitted by the insect vectors, usually members of the reduviidee family (kissing bug, Figure I.11), which are present throughout most of South and Central America[8], and extend across the Southern United States. The bugs preferred habitat is in crevices in the walls of poor-quality houses in rural areas. Prevalence of the disease is directly related to socio-economic factors. Another frequent mode of transmission is by blood transfusion. The disease can also be transmitted from mother to foetus[9,19].

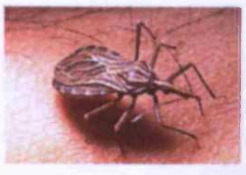

Figure I.11: Kissing bug[8]

[38] Chagas, C. Nova Entidad Morbida do Homen: Resumo General Dos Estudios Etiologicos e Clinicos. *Mem.Inst. Oswaldo Cruz* **1911**, *3*, 219-250.

There are two phases of the human disease:

- **The acute phase**: appears shortly after the infection; shows few clinical effects, and rarely lethal in adults whereas an infection with fever is soon followed by death from myocarditis or encephalitis for young children;

- **The chronic phase**: appears after a silent period that may last several years with damage to the heart and central nervous system usually irreversible.

I.3.2 Geographic distribution

The disease is endemic in 18 countries in Central and South America, but particularly Brazil, Argentina, Venezuela, Chile, Columbia, Peru and Mexico (Figure I.12).

Figure I.12: Geographic distribution of Chagas' disease[39]

[39] http/:www.uta.edu.

Because many small animals[40] act as natural reservoirs, the control of Chagas' disease is really problematic and a major concern in South America. The WHO started a plan in 1996 (Resolution WHA51.14) which consists of interrupting the transmission by the vector[8]. Deltamethrin, a pyrethroid insecticide has been used, and major reductions in human cases of disease were observed[41,42,43].

I.3.3 Life cycle

The life cycle for the *T. cruzi* has two phases (Figure I.13): one with sexual reproduction that occurs in the vector and one which is asexual inside the host[10]. Trypanosomes, which are taken up in a blood meal from the host by the vector change into epimastigotes and multiply before later transforming into metacyclic trypomastigotes (similar to trypomastigotes in human blood but smaller and infective to vertebrates). During a second blood meal, the insect defecates on the skin. Metacyclic trypomastigotes present in the faeces penetrate across the laceration in the skin. After infection, metacyclic trypomastigotes transform into the trypomastigote. These invade cells and multiply by binary fission, causing the rupture of the cells. The trypomastigotes can either infect new cells from a variety of tissues and transform into amastigotes in new infection sites, or remain available in the bloodstream being a reservoir for vector uptake and transmission.

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[40] Teixeira, A. R. L.; Monteiro, P. S.; Rebelo, J. M.; Arganaraz, E. R.; Vieira, D.; Lauria-Pires, L.; Nascimento, R.; Vexenat, C. A.; Silva, A. R.; Ault, S. K.; Costa, J. M. Emerging Chagas' Disease: Trophic Network and Cycle of Transmission of *Trypanosoma cruzi* from Palm Trees in the Amazon. *Emerg. Infect. Dis* **2001**, *7*, 100-112.

[41] Guillen, G.; Diaz, R.; Jemio, A.; Cassab, J. A.; Pinto, C. T.; Schofield, C. J. Chagas' Disease Vector Control in Tupiza, Southern Bolivia. *Mem. Inst. Oswaldo Cruz* **1997**, *92*, 1-8.

[42] Schmunis, G. A.; Zicker, F.; Moncayo, A. Interruption of Chagas' Disease Transmission through Vector Elimination. *Lancet* **1996**, *348*, 1171-1171.

[43] Schofield, C. J.; Dujardin, J. P. Chagas' Disease Vector Control in Central America. *Parasitol. Today* **1997**, *13*, 141-144.

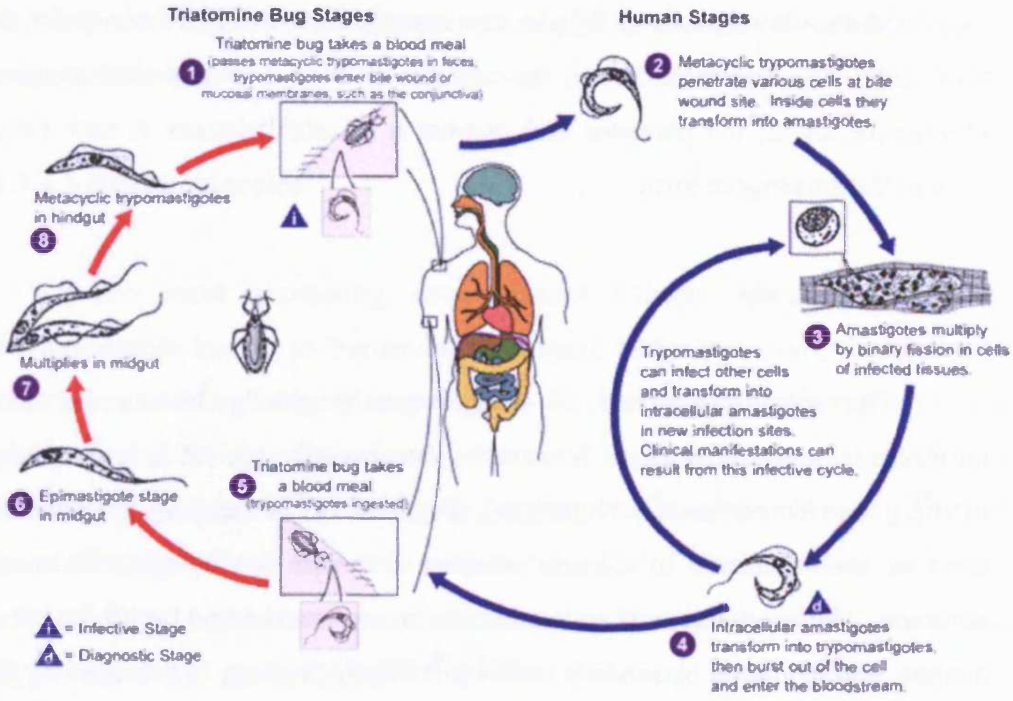

Figure I.13: Life cycle of *Trypanosoma cruzi*[12]

I.3.4 Drug treatment

Effective chemotherapy of Chagas' disease (*Trypanosoma cruzi*) does not exist. Two groups of drugs are registered to treat Chagas' disease, the nitrofurans (Nifurtimox, Figure I.14) and the nitroimidazoles (benznidazole, Figure I.14). Chagas' disease therapy[44] is limited to these two drugs. Their efficacy against acute forms is limited whereas they are ineffective against chronic forms.

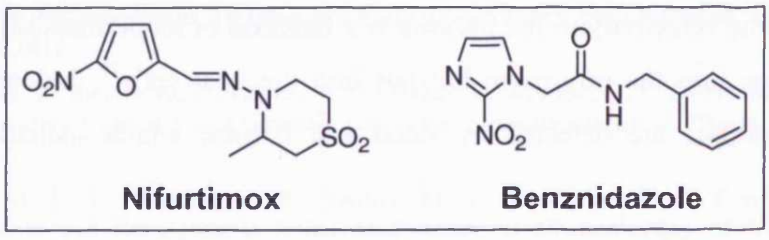

Figure I.14: Nifurtimox and benznidazole

[44] Barrett, M. P.; Gilbert, I. H. Perspectives for New Drugs against Trypanosomiasis and Leishmaniasis. *Current Topics in Medecinal Chemistry* **2002**, *2*, 471-482.

Moreover, the two molecules are associated with high toxicity for the host cells. The treatment has to be continuous over a long period to cause elimination of all the parasites and prevent a re-establishment at new tissue sites in the amastigote form.

I.3.4.1 Nitrofurans

The nitrofuran group of drugs were originally known for their antibacterial activity and have been tested successfully against several species of the genus *Trypanosoma*. Nifurtimox (Figure I.14) is the drug most widely used in the treatment of Chagas' disease although toxic side effects are common. The mechanism of action of nifurtimox seems to be by inhibition of nucleic acid synthesis by binding to DNA[19]. Firstly the drug is activated by the formation of reduced intermediates such as the corresponding nitro-aryl anion[45] radical ($ArNO_2^{\bullet -}$), which is oxidized to generate superoxide anions in first place, followed by production of hydrogen peroxide by the influence of superoxide dismutase (SOD). Ionizing hydroxyl radicals will be formed in the reaction of hydrogen peroxide with more superoxide anions (Scheme I.1)[10].

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H \xrightarrow{SOD} 2H_2O_2 + O_2^{-}$$

$$2H_2O_2 + O_2^{\bullet -} \xrightarrow{Fe} OH^{\bullet} + OH^{-} + O_2$$

Scheme I.1: Mechanism of action of nifurtimox

The selectivity to the parasite is a function of its permeability: the drug penetrates into the protozoan but not into the host cells[19]. Indeed only low concentrations are detected in blood and tissues, which indicates a rapid

[45] Maya, J. D.; Bollo, S.; Nunez-Vergara, L. J.; Squella, J. A.; Repetto, Y.; Morello, A.; Perie, J.; Chauviere, G. *Trypanosoma cruzi*: Effect and Mode of Action of Nitroimidazole and Nitrofuran Derivatives. *Biochem. Pharmacol.* **2003**, *65*, 999-1006.

formation of metabolites[10]. Combination of nifurtimox with other drugs is under research[46].

I.3.4.2 Nitroimidazoles

The most promising drug against Chagas' disease is the 2-nitroimidazole known as benznidazole (Figure I.14). Its activity is similar to nifurtimox, although the efficacy depends on the strain of the parasite and the patient[10]. A 60% cure rate of early chronic *T. cruzi* infection in children was recently demonstrated[47]. Also, new formulations[48] and combinations[49] have been investigated and proved to enhance efficacy of chemotherapy. But toxic effects from benznidazole are common, especially in adults, including hypersensitivity reactions affecting the skin and other effects due to interference with protein and RNA synthesis. Moreover, studies carried out in pregnant rats[50] showed that the drug is readily absorbed, crosses the placental barrier and reaches the foetuses, resulting in covalent binding of reactive metabolites to foetal and maternal proteins.

The mode of action was recently demonstrated[45]; nitroimidazoles derivatives are converted to nitroso forms, which are efficient thiol scavengers, and could react with the dithiol trypanothione, which is integral to protection of the parasite from oxidative damage.

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[46] Andrade, S. G.; Andrade, Z. A.; Sadigursky, M. Combined Treatment with a Nitrofuranic and a Corticoid in Experimental Chagas' Disease in the Dog. *Am. J. Trop. Med. Hyg.* **1980**, *29*, 766-773.

[47] deAndrade, A.; Zicker, F.; deOliveira, R. M.; Silva, S. A. E.; Luquetti, A.; Travassos, L. R.; Almeida, I. C.; deAndrade, S. S.; deAndrade, J. G.; Martelli, C. M. T. Randomised Trial of Efficacy of Benznidazole in Treatment of Early *Trypanosoma cruzi* Infection. *Lancet* **1996**, *348*, 1407-1413.

[48] Morilla, M. J.; Benavidez, P.; Lopez, M. O.; Bakas, L.; Romero, E. L. Development and *in vitro* Characterisation of a Benznidazole Liposomal Formulation. *Int. J. Pharm.* **2002**, *249*, 89-99.

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[50] de Toranzo, E. G. D.; Masana, M.; Castro, J. A. Administration of Benznidazole, a Chemotherapeutic Agent against Chagas' Disease, to Pregnant Rats. Covalent Binding of Reactive Metabolites to Fetal and Maternal Proteins. *Arch. Int. Pharmacodyn.* **1984**, *272*, 17-23.

I.4 Human African Trypanosomiasis (HAT) - Sleeping sickness

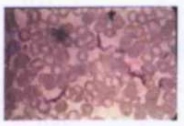

Figure I.15: *Trypanosoma gambiense* trypomastigote[8]

I.4.1 Transmission and infection

The two subspecies, *Trypanosoma brucei gambiense* (Figure I.15) and *Trypanosoma brucei rhodesiense* (see Table 1.1) are responsible for the Human African Trypanosomiasis (HAT) or sleeping sickness[10]. These species also infect wild animals and can be transmitted from these animals to humans (zoonotic infections). Other subspecies exist but are only infective for horses, cattle and other domestic animals[10]. Vectors transmit African trypanosomes and the most common one is the tsetse fly (*Glossina* sp., Figure I.16).

Figure I.16: Tsetse fly[8]

The two human species can generally be distinguished by different clinical patterns of disease:

- ***Trypanosoma brucei rhodesiense***: is found in East African countries; produces an acute infection with severe fever, headache and cardiovascular and endocrinological disorders. This is followed by rapid involvement of the central nervous system (CNS), with the characteristic signs of the disease (motor function impaired, mental alterations, sleep reversal patterns, coma) and culmination in death within a few months[10]. All these symptoms can be reversible using the appropriate treatment;

- ***Trypanosoma brucei gambiense***: is found in Western African countries; infection runs a more chronic course with less severe recurrent bouts of fever, and may even remain undiagnosed for years until the CNS is involved[10]. Because only a few drugs are capable of penetrating the CNS, treatment of the late stage of the disease is more difficult.

I.4.2 Geographic distribution

As their names imply, African trypanosomes are restricted to Africa (Figure I.17), although a few species have been imported into South America[8]. HAT is an endemic disease in 36 African countries, and corresponds to the areas where the tsetse fly lives. The disease is currently epidemic in Congo, Sudan and Angola. 60 million people are estimated to be at risk, and between 300000 and 500000 people are thought to be infected annually.

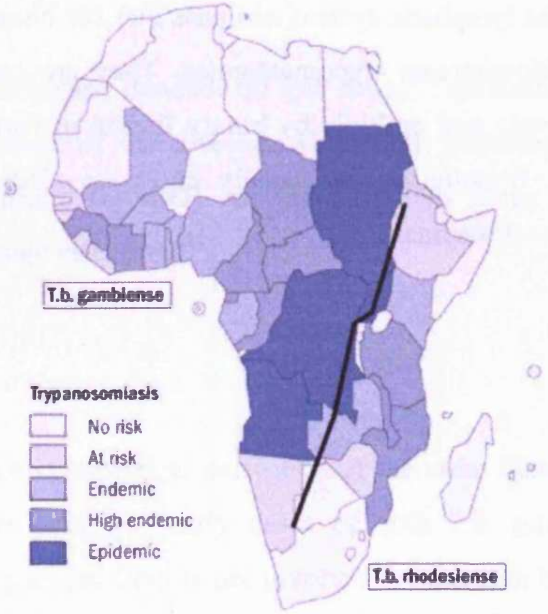

Figure I.17: Geographic distribution of HAT[8]

I.4.3 Life cycle

The blood forms of both subspecies are trypomastigotes, the stum forms being the most infective to the vector. The tsetse fly becomes infec with bloodstream trypomastigotes (Figure I.18) when taking a blood meal an infected mammalian host. In the vector's gut, they change morphologica into procyclic trypomastigotes, which divide and migrate to the inse salivary glands. Here they transform to epimastigotes and divide bef transforming into small infective metacyclic trypomastigotes. During a bl meal, the tsetse fly injects metacyclic trypomastigotes into skin tissue. T parasites enter the lymphatic system and pass into the bloodstream where t transform into bloodstream trypomastigotes. They are carried to other si throughout the body and multiply by binary fission in various body fluids. advanced cases, trypomastigotes usually enter the CNS and may then detected in the cerebrospinal fluid.

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[51] Seed, J. R. African Trypanosomiasis Research: 100 Years of Progress, but Questions Problems Still Remain. *Int. J. Parasit.* **2001**, *31*, 434-442.

[52] Ekwanzala, M.; Pepin, J.; Khonde, N.; Molisho, S.; Bruneel, H.; DeWals, P. In the Hear Darkness: Sleeping Sickness in Zaire. *Lancet* **1996**, *348*, 1427-1430.

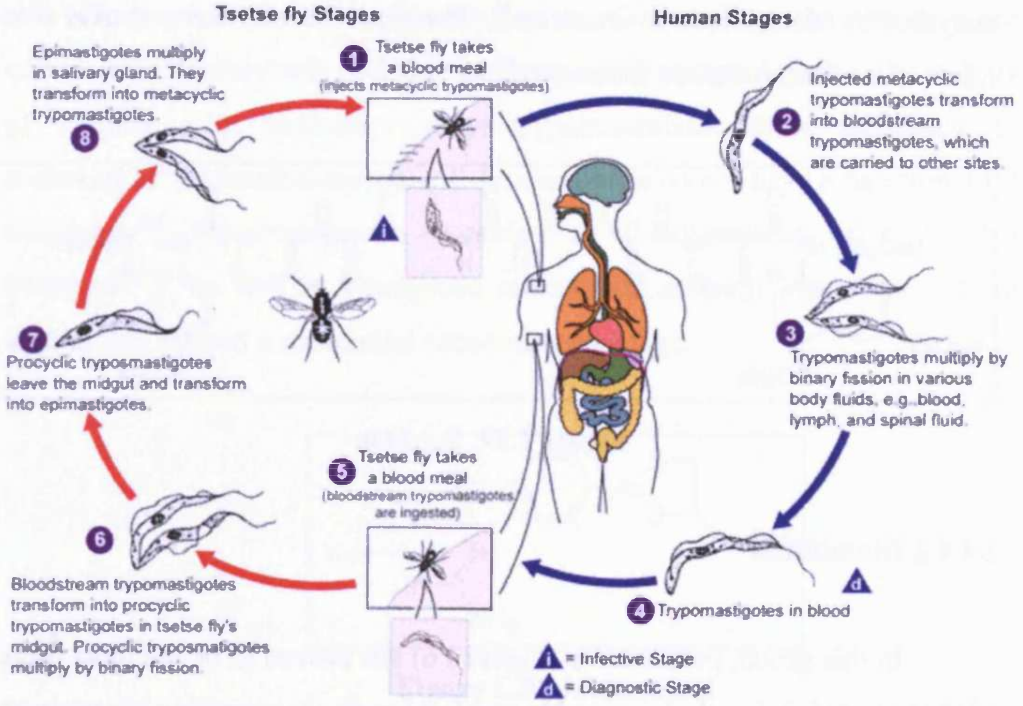

Figure I.18: Life cycle of *Trypanosoma brucei*[12]

I.4.4 Drug treatment

HAT chemotherapy depends on four drugs[53]: suramin and pentamidine, used in the first stage of the disease, while both melarsoprol (Mel B) and difluoromethylornithine (DFMO) are the only two drugs available for the treatment of late stage infections[52].

I.4.4.1 Suramin

Suramin is a symmetrical derivative of the urea. Suramin (Figure I.19) is a very effective drug for early cases of both *T.b. gambiense* and *T.b. rhodesiense* as long as the CNS is not involved[10]. Although the evidence is not conclusive, suramin seems to interfere with the carbohydrate metabolism[19]. At physiological pH, the drug bears six negative charges, and inhibits many

[53] Barrett, M. P.; Basselin, M.; Coombs, G. H. Emerging Therapeutic Targets in Parasitic Protozoa. *Emerging Therapeutic Targets* **1998**, *2*, 57-85.

enzymes by electrostatic interactions[54]. The most serious and common toxic effect of suramin is that on the kidney[10].

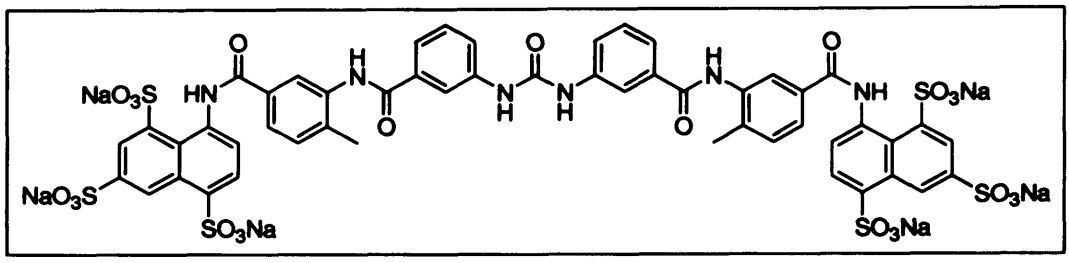

Figure I.19: Suramin

I.4.4.2 Diamidines

In this group, pentamidine (Figure I.6) has proved to be the most useful of those tested, being the most stable and least toxic. Pentamidine has probably several modes of action: it seems that pentamidine can enter *T. brucei* via the same P2 amino-purine transporter that accumulates melamino-phenyl arsenicals[54]; it is also known to interfere with nucleic acid synthesis[19]; and as a polycation, it interacts electrostatically with cellular polyanions. Its interaction is particularly tight with DNA molecules[54]. Pentamidine is very effective for the treatment of early cases of *T.b. gambiense.*

I.4.4.3 Arsenicals

Arsenicals were the first trypanocidal drugs to be introduced early in the 20th century to treat sleeping sickness. These compounds are capable of penetrating the CNS and are, therefore, effective in the late forms of the disease where there is CNS involvement. Melarsoprol (Figure I.20) is the standard treatment for sleeping sickness. Its mechanism of action is not known but seems to be by interfering with the energy metabolism[19]. It is accumulated by the amino-purine P2 transporter, as in the case of the diamidines[54]. Many

[54] Denise, H.; Barrett, M. P. Uptake and Mode of Action of Drugs Used Against Sleeping Sickness. *Biochem. Pharmacol.* **2001**, *61*, 1-5.

side effects are involved[10] and immediate reactions to drug administration are common and cause death of 5% of patients due to encephalopathy. Up to 20% of patients with secondary stage trypanosomiasis show resistance to melarsoprol[51]. Arsenical-resistant *T. brucei* strains do not have a functional P2 transporter[54]. New treatment schedules, a 10-day instead of a 26 day treatment[55,56], as well as synergestic effects with suramin[57] gave cure of the disease and offered a substantial economic advantage.

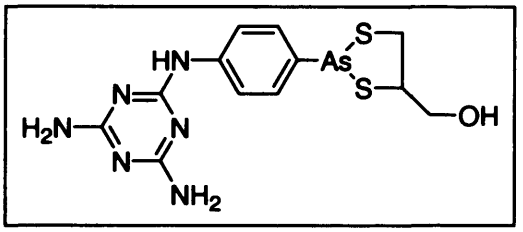

Figure I.20: Melarsoprol

I.4.4.4 Difluoromethylornithine

Difluoromethylornithine (DFMO, Figure I.21) is an analogue of ornithine, and is used for cases resistant to melarsoprol. The mechanism of action appears to be acting as a specific inhibitor of the key polyamine biosynthesis enzyme ornithine decarboxylase (ODC)[58,59,60]. Although this

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[55] Burri, C.; Nkunhu, S.; Merolle, A.; Smith, T.; Blum, J.; Brun, R. Efficacy of New, Concise Schedule for Melarsoprol in Treatment of Sleeping Sickness Caused by *Trypanosoma brucei gambiense*: a Randomised Trial. *Lancet* **2000**, *355*, 1419-1425.

[56] Schmid, C.; Nkunku, S.; Merolle, A.; Vounatsou, P.; Burri, C. Efficacy of 10-Day Melarsoprol Schedule 2 Years after Treatment for Late-Stage *gambiense* Sleeping Sickness. *Lancet* **2004**, *364*, 789-790.

[57] Jennings, F. W.; Rodgers, J.; Bradley, B.; Gettinby, G.; Kennedy, P. G. E.; Murray, M. Human African Trypanosomiasis: Potential Therapeutic Benefits of an Alternative Suramin and Melarsoprol Regimen. *Parasitol. Int.* **2002**, *51*, 381-388.

[58] McCann, P. P.; Pegg, A. E. Ornithine Decarboxylase as an Enzyme Target for Therapy. *Pharmacol. Ther.* **1992**, *54*, 195-215.

[59] Fairlamb, A. H.; Le Quesne, S. A. Polyamine Metabolism in Trypanosomes. In: Trypanosomiasis and Leishmaniasis. Biology and Control. CAB International, Wallington, UK **1997**, 149-162.

[60] Li, F.; Hua, S. B.; Wang, C. C.; Gottesdiener, K. M. *Trypanosoma brucei brucei*: Characterization of an ODC Null Bloodstream Form Mutant and the Action of Alpha-Difluoromethylornithine. *Exp. Parasitol.* **1998**, *88*, 255-257.

target enzyme is present in both parasite and host cells, the parasite ODC turn over is much less rapid than the mammalian version[61,62]. Thus, the host restores active enzyme quicker while the parasite does not. To be effective, the drug needs to be given in large doses[54].

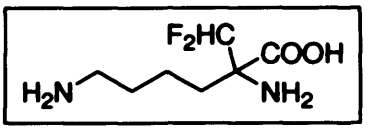

Figure I.21: Difluoromethylornithine

I.5 Summary

Only a limited range of drugs are available for the cure of leishmaniasis and trypanosomiasis. All drugs have toxic side effects and most of them need to be administrated parentally. Clearly, there is an urgent need for new and better drugs to replace and supplement those in present use.

[61] Ghoda, L.; Phillips, M. A.; Bass, K. E.; Wang, C. C.; Coffino, P. Trypanosome Ornithine Decarboxylase Is Stable Because It Lacks Sequences Found in the Carboxyl Terminus of the Mouse Enzyme Which Target the Latter for Intracellular Degradation. *J. Biol. Chem.* **1990**, *265*, 11823-11826.

[62] Iten, M.; Mett, H.; Evans, A.; Enyaru, J. C. K.; Brun, R.; Kaminsky, R. Alterations in Ornithine Decarboxylase Characteristics Account for Tolerance of *Trypanosoma brucei rhodesiense* to D,L-Alpha-Difluoromethylornithine. *Antimicrob. Agents Chemother.* **1997**, *41*, 1922-1925.

CHAPTER II

STEROL 24-METHYL-TRANSFERASE ENZYME

Biosynthesis of sterols is important for eukaryotes in general. The list of organisms requiring sterols includes yeast, fungi and pathogenic parasites. Sterols play vital roles in all living organisms and especially in the

maintenance of the membrane architecture and fluidity[63]. Sterol biosynthesis and its regulation vary between animals, plants and fungi; therefore, inhibition of sterol biosynthesis has potential for the treatment of parasitic diseases.

II.1 Sterol metabolism

In fungi and some parasites, including those in *Trypanosoma* and *Leishmania* genus, the major sterols present are ergosterol and related sterols; in mammalian systems the major sterol is cholesterol and in plants stigmasterol and β–sitosterol (Figure II.1). There are differences in the biosynthetic pathway for these different sterols and enzymes within the pathways which offers scope for selective inhibition and drug development. Indeed, sterol biosynthesis is a major target for anti-fungal drugs.

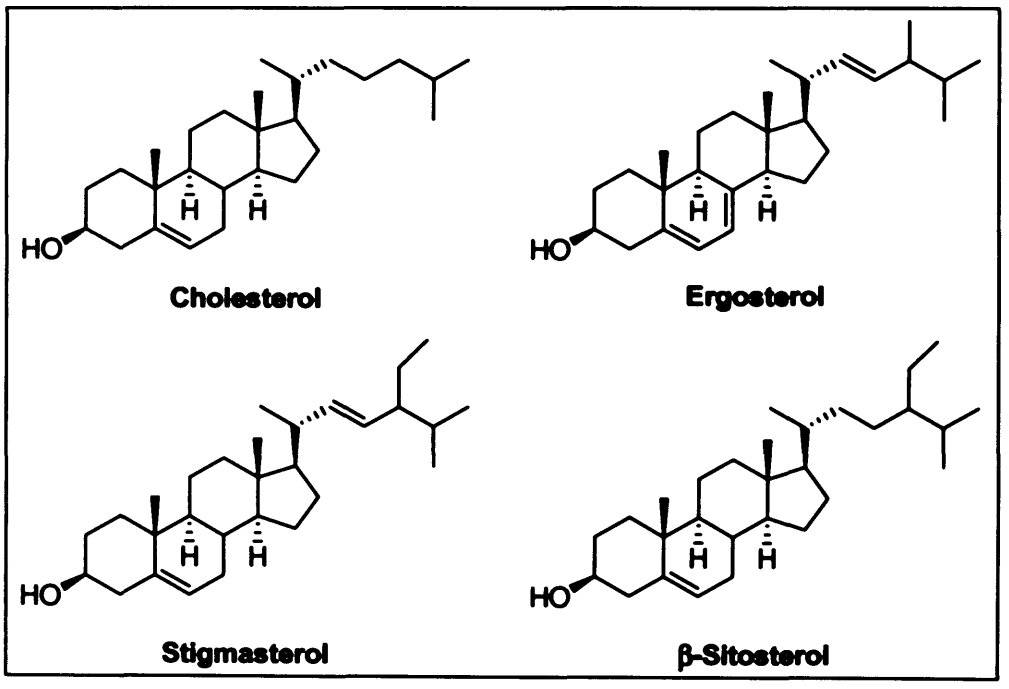

Figure II.1: Main sterols in fungi, mammalians and plants.

[63] Parks, L. W.; Lorenz, R. T.; Casey, W. M. Functions for Sterols in Yeast Membranes. *In: Emerging targets in antibacterial and antifungal chemotherapy, Chapman & Hall, New York* **1992**, *15*, 393-409.

Cholesterol and ergosterol differ in several ways: cholesterol has a Δ^5-unsaturation in the B ring and a fully saturated side chain without a methyl group at C-24; ergosterol has a conjugated Δ^5/Δ^7-unsaturation in the B ring, a Δ^{22}-unsaturated side chain and an additional methyl group at C-24. Figure II.2 shows the biosynthetic pathway leading to the production of cholesterol and ergosterol and the main enzymes involved in the different steps.

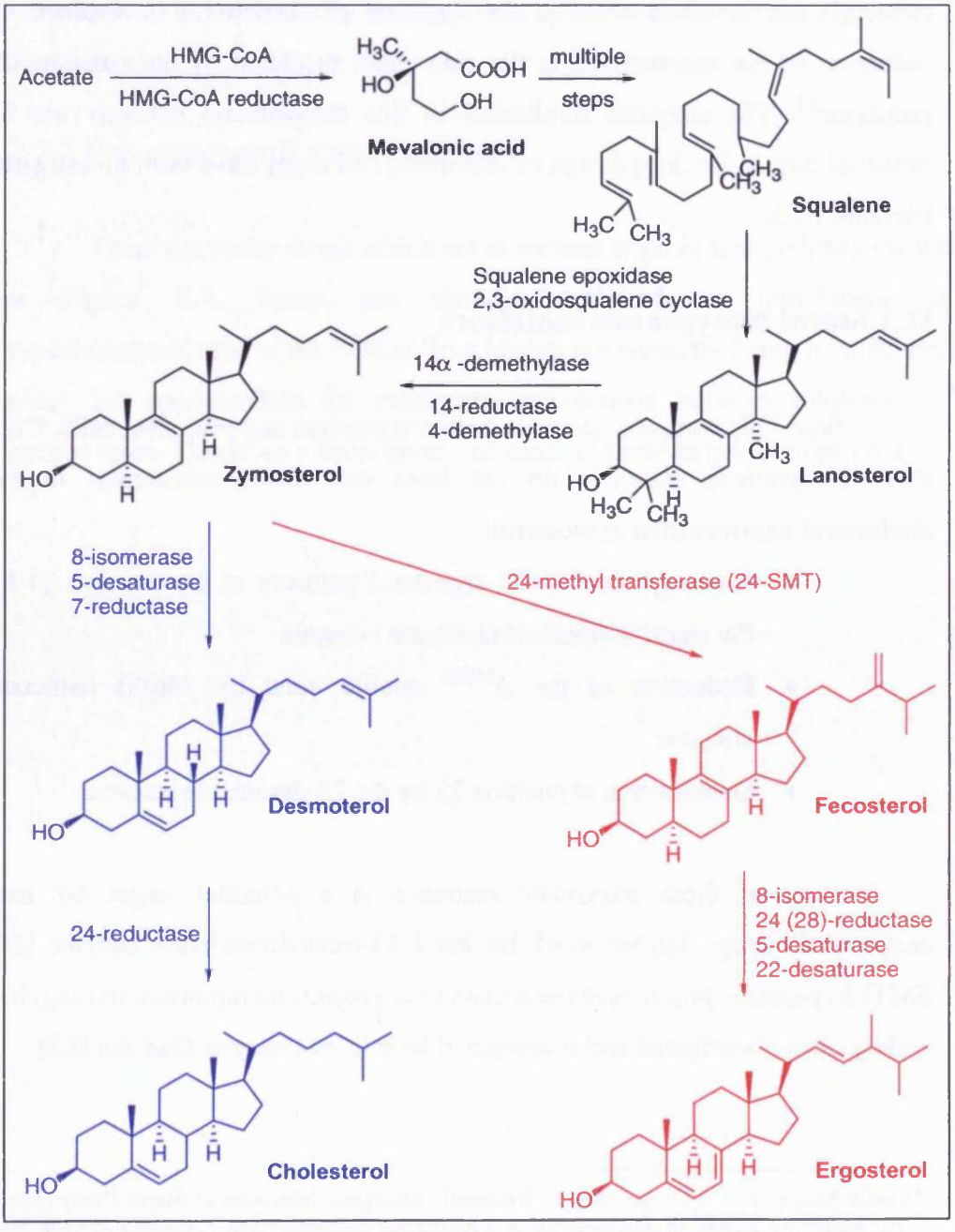

Figure II.2: Sterol biosynthetic pathways of cholesterol and ergosterol

Vertebrates and fungi share the same sterol biosynthetic pathway until zymosterol. Then, in fungi, zymosterol is alkylated at C-24 by the enzyme sterol 24-methyltransferase (24-SMT) to give fecosterol. Ergosterol biosynthesis is then mediated by the enzymes 8-isomerase, 24(28)-reductase, 5-desaturase and 22-desaturase. The precise order of reactions may vary from organism to organism. In vertebrates, zymosterol is transformed into desmosterol in three steps where the enzymes 8-isomerase, 5-desaturase and 7-reductase are involved. Finally, the synthesis of cholesterol is obtained by reduction of the unsaturation in the side chain mediated by the enzyme 24-reductase[64]. The enzymes implicated in this biosynthetic pathway are all potential targets for drug design of inhibitors, and many have been investigated (Section II.2).

II.2 Sterol biosynthesis inhibitors

Sterol biosynthesis occurs in both mammalian and protozoal cells. Only three biosynthetic reactions do not have any direct counterpart in the cholesterol pathway after zymosterol:

- Methenylation in the ergosterol pathway at the position 24 by the sterol 24-methyltransferase enzyme;
- Reduction of the $\Delta^{24(28)}$ double bond by 24(28) reductase enzyme;
- Desaturation at position 22 by the 22-desaturase enzyme.

Any of these enzymatic reactions is a potential target for new antiparasitic drugs. Inhibition of the sterol 24-methyltransferase enzyme (24-SMT) in parasitic protozoa is the focus of the project. Its inhibition in fungi has widely been investigated and is described later in this chapter (Section II.3).

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[64] Oehlschlager, A. C.; Czyzewska, E. Rationally Designed Inhibitors of Sterol Biosynthesis. *In: Emerging targets in antibacterial and antifungal chemotherapy, Chapman & Hall, New York* **1992**, *17*, 437-475.

the mammalian sterol biosynthesis pathway during inhibition of the p
pathway may not necessarily be a problem as humans also obtain cho
from diet.

II.2.1 Pre-zymosterol steps inhibitors

There are many drugs which act at various steps in this pathway
in Figure II.3. Some are already exploited as anti-fung
hypocholesterolemic agents. Other drug targets are currently being inves
which are opportunities for synergistic interactions between inhibi
various steps. Therefore a short review of some of these targets is presen

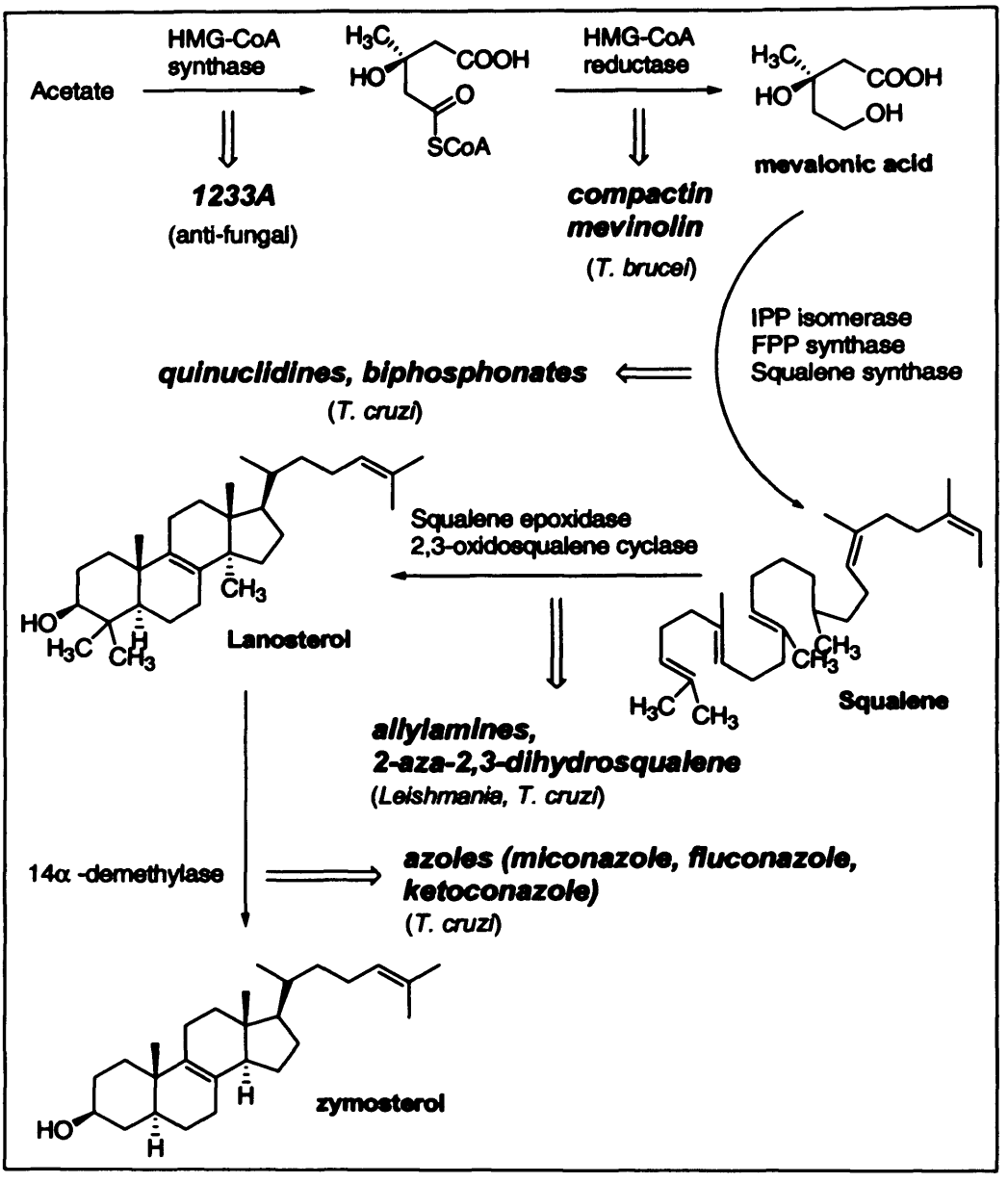

Figure II.3: Pre-zymosterol enzyme inhibitors

The entire skeleton of the sterol molecule derives from acetyl-CoA, except the C-24 methyl group in the ergosterol side chain. The beginning of the biosynthetic pathway occurs via a condensation of two acetyl-CoA units which form acetoacetyl-CoA, and then, addition of a third unit of acetyl-CoA forms 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is reduced by

NADPH to give mevalonic acid[65]. These three steps are catalyzed by three enzymes:

- Acetoacetyl-CoA thiolase and HMG-CoA synthase enzymes[65];
- HMG-CoA reductase enzyme, known as rate-limiting step in cholesterol biosynthesis[66,67].

II.2.1.1 HMG-CoA synthase

The β-lactone antibiotic 1233A (Figure II.4)[65] was determined as a specific inhibitor of the HMG-CoA synthase with an IC_{50} of 0.1μM. This compound has antifungal activity *in vitro*, but is not selective, and also inhibits the growth of mammalian cells.

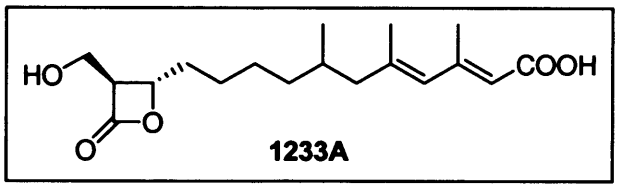

Figure II.4: HMG-CoA synthase inhibitor

II.2.1.2 HMG-CoA reductase

Compactin (Figure II.5), a sesquiterpene, was the first agent found to inhibit the HMG-CoA reductase enzyme. Its activity was demonstrated against this enzyme in insect tissue[68] and higher plant cells[69], but has, with other

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[65] Barrett-Bee, K.; Ryder, N. Biochemical Aspects of Ergosterol Biosynthesis Inhibition. *In: Emerging targets in antibacterial and antifungal chemotherapy, Chapman & Hall, New York* **1992**, *16*, 410-436.

[66] Heller, R. A.; Shrewsbury, M. A. 3-Hydroxy-3-Methylglutaryl Coenzyme-A Reductase from Rat-Liver - Its Purification, Properties, and Immunochemical Studies. *J. Biol. Chem.* **1976**, *251*, 3815-3822.

[67] Angelin, B.; Backman, L.; Einarsson, K.; Eriksson, L.; Ewerth, S. Hepatic Cholesterol-Metabolism in Obesity - Activity of Microsomal 3-Hydroxy-3-Methylglutaryl - Coenzyme-A Reductase. *J. Lipid Res.* **1982**, *23*, 770-773.

[68] Monger, D. J.; Lim, W. A.; Kezdy, F. J.; Law, J. H. Compactin Inhibits Insect HMG-CoA Reductase and Juvenile- Hormone Biosynthesis. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 1374-1380.

structurally related compounds like synvinolin and lovastatin, a very weak growth inhibitory activity against relevant fungi.

Synvinolin (Figure II.5) has also been shown to be active against both the procyclic and blood stream forms of *T. brucei*, presumably by inhibition of HMG-CoA reductase. *T. brucei* procyclic parasite biosynthesises ergosterol[70]. However blood stream form of *T. brucei* is reported to scavenge cholesterol from the host[70], so growth inhibition may be due to inhibition of biosynthesis of other isoprenoid products[71,72].

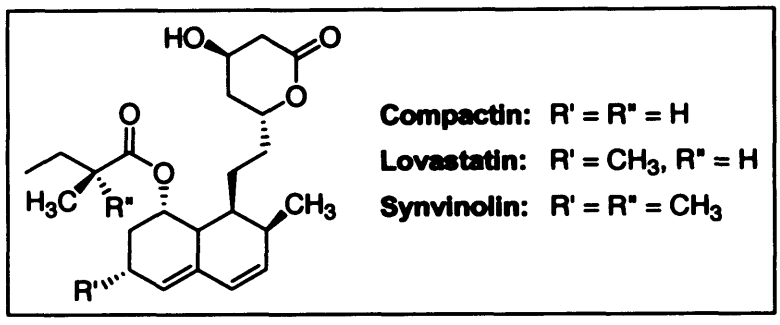

Figure II.5: HMG-CoA reductase inhibitors

II.2.1.3 Farnesyl diphosphate synthase

The sequence from mevalonic acid to squalene is carried out by three enzymes, the IPP isomerase, the farnesyl diphosphate synthase (FPPS, major drug target in cancer research[73]) and the squalene synthase[65]. Recently, several

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[69] Ryder, N. S.; John Goad, L. The Effect of the 3-Hydroxy-3-Methylglutaryl CoA Reductase Inhibitor ML-236B on Phytosterol Synthesis in Acer Pseudoplatanus Tiddue Culture. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1980**, *619*, 424-427.

[70] Coppens, I.; Courtoy, P. J. The Adaptative Mechanisms of *Trypanosoma brucei* for Sterol Homeostasis in its Different Life-Cycle Environments. *Annu. Rev. Microbiol.* **2000**, *54*, 129-156.

[71] Coppens, I.; Bastin, P.; Levade, T.; Courtoy, P. J. Activity, Pharmacological Inhibition and Biological Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme-A Reductase in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **1995**, *69*, 29-40.

[72] Coppens, I.; Courtoy, P. J. Exogenous and Endogenous Sources of Sterols in the Culture-Adapted Procyclic Trypomastigotes of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **1995**, *73*, 179-188.

[73] Yokoyama, K.; Zimmerman, K.; Scholten, J.; Gelb, M. H. Differential Prenyl Pyrophosphate Binding to Mammalian Protein Geranylgeranyltransferase-I and Protein Farnesyltransferase

biphosphonates such as pamidronate biphosphonate (Figure II.6)[74] were reported as FPPS inhibitors. A radical cure of cutaneous leishmaniasis in mice models was observed, although biphosphonate derivatives have been identified as poorly absorbable[75].

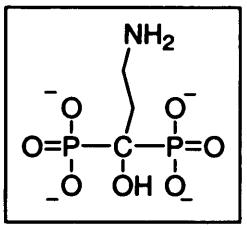

Figure II.6: Pamidronate biphosphonate, FPPS inhibitor

II.2.1.4 Squalene synthase

Squalene synthase inhibitors have been the widest studied. In the last decade, several compounds having a 4-phenoxyphenoxy skeleton have been designed, synthesised and evaluated as antiproliferative agents against *T. cruzi*[76,77]. The 4-phenoxyphenoxyethyl thiocyanate (WC-9, Figure II.7) proved to be an extremely active growth inhibitor of the epimastigote forms of *T. cruzi*, showing activities 4 times higher than that observed when using nifurtimox under the same conditions. The mechanism of action was thought to act at an early stage of the sterol biosynthetic pathway[76]. In fact, a primary mechanism is the depletion of essential endogenous sterols by a specific

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and its Consequence on the Specificity of Protein Prenylation. *J. Biol. Chem.* **1997**, *272*, 3944-3952.

[74] Rodriguez, N.; Bailey, B. N.; Martin, M. B.; Oldfield, E.; Urbina, J. A.; Docampo, R. Radical Cure of Experimental Cutaneous Leishmaniasis by the Bisphosphonate Pamidronate. *J. Infect. Dis.* **2002**, *186*, 138-140.

[75] Zmuidinavicius, D.; Didziapetris, R.; Japertas, P.; Avdeef, A.; Petrauskas, A. Classification Structure-Activity Relations (C-SAR) in Prediction of Human Intestinal Absorption. *J. Pharm. Sci.* **2003**, *92*, 621-633.

[76] Cinque, G. M.; Szajnman, S. H.; Zhong, L.; Docampo, R.; Schvartzapel, A. J.; Rodriguez, J. B.; Gros, E. G. Structure-Activity Relationship of New Growth Inhibitors of *Trypanosoma cruzi*. *J. Med. Chem.* **1998**, *41*, 1540-1554.

[77] Schvartzapel, A. J.; Zhong, L.; Docampo, R.; Rodriguez, J. B.; Gros, E. G. Design, Synthesis, and Biological Evaluation of New Growth Inhibitors of *Trypanosoma cruzi* (Epimastigotes). *J. Med. Chem.* **1997**, *40*, 2314-2322.

blockade of their *de* novo biosynthesis at the level of the squalene synthase[78]. This compound and related derivatives could then represent a new class of squalene synthase inhibitors.

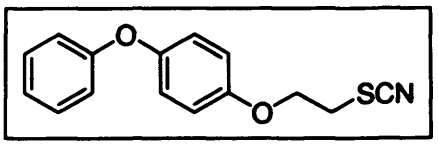

Figure II.7: 4-Phenoxyphenoxyethyl thiocyanate (WC-9)

Quinuclidine derivatives (Figure II.8) also showed some potency against *T. cruzi*[79]. ER5700 was potent against the growth of epimastigotes form of the parasite with an IC_{50} of 30nM[80], while BPQ-OH showed an IC_{50} of 24μM[81]. E5700 was also capable of providing a complete protection against *T. cruzi* infection in a murine model, orally administered. The mode of action seems to be by destabilisation of the parasite cell membranes.

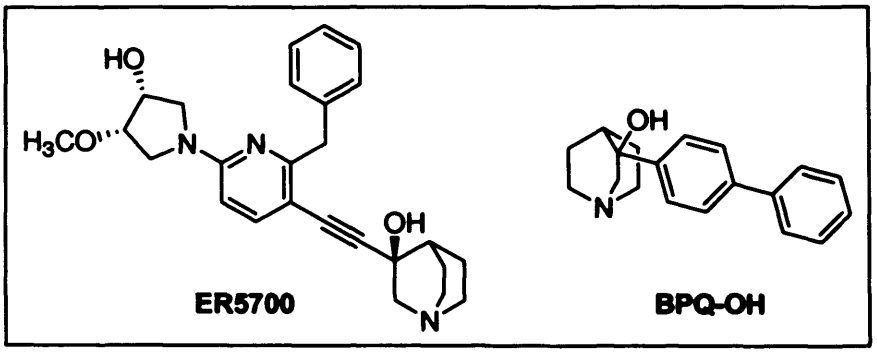

Figure II.8: Squalene synthase inhibitors

[78] Urbina, J. A.; Concepcion, J. L.; Montalvetti, A.; Rodriguez, J. B.; Docampo, R. Mechanism of Action of 4-Phenoxyphenoxyethyl Thiocyanate (WC-9) Against *Trypanosoma cruzi*, the Causative Agent of Chagas' Disease. *Antimicrob. Agents Chemother.* **2003**, *47*, 2047-2050.

[79] Lorente, S. O.; Gomez, R.; Jimenez, C.; Cammerer, S.; Yardley, V.; de Luca-Fradley, K.; Croft, S. L.; Ruiz Perez, L. M.; Urbina, J.; Pacanowska, D. G.; Gilbert, I. H. Biphenylquinuclidines as Inhibitors of Squalene Synthase and Growth of Parasitic Protozoa. *Bioorg. Med. Chem.* **2005**, *13*, 3519-3529.

[80] Urbina, J. A.; Concepcion, J. L.; Caldera, A.; Payares, G.; Sanoja, C.; Otomo, T.; Hiyoshi, H. *In vitro* and *in vivo* Activities of E5700 and ER-119884, Two Novel Orally Active Squalene Synthase Inhibitors, Against *Trypanosoma cruzi*. *Antimicrob. Agents Chemother.* **2004**, *48*, 2379-2387.

[81] Braga, M. V.; Urbina, J. A.; de Souza, W. Effects of Squalene Synthase Inhibitors on the Growth and Ultrastructure of *Trypanosoma cruzi*. *Int. J. Antimicrob. Agents* **2004**, *24*, 72-78.

II.2.1.5 Squalene epoxidase

Epoxidation at the 2,3-position is the first step in the conversion of squalene to lanosterol. Squalene epoxidase is the first enzyme in the pathway to require molecular oxygen and is of particular interest in fungi as the step at which ergosterol biosynthesis is modulated by availability of oxygen[65]. The allylamines, naftifine and terbinafine (Figure II.9) showed some good potency and selectivity against this enzyme, having a higher activity against the enzyme in yeast or fungi than against the enzyme in mammalians[82,83]. Terbinafine inhibits this enzyme in *L.m. mexicana* with an MIC of 34μM, and stops the parasite growth (epimastigote form) against *T. cruzi* at a concentration of 1μM in only 48h[84]. Nevertheless, the inhibition of the squalene epoxidase with terbinafine in tobacco cells was shown to up-regulate the HMG-CoA reductase[85], giving evidence for a feedback regulation of this enzyme in response to a selective depletion of endogenous sterols. However, another compound, FR194738[86], gave an IC_{50} of 5.4nM against squalene epoxidase and a minimal increase of HMG-CoA reductase activity.

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[82] Ryder, N. S. Squalene Epoxidase as the Target of Antifungal Allylamines. *Pestic. Sci.* **1987**, *21*, 281-288.

[83] Ryder, N. S.; Dupont, M. C. Inhibition of Squalene Epoxidase by Allylamine Antimycotic Compounds - A Comparative-Study of the Fungal and Mammalian Enzymes. *Biochem. J.* **1985**, *230*, 765-770.

[84] Van den Bossche, H. Antiprotozoal Activity of Ergosterol Biosynthesis Inhibitors: Focus on Trypanosomatidae and Plasmodiidae. *Microbiol. Eur.* **1993**, 20-28.

[85] Wentzinger, L. F.; Bach, T. J.; Hartmann, M. A. Inhibition of Squalene Synthase and Squalene Epoxidase in Tobacco Cells Triggers an Up-Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase. *Plant Physiol.* **2002**, *130*, 334-346.

[86] Sawada, M.; Washizuka, K.; Okumura, H. Synthesis and Biological Activity of a Novel Squalene Epoxidase Inhibitor, FR194738. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 633-637.

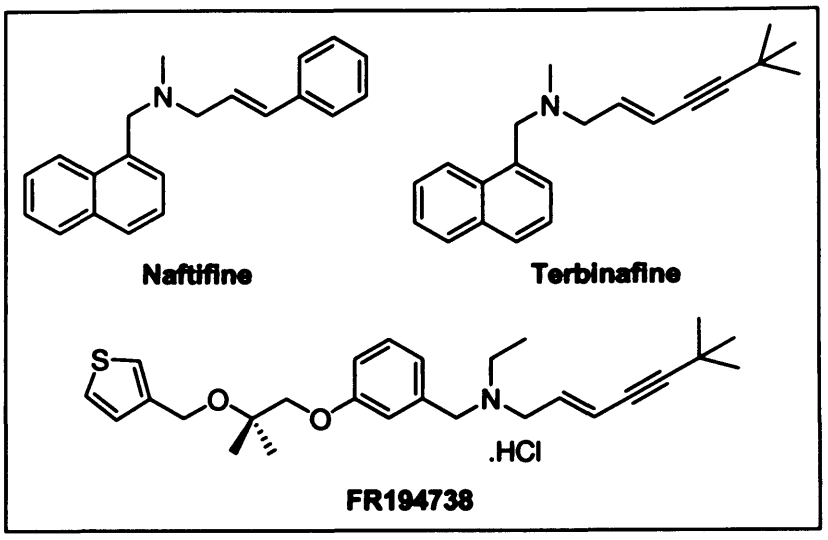

Figure II.9: Squalene epoxidase inhibitors

II.2.1.6 Squalene cyclase

The 2,3-oxido-squalene is cyclised to lanosterol in a reaction catalysed by the 2,3-oxidosqualene cyclase enzyme. The first series of inhibitors designed were azasqualene derivatives[87] (Figure II.10). They have been successfully synthesized as mimics of the carbocationic intermediate of the cyclisation[65]. Similar derivatives to the 2-aza-2,3-dihydrosqualene were evaluated against *T. cruzi* and showed some promising data with IC_{50}'s in the nanomolar range, more potent than the current drug (Benznidazole)[88]. The cause of the death of the parasite may be by accumulation of a toxic by-product or concomitant membrane disruption.

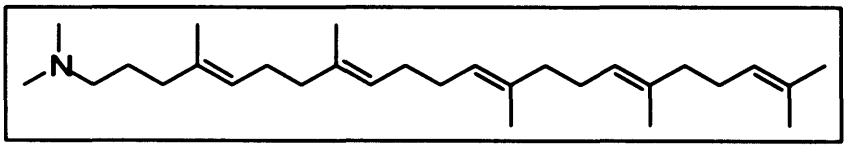

Figure II.10: Squalene cyclase inhibitor, 2-aza-2,3-dihydrosqualene

[87] Duriatti, A.; Bouviernave, P.; Benveniste, P.; Schuber, F.; Delprino, L.; Balliano, G.; Cattel, L. *In vitro* Inhibition of Animal and Higher-Plants 2,3- Oxidosqualene-Sterol Cyclases by 2-Aza-2,3-Dihydrosqualene and Derivatives, and by Other Ammonium-Containing Molecules. *Biochem. Pharmacol.* **1985**, *34*, 2765-2777.

[88] Buckner, F. S.; Griffin, J. H.; Wilson, A. J.; Van Voorhis, W. C. Potent Anti-*Trypanosoma cruzi* Activities of Oxidosqualene Cyclase Inhibitors. *Antimicrob. Agents Chemother.* **2001**, *45*, 1210-1215.

II.2.1.7 14α-Demethylase

The demethylation step utilises a cytochrome P-450. The removal of the methyl group is a two stage oxidative reaction. First, hydroxylation gives a hydromethyl derivative and then a second hydroxylation leads to the loss of water and the methyl group. Finally the produced double bond is reduced by the Δ^{14} reductase[65].

The major antifungal inhibitors designed to inhibit 14α-demethylase are azole derivatives (ketoconazole, fluconazole, posaconazole and itraconazole: Figure II.11). This is the most important class of sterol biosynthesis inhibitors used as antifungal. Interaction between the lone-pair of electrons which exist on the azole nitrogen atoms with the iron of the cytochrome unit of the enzyme is stabilized at the binding site. The trimethylated sterols that accumulate after inhibition are bulkier than ergosterol. Membranes function then less efficiently and there is interference with the function of membrane-bound enzyme[65].

Addition of ketoconazole or itraconazole to both promastigotes and amastigotes resulted in the decrease of ergosterol synthesis by inhibition of the P-450 dependant 14α-demethylase in *L. braziliensis* and *L. mexicana*. They also inhibit *T. cruzi* epimastigotes growth with an IC_{50} of 1.9 and 1.4μM, respectively[81]. Ketoconazole in combination with terbinafine (a squalene epoxidase inhibitor) showed some synergism against *L. braziliensis* which is naturally resistant to ketoconazole[89].

Posaconazole was recently developed, and showed *in vitro* activity against both proliferatives stages of *T. cruzi*[90], but also, good *in vivo* activity

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[89] Rangel, H.; Dagger, F.; Hernandez, A.; Liendo, A.; Urbina, J. A. Naturally Azole-Resistant *Leishmania braziliensis* Promastigotes Are Rendered Susceptible in the Presence of Terbinafine: Comparative Study with Azole-Susceptible *Leishmania mexicana* Promastigotes. *Antimicrob. Agents Chemother.* **1996**, *40*, 2785-2791.

[90] Urbina, J. A.; Payares, G.; Contreras, L. M.; Liendo, A.; Sanoja, C.; Molina, J.; Piras, M.; Piras, R.; Perez, N.; Wincker, P.; Loebenberg, D. Antiproliferative Effects and Mechanism of Action of SCH 56592 Against *Trypanosoma (Schizotrypanum) cruzi*: *In Vitro* and *in Vivo* Studies. *Antimicrob. Agents Chemother.* **1998**, *42*, 1771-1777.

with benznidazole-, nifurtimox- and ketoconazole-resistant organisms in murine models of both acute and chronic Chagas' disease[91].

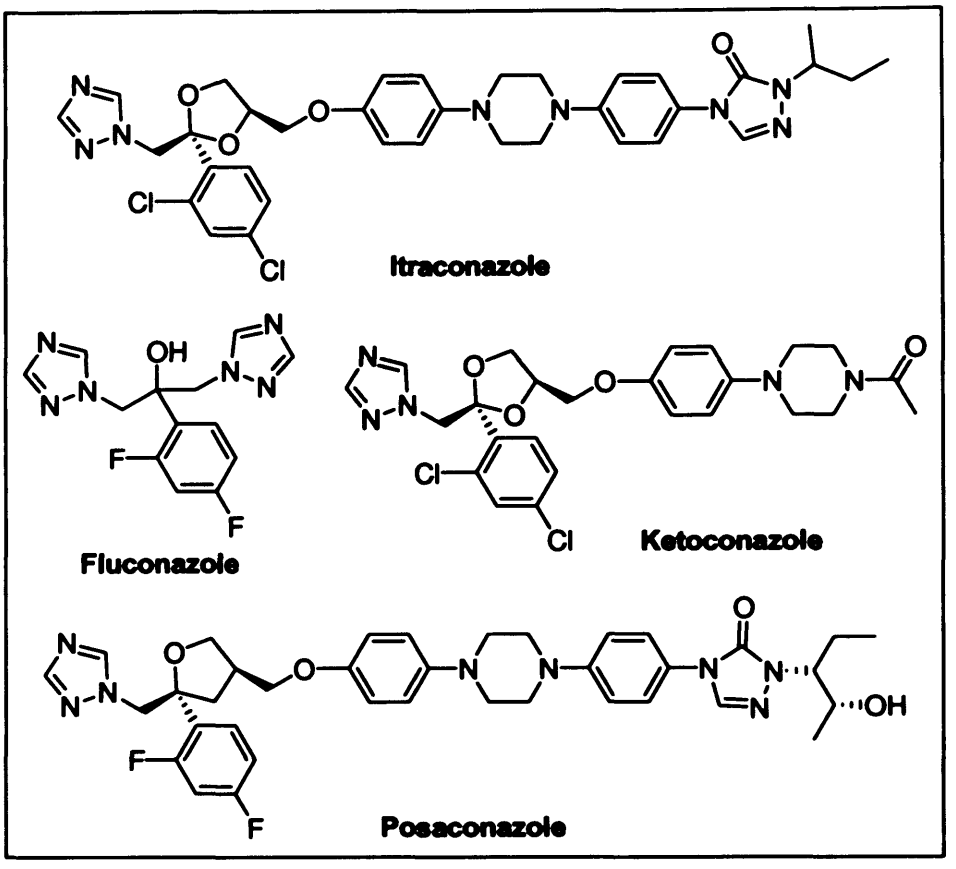

Figure II.11: 14α -Demethylase inhibitors

The bis(triazole) derivative ICI 195739 (Figure II.12), is a racemic compound that has also been tested as a 14α-demethylase inhibitor. This mixture showed antifungal activities against animal models and especially against *T. cruzi* in murine models[92]. D0870, the *R*(+)-enantiomer of ICI 195739 is responsible for the activity and was shown to be more effective against both

[91] Molina, J.; Martins, O.; Brener, Z.; Romanha, A. J.; Loebenberg, D.; Urbina, J. A. Activities of the Triazole Derivative SCH 56592 (Posaconazole) Against Drug-Resistant Strains of the Protozoan Parasite *Trypanosoma (Schizotrypanum) cruzi* in Immunocompetent and Immunosuppressed Murine Hosts. *Antimicrob. Agents Chemother.* **2000**, *44*, 150-155.

[92] Urbina, J. A.; Payares, G.; Molina, J.; Sanoja, C.; Liendo, A.; Lazardi, K.; Piras, M. M.; Piras, R.; Perez, N.; Wincker, P.; Ryley, J. F. Cure of Short- and Long-Term Experimental Chagas' Disease Using D 0870. *Science* **1996**, *273*, 969-971.

epimastigote and amastigote forms of *T. cruzi*[93] in both acute and chronic models[94]. The mechanism of action suggested for this compound involves a dual effect, an inhibition of the 14α-demethylase and a blockade of the cell cycle, leading to growth arrest and cell lysis. Unfortunately the compound showed low solubility, a feature that was improved by the incorporation of the compound into polyethyleneglycol-polylactide (PEG-PLA) nanospheres[95]. But unfortunately the use of this compound did not progress due to toxicity.

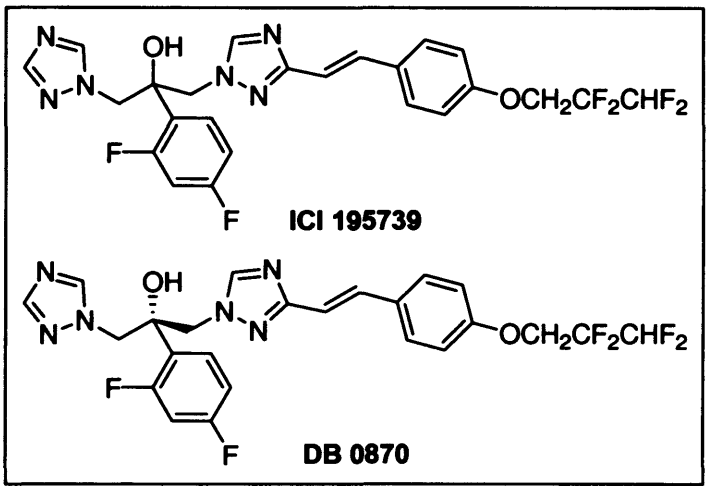

Figure II.12: Bis(triazole) ICI 195739 and (R)-D 0870

II.2.2 Post-zymosterol steps inhibitors.

As previously described, three enzymatic reactions do not have any direct counterpart in the mammalian sterol biosynthetic pathway (Figure II.13).

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[93] Liendo, A.; Lazardi, K.; Urbina, J. A. *In Vitro* Antiproliferative Effects and Mechanism of Action of the Bis-Triazole D 0870 and its S(-)Enantiomer Against *Trypanosoma cruzi*. *J. Antimicrob. Chemother.* **1998**, *41*, 197-205.

[94] Molina, J.; Brener, Z.; Romanha, A. J.; Urbina, J. A. *In Vivo* Activity of the Bis-Triazole D 0870 Against Drug-Susceptible and Drug-Resistant Strains of the Protozoan Parasite *Trypanosoma cruzi*. *J. Antimicrob. Chemother.* **2000**, *46*, 137-140.

[95] Molina, J.; Urbina, J.; Gref, R.; Brener, Z.; Rodriguez, J. M. Cure of Experimental Chagas' Disease by the Bis-Triazole D O870 Incorporated into 'Stealth' Polyethyleneglycol-polylactide Nanospheres. *J. Antimicrob. Chemother.* **2001**, *47*, 101-104.

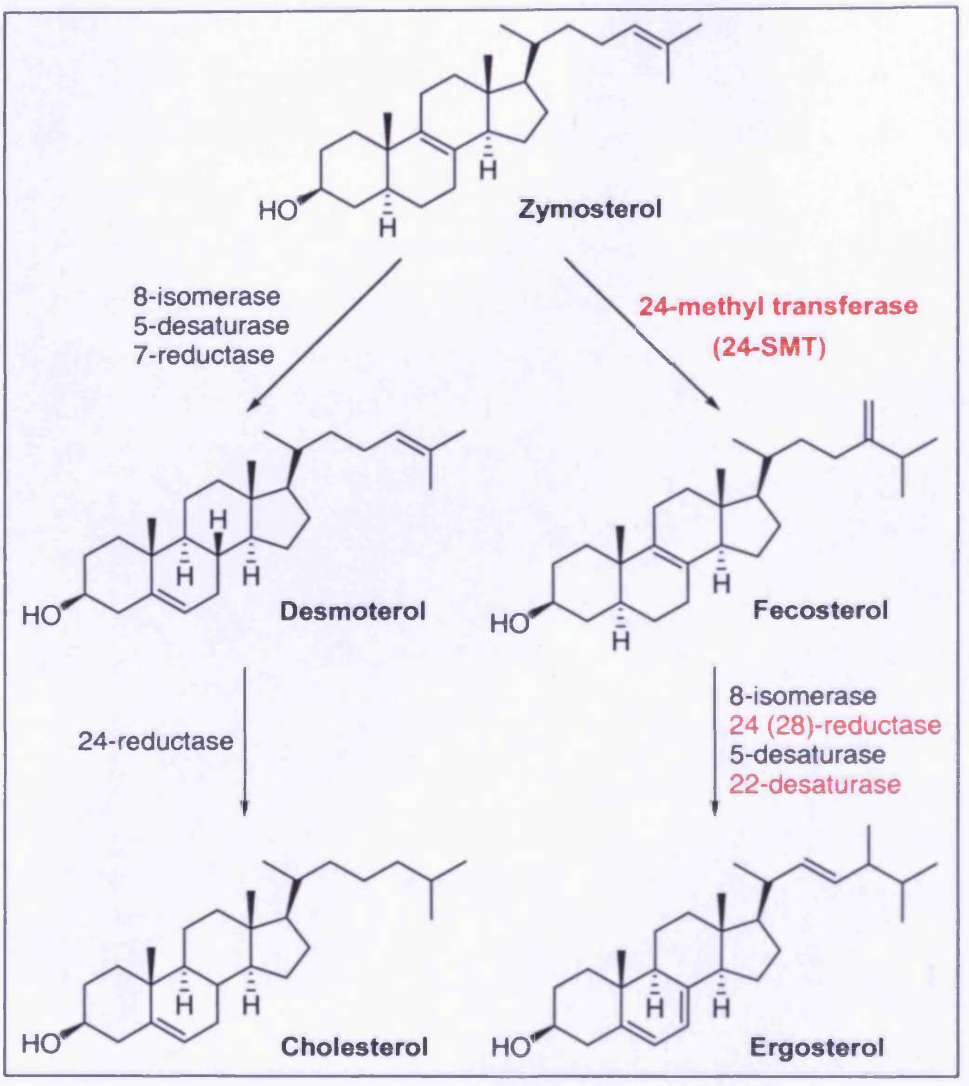

Figure II.13: Post-zymosterol enzymes

Therefore they constitute potential targets for new antiparasitic drugs since inhibitors of these enzymes should have no effect on a mammalian host.

Azasterols, sterols with nitrogen substitution in the side chain, have been the widest studied as inhibitors of 24-SMT. Some of them also showed activity against the 24(28)-reductase. No specific reductors have been found for the 22-desaturase yet[65].

II.3 Sterol 24-methyltransferase inhibition

Inhibition of the methenylation reaction at C-24 is the focus of this project. An outline of the enzyme and mechanism of action is presented in section II.3.1, before an outline of 24-SMT's inhibitors in section II.3.2.

II.3.1 The sterol 24-methyltransferase enzyme

24-Methyltransferase (24-SMT) is a membrane-bound protein present in plants and fungi. This enzyme has been partially purified in the late seventies from yeast (*Saccharomyces cerevisiae*)[96], and characterised in the late eighties from *Candida albicans* and *Candida tropicalis*[97], with an apparent molecular weight of approximately 150kD. The 24-SMT enzyme from *Saccharomyces cerevisiae* has been purified to homogeneity, and is a tetramer with a molecular weight of 172kD[98]. Unfortunately, to date, no 24-SMT has been crystallised; therefore, no three-dimensional structure is available for drug design.

In order to develop new drugs against the 24-SMT, we need to know which substrates are used by this enzyme and the overall mechanism of action used for the alkylation reaction. The preferred substrates in fungi are zymosterol and *S*-adenosyl-$_L$-methionine (SAM), while in plants the preferred sterol is cycloartenol (Figure II.14).

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[96] Moore, J. T., Jr.; Gaylor, J. L. Isolation and Purification of an S-Adenosylmethionine: Δ^{24}-Sterol Methyltransferase from Yeast. *J. Biol. Chem.* **1969**, *244*, 6334-6340.

[97] Ator, M. A.; Schmidt, S. J.; Adams, J. L.; Dolle, R. E. Mechanism and Inhibition of Δ^{24}-Sterol Methyltransferase from *Candida-Albicans* and *Candida-Tropicalis*. *Biochemistry* **1989**, *28*, 9633-9640.

[98] Nes, W. D.; Marshall, J. A.; Jia, Z. H.; Jaradat, T. T.; Song, Z. H.; Jayasimha, P. Active Site Mapping and Substrate Channeling in the Sterol Methyltransferase Pathway. *J. Biol. Chem.* **2002**, *277*, 42549-42556.

The features for sterol binding to the fungal enzyme (yeast) may not be significantly different from the features recognized by the plant enzyme[99], although two classes of 24-SMTs have been reported in plants[1,100].

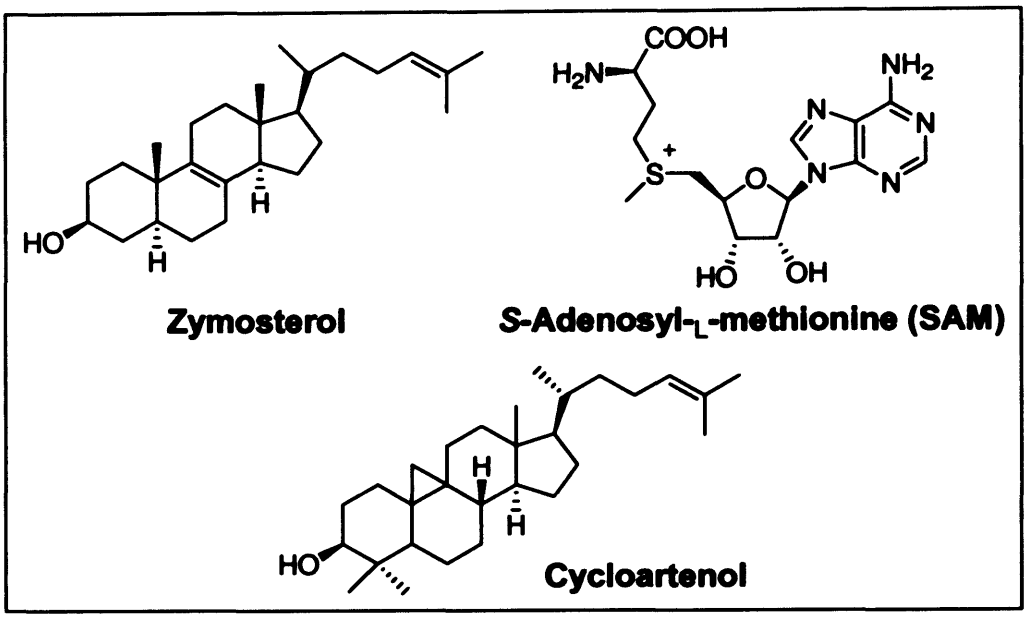

Figure II.14: Substrates of the 24-SMT in fungi and plants

Different classes of sterol methylation of inhibitors have been tested in order to determine the main features for the alkylation mechanism. The analysis led to an active-site model, referred to the "steric-electric plug" model[101]. The transfer of the methyl attached to the side chain proceeds by the formation of a 24-methyl carbonium ion intermediate[102]. Mechanistically this occurs by a nucleophilic attack by the π electrons of the Δ^{24} double bond on the *S*-methyl group of SAM as depicted in Figure II.15. The resulting bridged

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[99] Venkatramesh, M.; Guo, D.-a.; Jia, Z.; David Nes, W. Mechanism and Structural Requirements for Transformation of Substrates by the Methyl Transferase from *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1996**, *1299*, 313-324.

[100] Sitbon, F.; Jonsson, L. Sterol Composition and Growth of Transgenic Tobacco Plants Expressing Type-1 and Type-2 Sterol Methyltransferases. *Planta* **2001**, *212*, 568-572.

[101] Nes, W. D.; Guo, D. A.; Zhou, W. Substrate-Based Inhibitors of the (*S*)-Adenosyl-L-Methionine:$\Delta^{24(25)}$- to $\Delta^{24(28)}$-Sterol Methyl Transferase from *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **1997**, *342*, 68-81.

[102] Nes, W. D. Sterol Methyl Transferase: Enzymology and Inhibition. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids **2000**, 1529, 63-88.

carbonium ion intermediate gives rise to a nucleophilic rearrangement in which H-24 migrates to C-25. The introduction of ^{13}C nuclear magnetic resonance (NMR) spectroscopy has had a significant impact on the study of phytosterol biosynthesis, and support for this mechanism derived from ^{13}C-labeling studies[103].

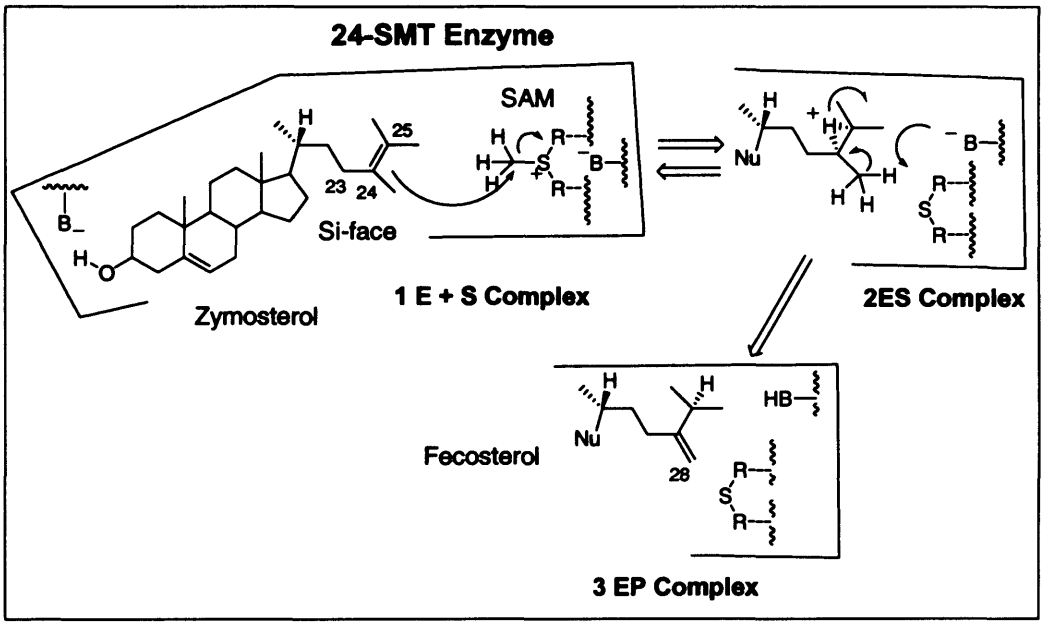

Figure II.15: Proposed methenylation mechanism by the 24-SMT enzyme

It has also been shown that in yeast[104] and fungi[105], the methyl transfer occurred via inversion of configuration, consistant with the nucleophilic attack happening from the Si-face followed by migration of the H across the opposite

[103] Tong, Y.; McCourt, B. S.; Guo, D. A.; Mangla, A. T.; Zhou, W. X.; Jenkins, M. D.; Zhou, W.; Lopez, M.; Nes, W. D. Stereochemical Features of C-Methylations on the Path to Δ $^{24(28)}$-Methylene and Δ$^{24(28)}$-Ethylidene Sterols: Studies on the Recombinant Phytosterol Methyl Transferase from *Arabidopsis thaliana*. *Tetrahedron Lett.* **1997**, *38*, 6115-6118.

[104] Nes, W. D.; Marshall, J. A.; Zhou, W.; He, L.; Dennis, A. L. Mechanism-Based Active Site Modification of Sterol Methyl Transferase by Tritium-Labeled 26-Homocholesta-8,14,24-trien- 26-yn-3beta-ol. *Tetrahedron Lett.* **1998**, *39*, 8575-8578.

[105] Marshall, J. A.; Nes, W. D. Isolation and Characterization of an Active-Site Peptide from a Sterol Methyl Transferase with a Mechanism-Based Inhibitor. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1533-1536.

Re-face to the carbon 25. Finally, the H on the carbon 28 is also lost from the Re-face[106].

From these studies, structural modifications of the substrates on SMT activity were assessed. Four domains of the sterol acceptor molecule were evaluated for their relevance to substrate acceptability[102,107] (Figure II.16).

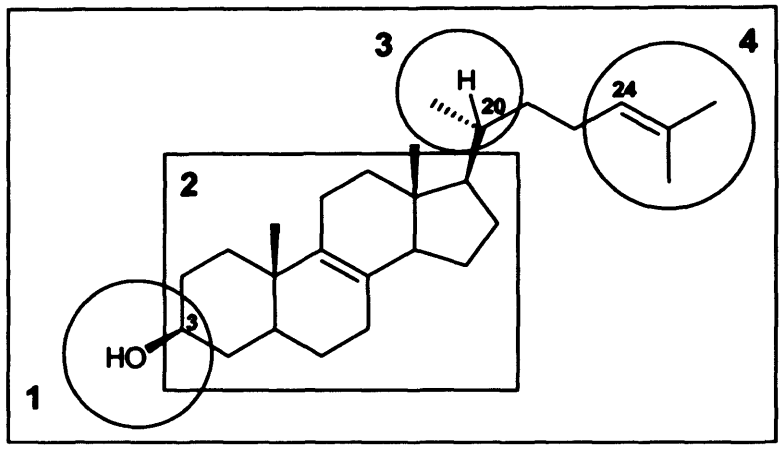

Figure II.16: Domains of sterol recognized by the 24-SMT

The principal determinants for binding were shown to be the 3 and 24 positions on the sterol, represented by domains 1 and 4. The absolute configuration at the carbon 20 also has an influence on the side chain and therefore on the nucleophilic attack occurring via the double bond (Domain 3). Finally, the nucleus has to be flat at initial binding which can be affected by displacement of the double bond in the nucleus (Domain 2).

II.3.2 Inhibitors of the 24-SMT

Carbocations mimic the high energy intermediate and have been shown to inhibit the enzyme. Therefore, isoelectronic and isosteric inhibitor intermediates formed in the enzymatic reaction are expected to bind tightly to

[106] Oehlschlager, A. C.; Angus, R. H.; Pierce, A. M.; Pierce, H. D.; Srinivasan, R. Azasterol Inhibition of Δ^{24}-Sterol Methyltransferase in *Saccharomyces-Cerevisiae*. *Biochemistry* **1984**, *23*, 3582-3589.

[107] Mangla, A. T.; Nes, W. D. Sterol C-Methyl Transferase from *Prototheca wickerhamii* Mechanism, Sterol Specificity and Inhibition. *Bioorg. Med. Chem.* **2000**, *8*, 925-936.

the enzyme through the same interactions as with SAM and zymosterol. An attractive strategy for the inhibition of the 24-SMT enzyme involves the design of sterol analogues of these highly reactive carbocation intermediates. Ammonium, sulphonium and arsonium analogues, which are positively charged, and derivatives which require protonation to yield a positive charge, such as amino or mercapto analogues, have been widely studied (Figure II.17; St: Sterol nucleus)[1,64,65,97,101,108,109,110,111,112,113].

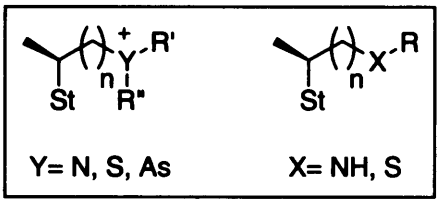

Figure II.17: Mimics of carbocation intermediates

The first synthesis of cholesterol analogues with an atom of nitrogen at positions 23, 24 or 25 of the side chain was carried out in the sixties as anti-hypocholesterolemic agents (Figure II.18)[114]. They were shown to inhibit the endogenous synthesis of cholesterol in rats and humans. This is probably due to inhibition of the 24-reductase. The minimal effective dose (MED) was the

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[108] Ator, M. A.; Schmidt, S. J.; Adams, J. L.; Dolle, R. E.; Kruse, L. I.; Frey, C. L.; Barone, J. M. Synthesis, Specificity, and Antifungal Activity of Inhibitors of the *Candida albicans* Δ^{24}-Sterol Methyltransferase. *J. Med. Chem.* **1992**, *35*, 100-106.

[109] Rahier, A.; Genot, J. C.; Schuber, F.; Benveniste, P.; Narula, A. S. Inhibition of *S*-Adenosyl-$_L$-Methionine Sterol-C-24- Methyltransferase by Analogs of a Carbocationic Ion High-Energy Intermediate - Structure Activity Relationships for C-25 Heteroatoms (N,As,S) Substituted Triterpenoid Derivatives. *J. Biol. Chem.* **1984**, *259*, 15215-15223.

[110] Rahman, M. D.; Seidel, H. M.; Pascal, R. A. Synthesis of 24-Heteroatom-Substituted Cholestanols. *J. Lipid Res.* **1988**, *29*, 1543-1548.

[111] Rahman, M. D.; Pascal, R. A. Inhibitors of Ergosterol Biosynthesis and Growth of the Trypanosomatid Protozoan *Crithidia fasciculata*. *J. Biol. Chem.* **1990**, *265*, 4989-4996.

[112] Urbina, J. A.; Vivas, J.; Lazardi, K.; Molina, J.; Payares, G.; Piras, M. M.; Piras, R. Antiproliferative Effects of $\Delta^{24(25)}$ Sterol Methyl Transferase Inhibitors on *Trypanosoma (Schizotrypanum) cruzi*: *In Vitro* and *in Vivo* Studies. *Chemotherapy* **1996**, *42*, 294-307.

[113] Pierce, J., H. D.; Pierce, A. M.; Srinivasan, R.; Unrau, A. M.; Oehlschlager, A. C. Azasterol Inhibitors in Yeast: Inhibition of the 24-Methylene Sterol $\Delta^{24(28)}$-Reductase and Δ^{24}-Sterol Methyltransferase of *Saccharomyces cerevisiae* by 23-Azacholesterol. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1978**, *529*, 429-437.

[114] Counsell, R. E.; Klimstra, P. D.; Nysted, L. N.; Ranney, R. E. Hypocholesterolemic Agents. V. Isomeric Azacholesterols. *J. Med. Chem.* **1965**, *8*, 45-48.

criterion of activity. Compounds having nitrogen at positions 20 and 22 resulted in no activity. On the other hand, successive substitution at positions 23, 24 and 25 progressively increased hypocholesterolemic activity. Clearly, the position of nitrogen in the side chain was already highlighted as important for the design of inhibitors. Further studies with 25-azasterol showed accumulation of desmosterol which was consistent with inhibition of the 24-sterol reductase.

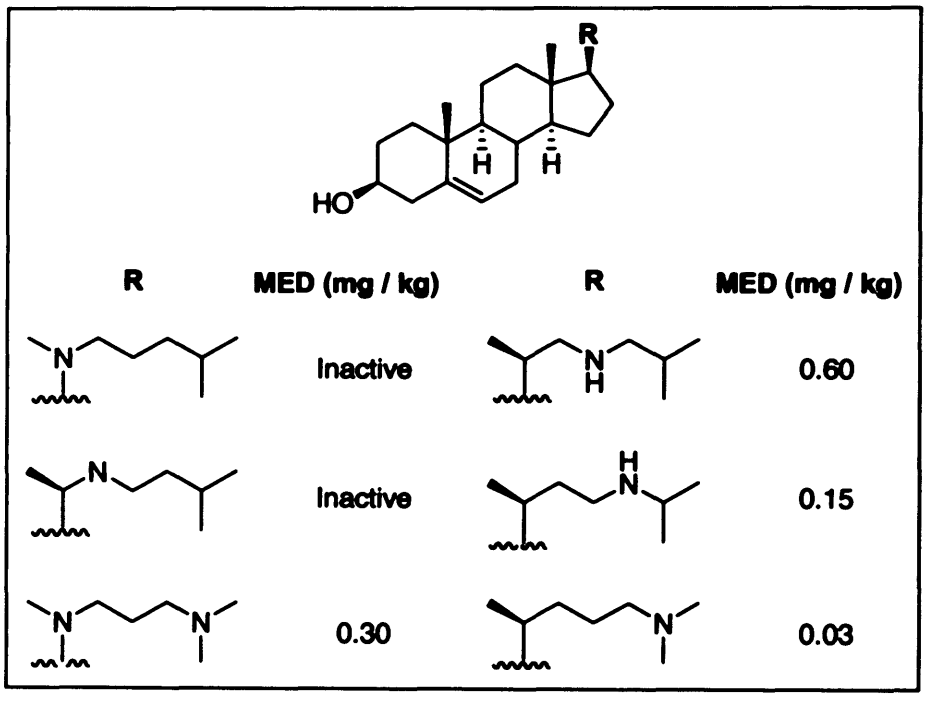

Figure II.18: Hypocholesterolemic inhibitors

A series of azacycloartanols and aza-cholesterols were tested against the 24-SMT enzyme isolated from higher plants[1,109]. The inhibition assays were conducted with the partially purified enzymes with cycloartenol, SAM and different concentration of inhibitors. The Ki constants are summarised in Figure II.19.

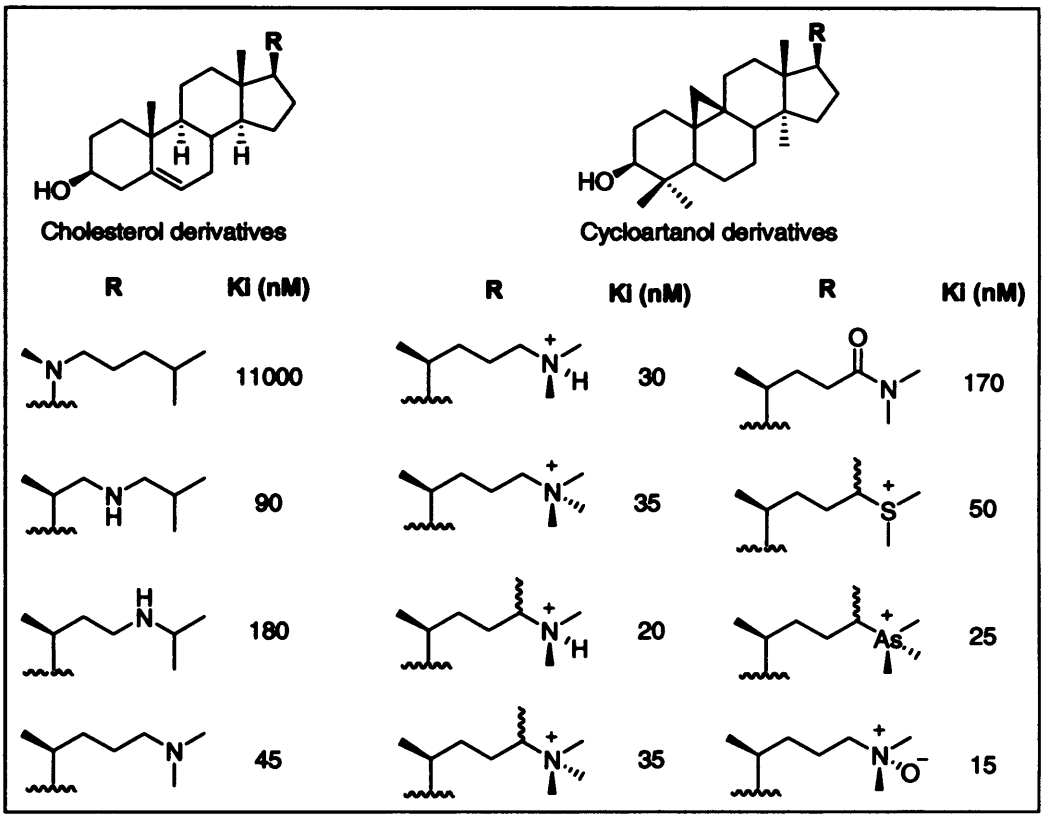

Figure II.19: Inhibitors of the 24-SMT enzyme isolated from higher plants

Again, the 25-azasterol was the most active of the series, which could agree with the bridged positive charge in the intermediate formed during the mechanism of the enzyme (Figure II.15). The 23-azacholesterol analogue was more active than the 24-azacholesterol, presumably due to the flexibility of the side chain. Tertiary amines and quaternary ammonium salts showed similar inhibition values. This is the evidence that tertiary amines are protonated at physiological pH. Derivatives with a hydrogen or a methyl group at position 24 also showed similar values. Sulfonium and arsonium sterols gave similar results to ammonium sterols. The N-oxide derivative, which has a dipolar moment with a strong positive charge on the nitrogen, was the most potent with a Ki of 15nM. It shows that the high energy intermediate carbocation is important in the inhibition mechanism.

The free hydroxyl group in position 3 of the sterol seemed to play an important role in the affinity of the substrate to the enzyme (Figure II.15), acting as a binding site. Nevertheless, the acetate derivative of the 25-

azacycloartanol proved to be as inhibitory as 25-azacycloartanol itself (Figure II.20)[109]. It has been previously reported[115] that the molecular features which determine the affinity of transition state analogues could be different from those affecting the recognition of the substrate in their ground state. So, the free hydroxyl group, which is absolutely required for the substrate to be transformed, might not be required for the inhibitor to be active.

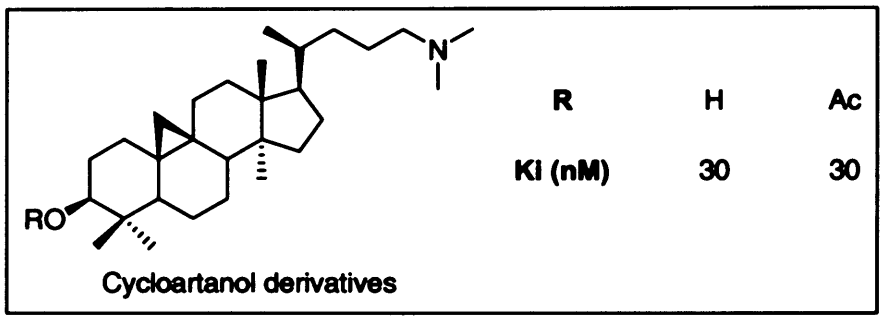

Figure II.20: Inhibitors of the 24-SMT enzyme isolated from higher plants

Oehlschlager *et al.* investigated the inhibition of the 24-SMT of yeast *Saccharomyces cerevisiae* with azasterols and sulfonium derivatives (Figure II.21)[64,106]. The IC_{50} values revealed that 25-azasterol was once again the more potent with a value of 0.14μM. Sulfonium cholesterol derivatives gave similar results as azasterols, while sulfonium cholestanol derivatives were less effective. By the analysis of the sterol composition, 23- and 24-azasterols were shown to also inhibit the 24(28)-reductase enzyme in yeast[106].

[115] Stein, R. L.; Elrod, J. P.; Schowen, R. L. Correlative Variations in Enzyme-Derived and Substrate-Derived Structures of Catalytic Transition-States - Implications for the Catalytic Strategy of Acyl-Transfer Enzymes. *J. Am. Chem. Soc.* **1983**, *105*, 2446-2452.

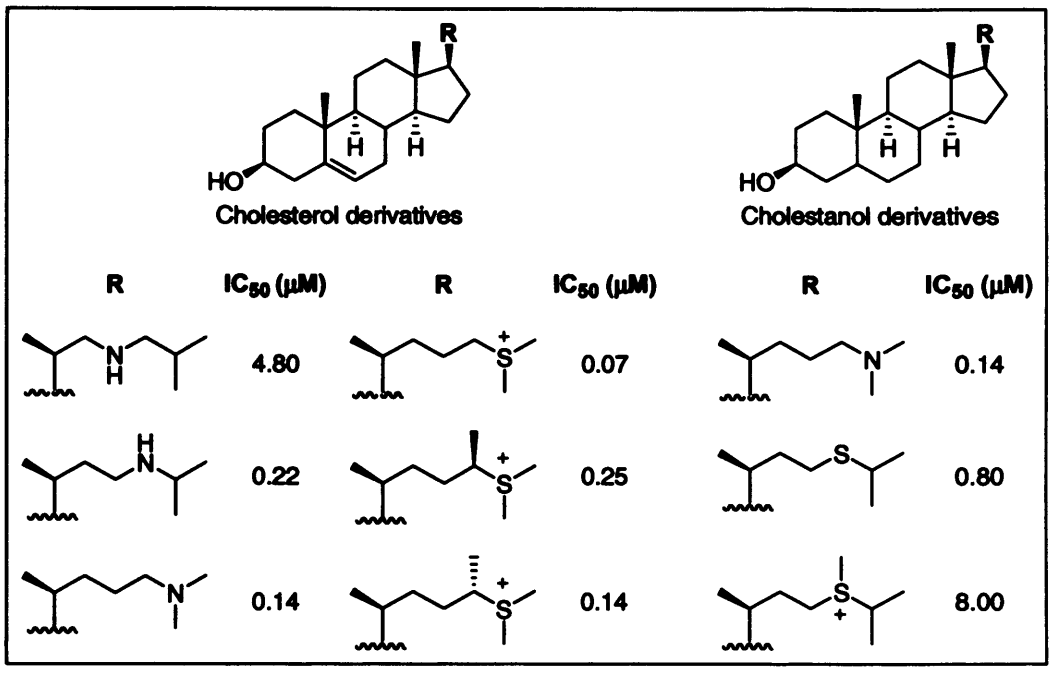

Figure II.21: Inhibitors of *S. cerevisiae* 24-SMT

The inhibition of the 24-SMT enzyme isolated from fungi was also studied using aza- and thio-cholesterols, and thio-, amidine- and imidazole-lanosterols[97,108]. The inhibition constants Ki were as shown Figure II.22. In the cholesterol analogues, derivatives with nitrogen or a sulphur atom gave the best activities, in the nanomolar range. Lanosterol was a poor substrate for the enzyme, and the 24-thiolanosterol derivative showed a poor activity of 72μM. On the other hand, in the presence of positively charged side chains, lanosterol derivatives showed good potency. In fact, the strong interactions between the charged side chain and the enzyme allowed the binding.

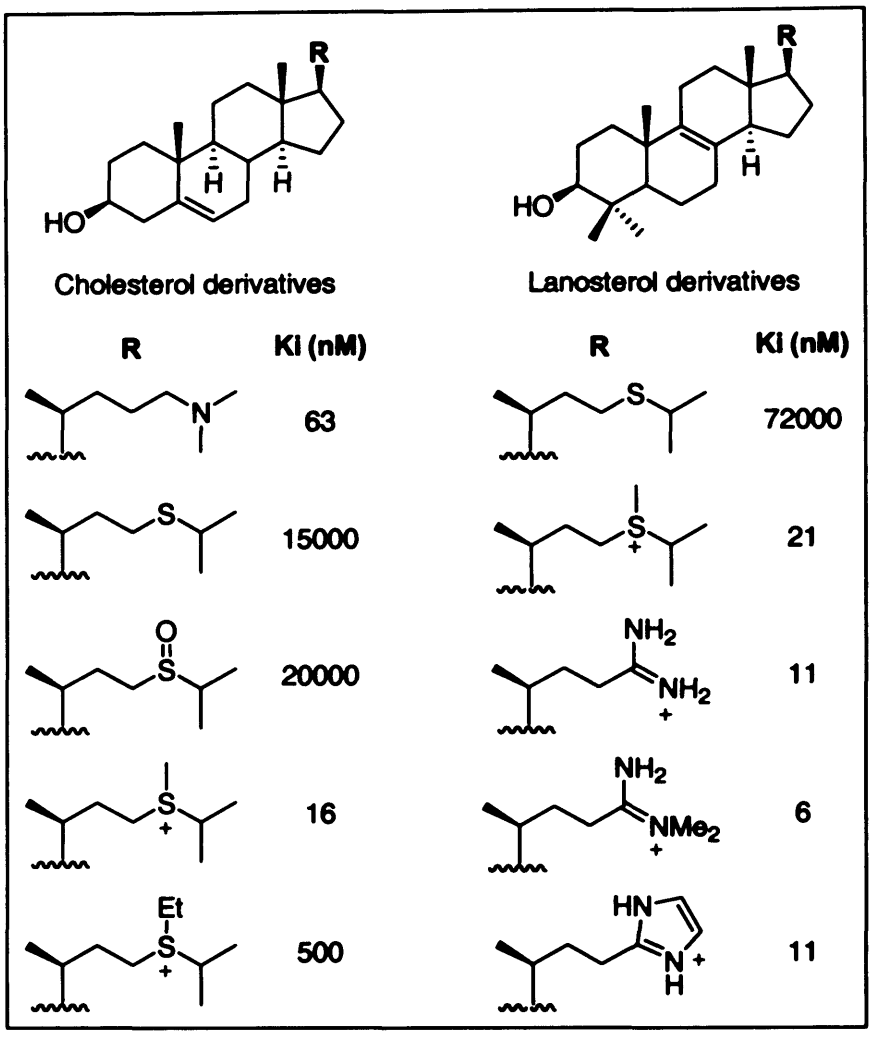

Figure II.22: Inhibitors of *Candida spp* 24-SMT

Different cholestanol derivatives have also been investigated against *Candida albicans*, (Figure II.23)[65]. Secondary and tertiary amines with a β-OH on carbon 20 gave the best inhibitions in the nanomolar range. The introduction of aromaticity via a pyridine group produced less activity compared to the piperidine derivatives. The removal of C-21 also led to a loss of activity. Finally, the natural product tomatidine showed a good growth inhibion with an IC_{50} of 98nM.

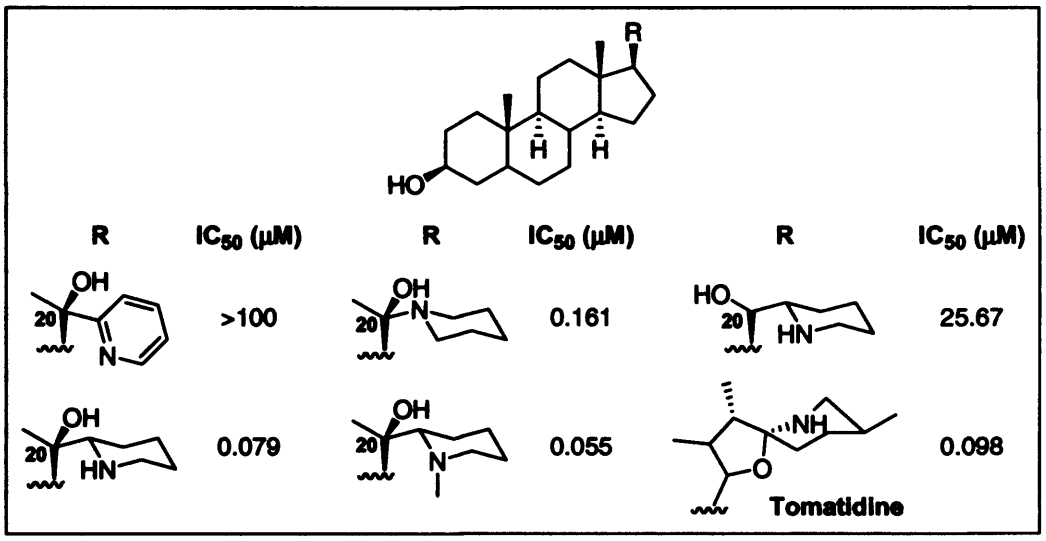

Figure II.23: Growth inhibitors of *Candida albicans*

Similar cholestanol derivatives (Figure II.24) were recently studied against another type of fungus, *Paracoccidioides brasiliensis*, responsible for human systemic mycosis in Latin America[116], and sensitive to sterol biosynthesis inhibitors. AZA-3 was shown to be the most potent of the three derivatives with a complete growth inhibition at 0.5μM. AZA-2 was the least active with 60% growth inhibition at 10μM. A composition analysis revealed that AZA-3 was acting as a 24-SMT inhibitor whereas AZA-1 and AZA-2 were acting as 24(28) reductase inhibitors.

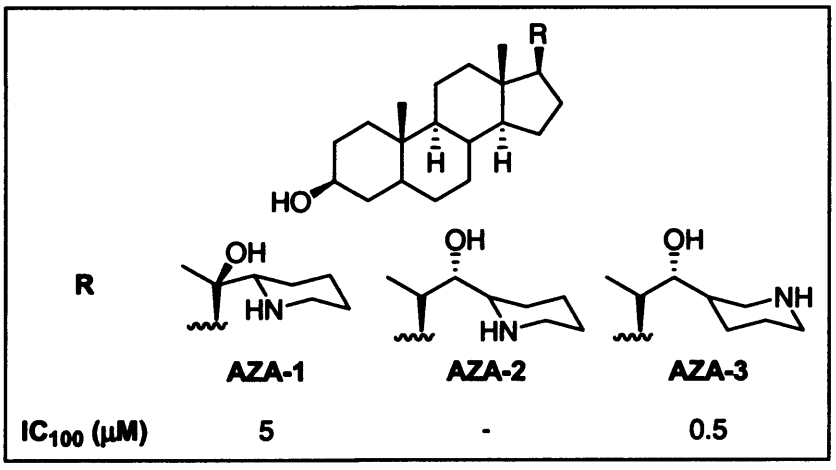

Figure II.24: Growth inhibitors of *Paracoccidioides brasiliensis*

[116] Visbal, G.; Alvarez, A.; Moreno, B.; San-Blas, G. *S*-adenosyl-L-Methionine Inhibitors Δ^{24}-Sterol Methyltransferase and $\Delta^{24(28)}$-Sterol Methylreductase as Possible Agents Against *Paracoccidioides brasiliensis*. *Antimicrob. Agents Chemother.* **2003**, *47*, 2966-2970.

Cholestanol derivatives (Figure II.25) were also synthesised and evaluated as inhibitors of ergosterol biosynthesis in the trypanosomatid protozoan *Crithidia fasciculate*[110,111]. They have been reported to be quite potent inhibitors with IC_{50}'s in the submicromolar range. The position of the nitrogen or sulphur atoms was again of importance. The 24-azasterol displayed the greatest activity while the 22-azasterol was 15 fold less active. The thiasterols were generally less active. 22- and 23 –Azasterols were active against the 24(28) reductase while 24-azasterol and 24-thiasterol were active against the 24-SMT suggesting that substitution at position 24 was preferred for higher activities.

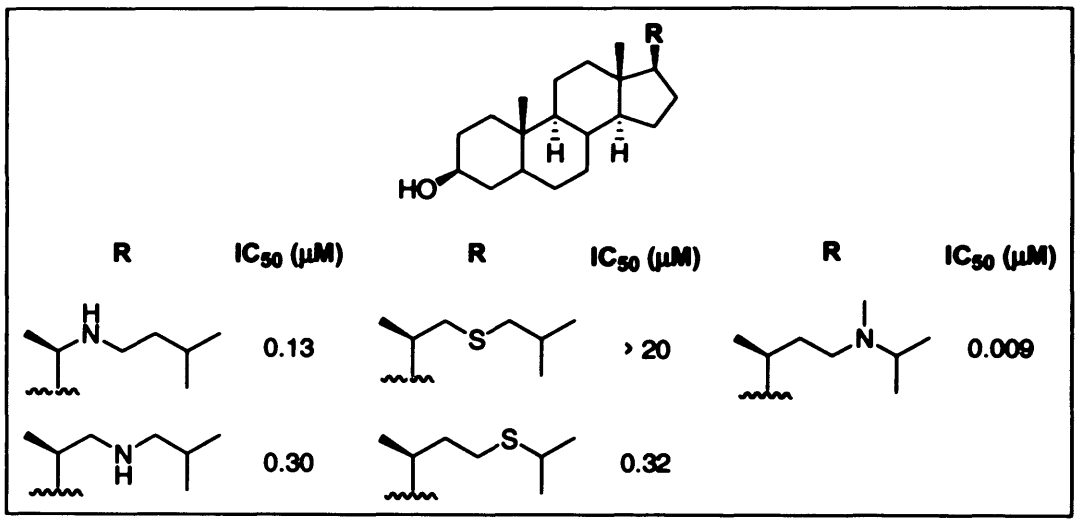

Figure II.25: Growth inhibition of *Crithidia fasciculata* by 24-SMT inhibitors

AZA-1 (Figure II.24) with a 6-membered aza ring as a side chain (22,26-azasterol or AZA-1) and 24-(R,S)-25-epiminolanosterol (EIL) have shown potent inhibitory properties *in vitro* and *in vivo* against the enzyme 24-SMT of *Trypanosoma cruzi* (Figure II.26)[112]. The 22,26-azacholestanol was tested *in vitro* against both the epimastigote and amastigote forms of the *T. cruzi*. The clinically relevant intracellular amastigote form was more affected at a concentration 100-fold lower than that required for the complete growth arrest of epimastigote. A comparable result was observed with EIL as a concentration 20-fold lower was necessary for a complete growth arrest of the amastigote form. *In vivo* studies with AZA-1 resulted in 100% survival 25 days

after treatment. Synergism was also observed when AZA-1 was used in combination with ketoconazole[112,117,118]. Survival levels were further increased which led to an almost total disappearance of circulating parasites, not observed using AZA-1 or ketoconazole alone.

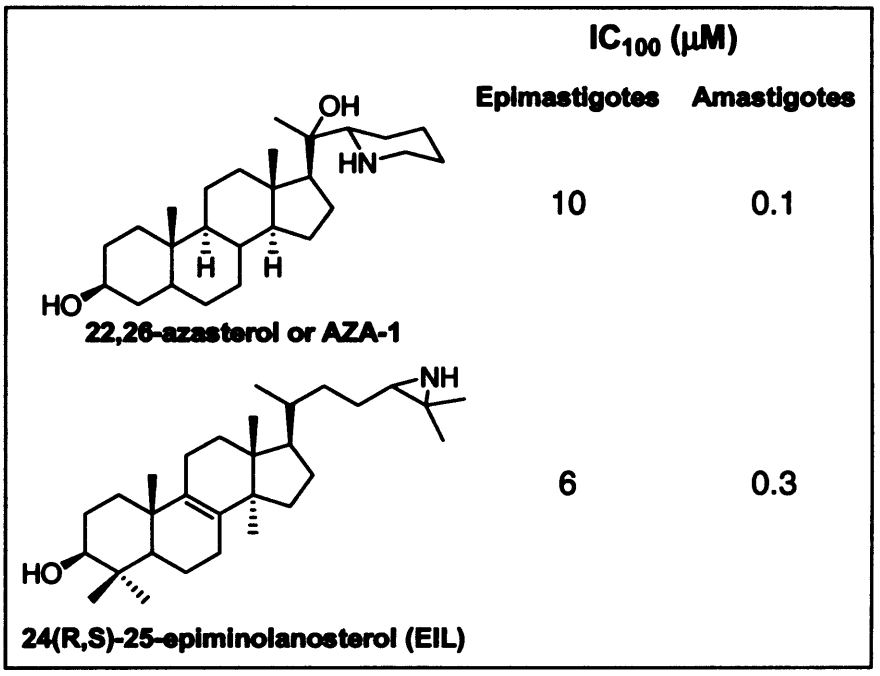

Figure II.26: Growth inhibitors of *T. cruzi*

22,26-Azasterol also showed good *in vitro* activities against both *L. amazonensis* and *T. cruzi*[119]. *L. donovani* promastigotes were also cultured in the presence of AZA-1[120]. Growth inhibition was gradually observed up to

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[117] Urbina, J. A.; Vivas, J.; Visbal, G.; Contreras, L. M. Modification of the Sterol Composition of *Trypanosoma (Schizotrypanum) cruzi* Epimastigotes by $\Delta^{24(25)}$-Sterol Methyl Transferase Inhibitors and Their Combinations with Ketoconazole. *Mol. Biochem. Parasitol.* **1995**, *73*, 199-210.

[118] Vivas, J.; Urbina, J. A.; deSouza, W. Ultrastructural Alterations in *Trypanosoma (Schizotrypanum) cruzi* Induced by $\Delta^{24(25)}$-Sterol Methyl Transferase Inhibitors and Their Combinations with Ketoconazole. *Int. J. Antimicrob. Agents* **1997**, *8*, 1-6.

[119] Magaraci, F.; Jimenez, C. J.; Rodrigues, C.; Rodrigues, J. C. F.; Braga, M. V.; Yardley, V.; de Luca-Fradley, K.; Croft, S. L.; de Souza, W.; Ruiz-Perez, L. M.; Urbina, J.; Pacanowska, D. G.; Gilbert, I. H. Azasterols as Inhibitors of Sterol 24-Methyltransferase in Leishmania Species and *Trypanosoma cruzi*. *J. Med. Chem.* **2003**, *46*, 4714-4727.

[120] Haughan, P. A.; Chance, M. L.; Goad, L. J. Effects of an Azasterol Inhibitor of Sterol 24-Transmethylation on Sterol Biosynthesis and Growth of *Leishmania donovani* Promastigotes. *Biochem. J.* **1995**, *308*, 31-38.

5μM. Concentrations exceeding 10μM were lethal. The ultrastructure effects of AZA-1 on both amastigote and epimastigote form of *L. amazonensis* showed that the growth inhibition was acting by alteration of the sterol compositions leading to cell lysis[121].

II.4 Summary

The sterol 24-methyltransferase (24-SMT) is a key enzyme in the parasite life cycle. Its inhibition leads to cell lysis and death of the parasite cells. As no counterpart exists in the sterol biosynthetic mammalian pathway, inhibitors designed for the 24-SMT should be selective, and not be active against the 24-reductase.

New sterol derivatives have been described in the literature as antiparasitic agents. The most promising seem to be azasterols bearing a nitrogen atom in the side chain, which can be protonated at physiological pH and therefore act as the high energy intermediate. An approach to inhibitors of 24-SMTs would be to design structures containing SAM moieties with suspected 24-SMT transition state inhibitors.

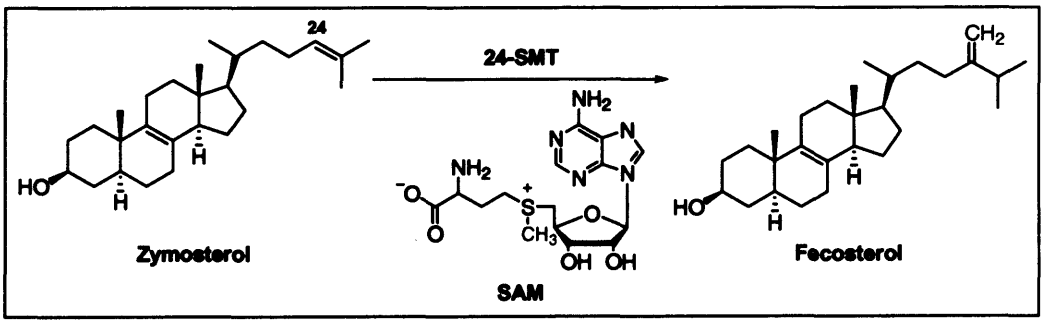
[121] Rodrigues, J. C. F.; Attias, M.; Rodriguez, C.; Urbina, J. A.; de Souza, W. Ultrastructural and Biochemical Alterations Induced by 22,26- Azasterol, a $\Delta^{24(25)}$-Sterol Methyltransferase Inhibitor, on Promastigote and Amastigote Forms of *Leishmania amazonensis*. *Antimicrob. Agents Chemother.* **2002**, *46*, 487-499.

CHAPTER III

AIMS AND OBJECTIVES

III.1 Synthesis of selective inhibitors of 24-SMT

The first aim of the project was to synthesize new targets for the inhibition of the 24-SMT enzyme. This enzyme catalyses the addition of a methyl group at position 24 on zymosterol (Scheme III.1). This is the first step toward ergosterol synthesis that does not have a counterpart in the mammalian pathway.



Scheme III.1: Fecosterol synthesis

The natural substrates are zymosterol and *S*-adenosyl-$_L$-methionine (SAM). The structure of the coenzyme factor SAM (Figure III.1) shows two distinct parts, a molecule of adenosine and the amino acid L-methionine.

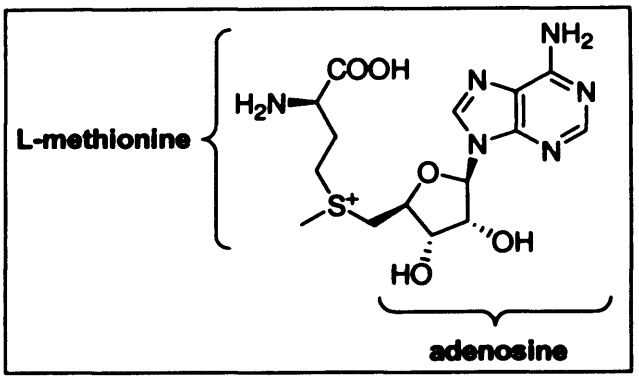


Figure III.1: *S*-adenosyl-$_L$-methionine (SAM)

We proposed to design novel inhibitors of 24-SMT by attaching SAM moieties to the sterol. These compounds should bind in both the sterol binding pocket and the SAM binding pocket. The SAM moieties could be attached to the sterol by either an amide or amine bond. The attachment of the whole structure of SAM has not been carried out because of the complexity of the synthetic route, which would lead to a large molecule which is not drug-like. Therefore, only moieties of SAM will be attached to azasterol derivatives. They should be selective against the 24-SMT and not affect the 24-reductase enzyme (Figure II.2) involved in the synthesis of cholesterol in humans, which does not have a SAM binding site. Functionalised inhibitors can be amide or amine derivatives (Figure III.2).

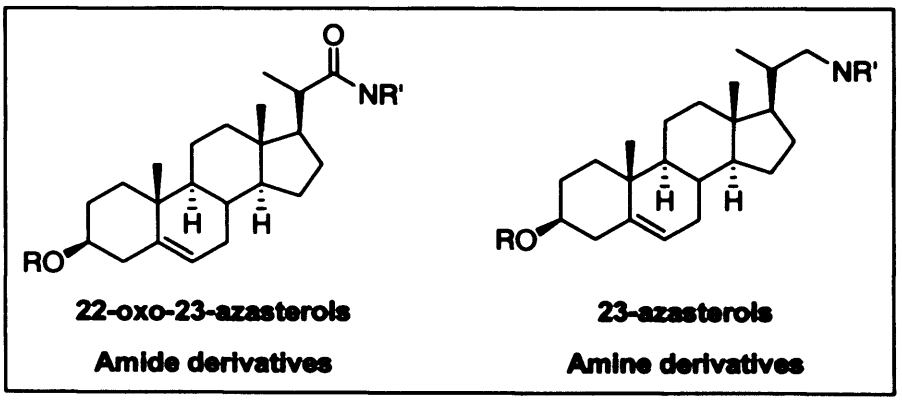
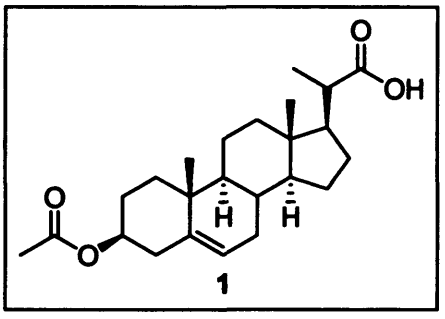


Figure III.2: Targets inhibitors

All the synthetic routes for the preparation of the sterols were undertaken using commercially available sterol 3β-acetoxy-23,24-bisnor-5-cholenic acid **1** (Figure III.3) for different reasons:

- The cost of drugs for parasitic diseases is a key question; where some sterol precursors are expensive, starting material **1** is relatively cheap;
- They are the easiest and more straightforward synthetic targets from compound **1** although N being in position 23 rather than 24 and 25;
- Synthetic studies using **1** have already been undertaken in this laboratory; the well-established chemistry led to compounds with *in vitro* activities.



1

Figure III.3: 3β -acetoxy-23, 24-bisnor- 5-cholenic acid **1**

The targets have been divided into two main groups (Figure III.2):

- 22-oxo-23-azasterols or amide derivatives;
- 23-azasterols or amine derivatives.

III.1.1 22-Oxo-23-azasterols or amide derivatives

The amide was selected as a potential target because some have been found to be inhibitors of plant/yeast 24-SMTs, although they do not have a full positive charge on this position (Figure II.17). Amide derivatives proposed here should bind to both sterol and SAM binding motifs. Different series of compounds will be prepared, with varying length of the side chain to optimise

the activity (Figure III.4). Position 3 could be either protected as an acetate group or free. At position 22, a carbonyl group will be present.

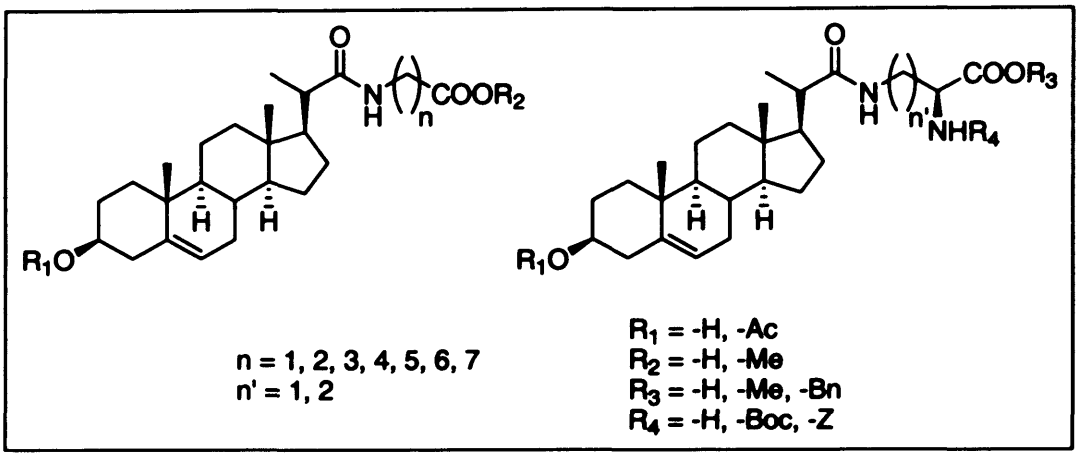


Figure III.4: Proposed amide derivative targets

Previously in this laboratory, derivatives with an amino function at the end of the side chain were synthesized (Figure III.5). This time, as side chains, series with a linear chain having a methyl ester or a carboxylic acid function will be prepared. Another series of compounds would be the attachment of α-amino acid moieties as side chains mimicking the L-methionine moiety of the SAM.

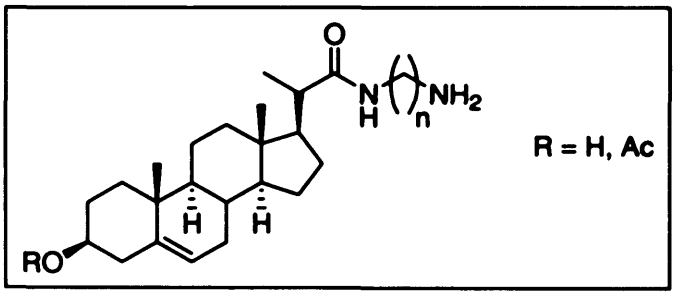


Figure III.5: Amide derivatives previously synthesized in this laboratory

The synthetic approach will be by activation of the carboxylic acid in the starting material 1 before coupling with the amino group of the side chain (Figure III.6).

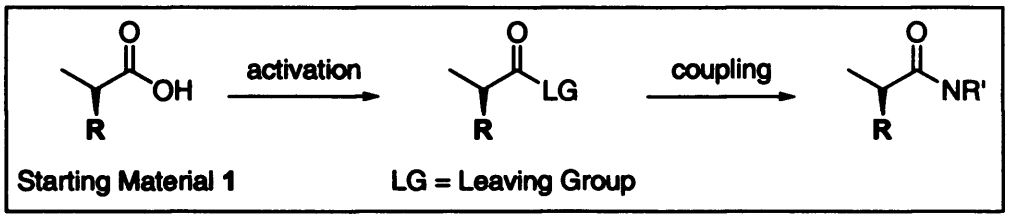


Figure III.6: Synthetic approach of the amide derivatives

III.1.2 23-Azasterols or amine derivatives

Amine derivatives (Figure III.2) will contain motifs which bind to both the sterol and SAM binding sites and also contain a positively charged side chain. Therefore, they have an additional interaction compared to amide derivatives and are expected to be more active. Similar side chains as for the amide derivatives will be attached (Figure III.7).

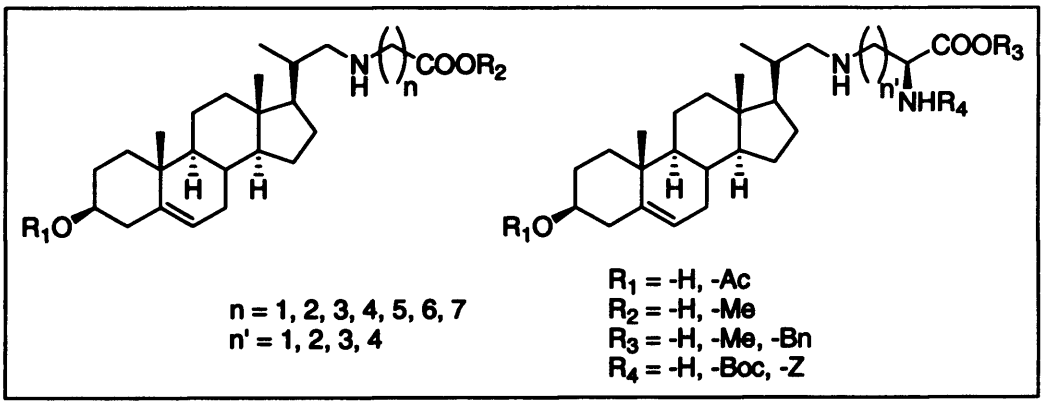


Figure III.7: Proposed amine derivative targets

For the formation of the amine derivatives, a retro synthetic route led to a nucleophilic substitution from a tosylate intermediate. The tosylate could be prepared from the alcohol, synthesised by reduction of the carboxylic acid in the starting material 1 (Figure III.8).

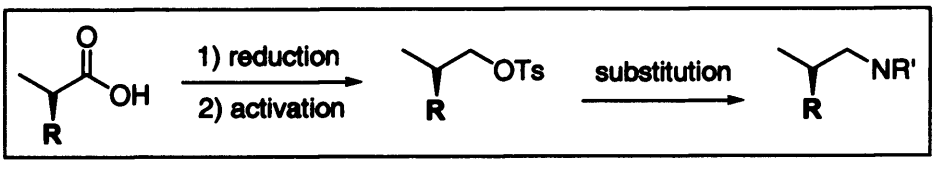


Figure III.8: Synthetic approach of the amine derivatives

III.2 Chemical proteomic approach

Studies during this project revealed that the mechanism of action of these compounds was not as simple as predicted. Chemical proteomics is a new technique which uses chemical probes to purify and identify proteins and to determine protein interaction and activity. In order to investigate the mode of action of the compounds, a chemical probe synthesis will be carried out using Biotin as a tag, and hexamethylenediamine or 6-aminocaproic acid as linkers (Figure III.9).

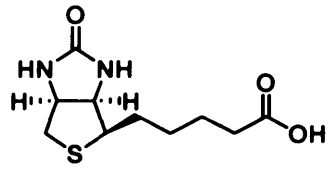
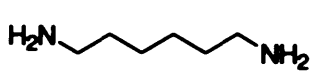
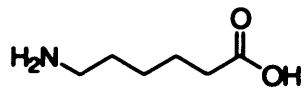
Tag	Linkers
	 Hexamethylenediamine  6-aminocaproic acid

Figure III.9: Tag and linkers proposed for the proteomic probe

The use of Biotin as a tag will allow the purification of the protein using affinity chromatography, whereas hexamethylenediamine and 6-aminocaproic acid will be the choice of linkers as they are believed to be long enough to minimize steric hindrance. The ligands used will be the azasterols showing good activity. Different points of attachment will be investigated.

III.3 Molecular biology and enzyme assays with the *T.b. brucei* 24-SMT

Biological evaluations showed that azasterols are active against blood stream form of the *T. brucei*. In this section, the aim was to investigate if 24-SMT is present in blood stream form of *T. brucei brucei* and if so to see if the azasterols inhibit the enzyme.

This work was carried out in the Instituto de Parasitologia y Biomedicina "Lopez-Neyra" in Granada (Spain) through a Marie-Curie

training fellowship. It concerned the over expression of the 24-SMT *T. brucei brucei* protein through different steps:

- Identify if transcription of the gene occurs in the blood stream form of *T.b. brucei*;
- Clone and over express the enzyme:
  - ✓ Identify the *T.b. brucei* 24-SMT DNA sequence;
  - ✓ Amplify and clone the *T.b. brucei* SMT DNA in an expression vector;
  - ✓ Introduce the resultant plasmid in *E. coli* cells for over expression;
  - ✓ Over express and purify the protein;
  - ✓ Measure the specific activity of the protein;
  - ✓ Carry out enzymatic assays with inhibitors against the recombinant *T.b. brucei* 24-SMT and also against the recombinant *L. major* 24-SMT, which had already been cloned and over expressed in Granada.
- Analysis of both procyclic and blood stream forms of the *T.b. brucei* parasite shapes in culture with azasterols by electron microscopy.

CHAPTER IV

SYNTHESIS OF THE AMIDE DERIVATIVES

Synthesis and inhibitory activity of azasterols against sterol 24-methyltransferase (24-SMT) has been discussed in Chapter II. Most of the inhibitors of 24-SMT reported have a positively charged group. Less research has been undertaken for amide derivatives as potential inhibitors of 24-SMT. There is an example in the literature of an amide derivative of cycloartanol, 24-

oxo-25-azacycloartanol, a sterol found in higher plants (Figure IV.1), which proved to be a potent inhibitor of the 24-SMT enzyme isolated from maize[109].

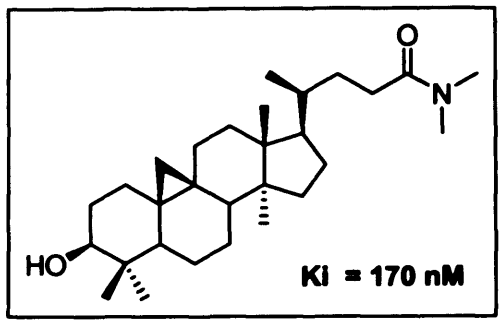


Figure IV.1: 24-Oxo-25-azacycloartanol

The activity of these compounds may be due to delocalisation of the carbonyl group leading to a partial positive charge on the nitrogen (Figure IV.2). This partial positive charge then perhaps acts as a mimic of the carbocation centre of the high energy intermediate.

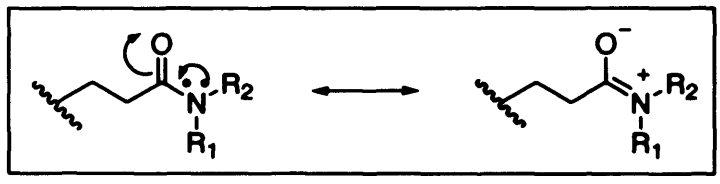


Figure IV.2: Polarisation of the carbonyl group on amide derivatives

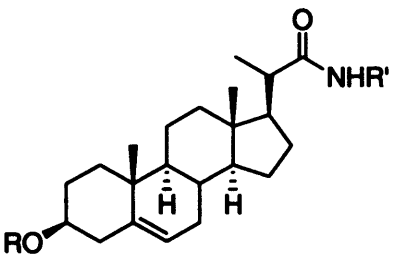
Interestingly, synthesis of squalamine analogues has been recently reported[122,123]. Squalamine is a novel sterol-spermidine conjugate that has been isolated from the stomach extracts of the dogfish shark which has been shown to have antimicrobial activity. Squalamine analogues with an amide function at position 22 have been designed and synthesised as a synthetic ionophore[124].

[122] Zhou, X. D.; Cai, F.; Zhou, W. S. A Stereoselective Synthesis of Squalamine. *Tetrahedron* **2002**, *58*, 10293-10299.

[123] Kim, H. S.; Choi, B. S.; Kwon, K. C.; Lee, S. O.; Kwak, H. J.; Lee, C. H. Synthesis and Antimicrobial Activity of Squalamine Analogue. *Bioorg. Med. Chem.* **2000**, *8*, 2059-2065.

[124] Merritt, M.; Lanier, M.; Deng, G.; Regen, S. L. Sterol-Polyamine Conjugates as Synthetic Ionophores. *J. Am. Chem. Soc.* **1998**, *120*, 8494-8501.

They were also recently tested against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei* strains (Table IV.1)[125].



R	R'	ED_{50} (μM)			
		L.dono.	*T.cruzi*	*T.brucei*	Tox.
Ac	$(CH_2)_3N(Boc)(CH_2)_4NHBoc$	17.3	>42	2.6	111
H	$(CH_2)_3N(Boc)(CH_2)_4NHBoc$	25.7	>15	4.7	65
H	$(CH_2)_3NH(CH_2)_4NH_2$	44.7	>63	1.9	3.3
Ac	$(CH_2)_3N(Boc)(CH_2)_4NBoc(CH_2)_3NHBoc$	4.7	>34	1.1	31
H	$(CH_2)_3N(Boc)(CH_2)_4NBoc(CH_2)_3NHBoc$	12.6	>36	0.60	27
H	$(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	5.0	>57	0.56	15

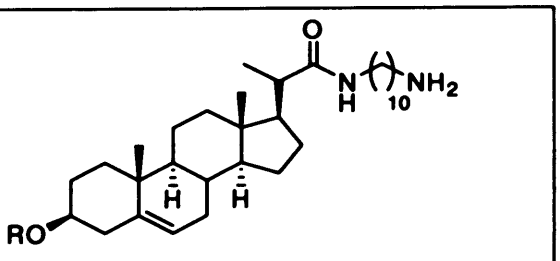
Table IV.1: ED_{50} values for the activity of squalamine analogues against trypanosomes and leishmania

The compounds showed the greatest activity against *T. brucei* with ED_{50} values close to 1μM. An *in vivo* study with the most active derivative led to no effect against *T.b. rhodesiense*, but a 16% reduction of parasite load in mice infected with *L. donovani*. It is possible these compounds could be inhibiting 24-SMT.

In this project, we proposed to investigate the effect of attaching SAM moieties to the sterol, via an amide bond. These compounds should have interaction with both the sterol and SAM binding sites. Some initial syntheses of novel amide derivatives (22-oxo-azasterol) were carried out previously in

[125] Khabnadideh, S.; Tan, C. L.; Croft, S. L.; Kendrick, H.; Yardley, V.; Gilbert, I. H. Squalamine Analogues as Potential Anti-Trypanosomal and Anti-Leishmanial Compounds. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1237-1239.

this laboratory (Table IV.2)[126,127,128]. Amide derivatives bearing an amino group at the end of the chain length were synthesised and tested. The derivatives with an acetate function at position 3 of the sterol were the most potent against *L. donovani* and *T. brucei*.



R	ED_{50} (μM)	
	L. donovani	***T. brucei***
Ac	10.63	2.52
H	16.11	› 20.00

Table IV.2: ED_{50} values for representative examples of amide derivatives

We proposed (Chapter III) to prepare amide derivatives in which the sterol nucleus was coupled to moieties of the SAM co-factor. In Figure III.4, target inhibitors are depicted, and reported synthetic routes were reinvestigated for their preparation.

[126] Lorente, S. O. Sterol 24-Methyltransferase Inhibitors as Potential Antimicrobials. *Thesis. Cardiff University* **2002**.

[127] Lorente, S. O.; Rodrigues, J. C. F.; Jimenez, C. J.; Joyce-Menekse, M.; Rodrigues, C.; Croft, S. L.; Yardley, V.; de Luca-Fradley, K.; Ruiz-Perez, L. M.; Urbina, J.; de Souza, W.; Pacanowska, D. G.; Gilbert, I. H. Novel Azasterols as Potential Agents for Treatment of Leishmaniasis and Trypanosomiasis. *Antimicrob. Agents Chemother.* **2004**, *48*, 2937-2950.

[128] Lorente, S. O.; Jimenez, C. J.; Gros, L.; Yardley, V.; de Luca-Fradley, K.; Croft, S. L.; Urbina, J.; Ruiz-Perez, L. M.; Pacanowska, D. G.; Gilbert, I. H. Preparation of Transition-State Analogues of Sterol 24-Methyl Transferase as Potential Anti-Parasitics. *Bioorganic & Medicinal Chemistry (in press)* **2005**.

IV.1 Preparation of the 3β-acetoxy-23,24-bisnor-5-en-22-(methyl ester alkyloate) amides (4)

In this work, the first series of compounds prepared were the amide derivatives with a linear chain (Figure IV.3). The major step of this synthesis, as described in Chapter III, should be a coupling reaction between the sterol 3β-acetoxy-23,24-bisnor-5-cholenic acid **1** (Figure III.3) and commercially available amino acids **2** (Figure IV.3).

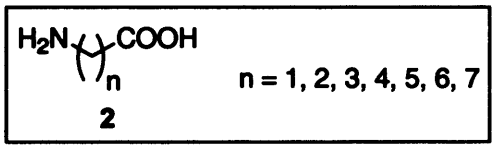


Figure IV.3: Amino acids **2** commercially available

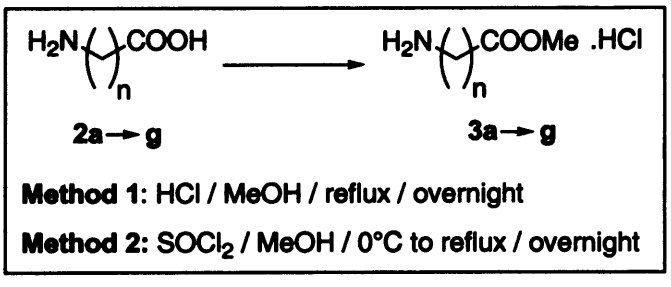
In order to simplify the following discussion, the different chain length will be denoted using letters as shown in Table IV.3.

n	1	2	3	4	5	6	7
Letter	a	b	c	d	e	f	g

Table IV.3: Lettering of the compounds

IV.1.1 Synthesis of the methyl amino esters (3)

Before undertaking the coupling reaction, the protection of the carboxylic acid function of the amino acids **2** was carried out (Scheme IV.1) to avoid a self-condensation of the amino acids during the coupling reaction.



Scheme IV.1: Synthesis of the methyl amino ester **3**

In the first method, the amino acid **2** was reacted with hydrochloric acid in methanol (solvent and reagent). This reaction was attempted with 6-aminocaproic acid **2e** (n=5), but did not occur and only starting material was recovered. The carboxylic acid was not sufficiently activated under this acidic condition and the esterification did not occur.

In the second method, as described in the literature[129,130], thionyl chloride was added to the amino acid **2** in methanol at 0°C, and then heated to reflux overnight. The methyl amino esters **3** were obtained in essentially quantitative yields (Table IV.4) as hydrochloride salts[131,132].

Compounds	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**	**3g**
Yields (%)	98	96	99	97	99	99	100

Table IV.4: Yields of the synthesis of the methyl amino esters **3**

IV.1.2 Synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(methyl ester alkyloate) amides (4)

The next step was the coupling between the methyl amino esters **3** and the carboxylic acid function of 3-acetoxy-23,24-bisnor-5-cholenic acid **1** (Scheme IV.2). A wide range of coupling agents used in peptide synthesis are available (Figure IV.4)[126,133,134,135,136,137].

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[129] Labaree, D. C.; Reynolds, T. Y.; Hochberg, R. B. Estradiol-16α-Carboxylic Acid Esters as Locally Active Estrogens. *J. Med. Chem.* **2001**, *44*, 1802-1814.

[130] Thottumkara, A. P.; Vinod, T. K. Synthesis and Oxidation Reactions of a User- and Eco-Friendly Hypervalent Iodine Reagent. *Tetrahedron Lett.* **2002**, *43*, 569-572.

[131] Soler, F.; Poujade, C.; Evers, M.; Carry, J.-C.; Henin, Y.; Bousseau, A.; Huet, T.; Pauwels, R.; De Clercq, E.; Mayaux, J.-F.; Le Pecq, J.-B.; Dereu, N. Betulinic Acid Derivatives: A New Class of Specific Inhibitors of Human Immunodeficiency Virus Type 1 Entry. *J. Med. Chem.* **1996**, *39*, 1069-1083.

[132] Humphlett, W. J.; Wilson, C. V. Formation and Properties of Isocyanates Derived from Amino Ester Hydrochlorides. *J. Org. Chem* **1961**, *26*, 2507-2510.

[133] Otoski, R. M.; Wilcox, C. S. An Approach to Lipophilic Nucleotide Phosphate Analogs - Synthesis of a Lipophilic Isostere of ATP. *Tetrahedron Lett.* **1988**, *29*, 2615-2618.

[134] Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. New Coupling Reagents in Peptide Chemistry. *Tetrahedron Lett.* **1989**, *30*, 1927-1930.

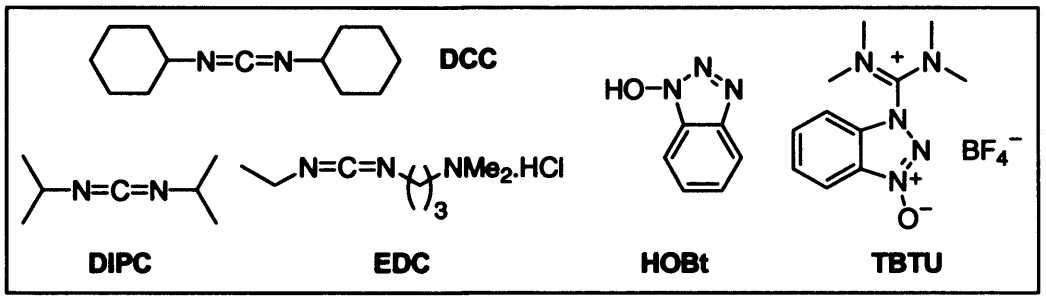
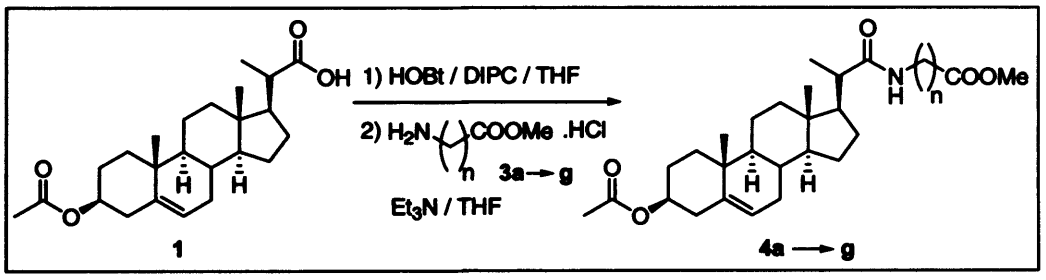


Figure IV.4: Coupling agents used in peptide synthesis

The coupling reaction was first attempted with DIPC and HOBt (Scheme IV.2). The carboxylic acid function of 3β-acetoxy-23, 24-bisnor-5-cholenic acid **1** was first activated with HOBt and DIPC for 30 minutes in THF (Solution A). At the same time, the methyl amino ester **3** was stirred with triethylamine in THF (Solution B). Then, Solution A was added to Solution B, and stirred overnight. After purification by column chromatography, a mixture of compound **4** and diisopropylurea **7** was obtained (Scheme IV.3).



Scheme IV.2: Synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(methyl ester alkyloate) amides **4**

The mechanism of the coupling reaction is depicted in Scheme IV.3. All compounds of the series were contaminated by diisopropyl urea (DIPU) **7**, 3 to 18% of the total weight of product obtained. Due to the complexicity of the ^{1}H NMR spectrum, this impurity was hard to detect. A further purification

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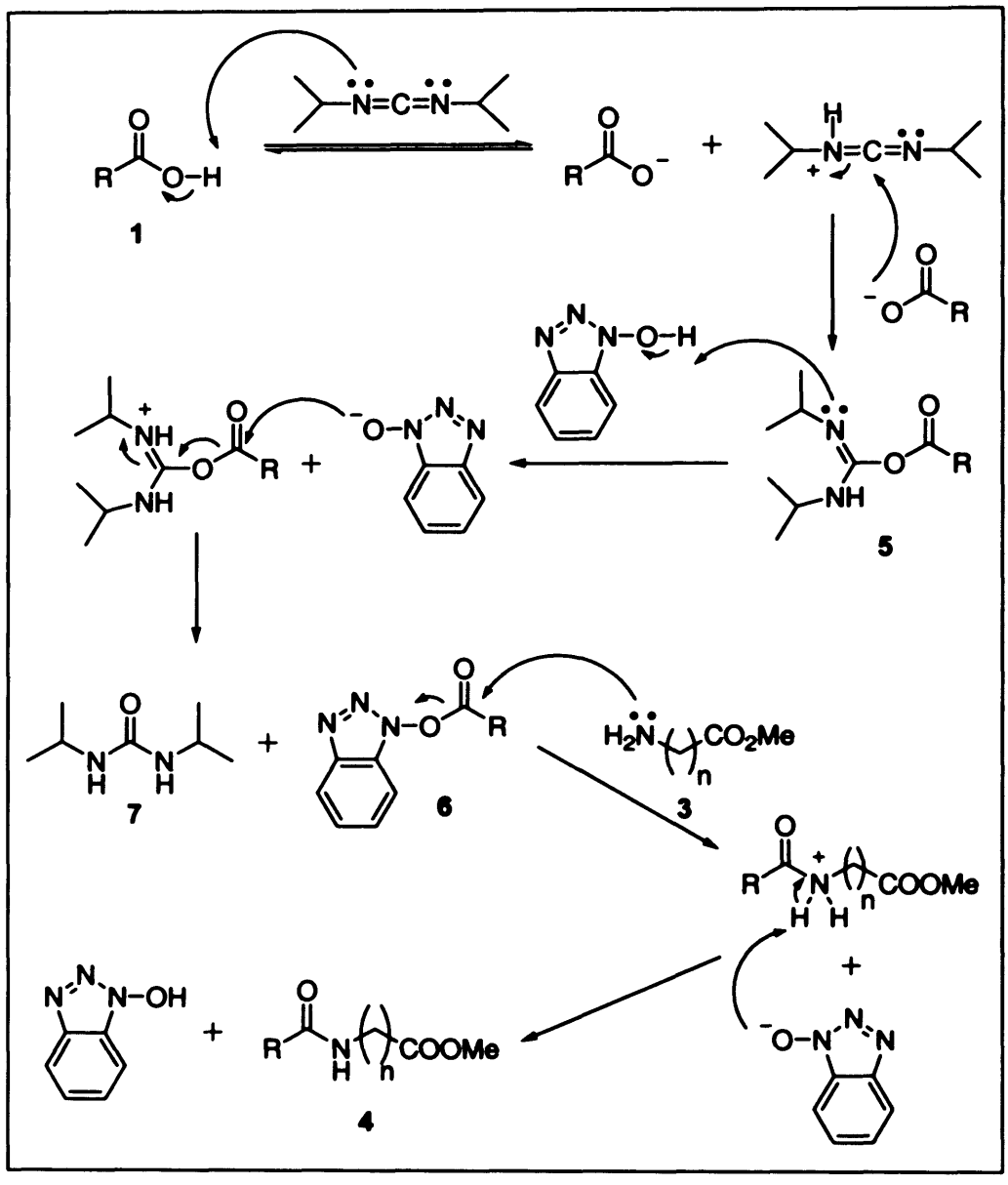
[135] Gilbert, I. H.; Rees, D. C.; Crockett, A. K.; Jones, R. C. F. Imidazolines as Amide Bond Replacements. *Tetrahedron* **1995**, *51*, 6315-6336.

[136] Bailey, P. D. An Introduction to Peptide Chemistry. *Wiley Medical Publication, Chichester A.* **1990**.

[137] Bodensky, M. Principles of Peptide Synthesis. *2nd Edition, Springler-Verlag, Berlin* **1993**.

by column chromatography did not give a pure product, presumably, the urea and the product co-run.

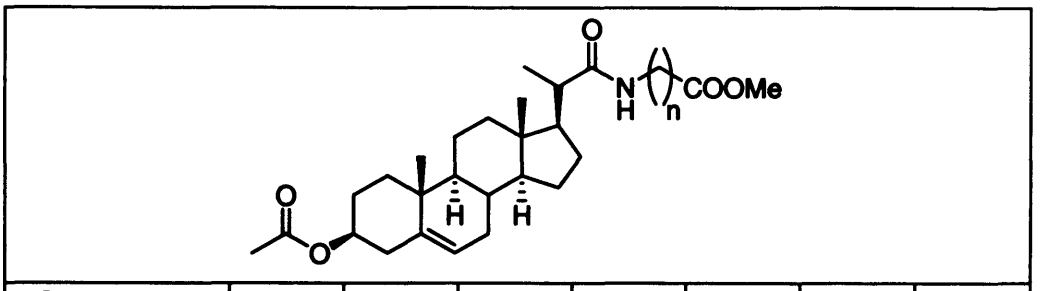


Scheme IV.3: Mechanism of the coupling reaction

In order to get compounds **4** pure, it was decided to change the coupling agent and use EDC instead of DIPC. The byproduct 1-(3-dimethylaminopropyl)-3-ethylurea (EDU) is water soluble[138] and so the mixture should be easier to purify. Yields are summarised in Table IV.5.

[138] Sheehan, J. C.; Cruickshank, P. A.; Broshart, G. L. A Convenient Synthesis of Water-Soluble Carbodiimides. *J. Org. Chem.* **1961**, *26*, 2525-2528.

Yields obtained using EDC were lower, 20-30%, but the compounds **4** were obtained pure which is essential for *in vitro* assays.

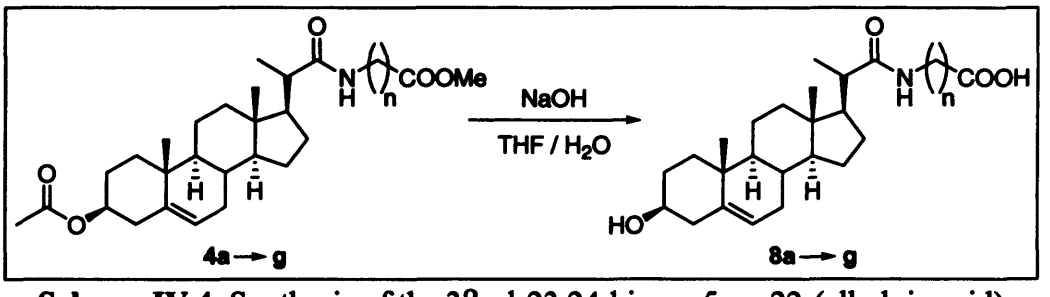


Compounds	4a	4b	4c	4d	4e	4f	4g
n	1	2	3	4	5	6	7
% with EDC	20	22	34	22	31	20	21

Table IV.5: Yields of the synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(methyl ester alkyloate) amides **4**

IV.2 Preparation of the 3β-ol-23,24-bisnor-5-en-22-(alkyloic acid) amides (8)

As previously mentioned, one objective of this thesis is to understand the exact role of the 3-position on the sterol. To investigate the necessity of the esters on the 3-OH and the carboxylic acid, they were removed by hydrolysis of the compounds **4** (Scheme IV.4).



Scheme IV.4: Synthesis of the 3β-ol-23,24-bisnor-5-en-22-(alkyloic acid) amides **8**

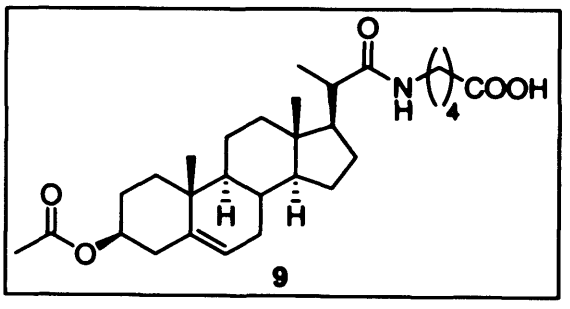
The hydrolysis was carried out with sodium hydroxide in a THF / H_2O (3:1) solution. According to literature precedents[130], this reaction should take

between 30 minutes and 1 hour to afford quantitative yields. Various reaction times were investigated (Table IV.6).

Entry	Compounds	Time (h)	Yield (%)
I	**7d**	1	0
II	**7e**	5	19
III	**7d**	o/n	8
IV	**7d**	24	53
V	**7d**	36	53

Table IV.6: First study of the synthesis of the 3β-ol-23,24-bisnor-5-en-22-(alkyloic acid) amide **8**

Entry I shows that after one hour reaction, there was no reaction and only starting material was recovered. Five hours reaction time (Entry II) gave 19% of the product **7e**, while an overnight reaction (Entry III) gave only 8% of the desired compound **7d**, 2% of starting material and 40% of the 3β-acetoxy-23,24-bisnor-5-en-22-(pentanoic acid) amide **9** (Figure IV.5). Then, the reaction time was increased to 24 or 36 hours and this finally gave 53% of compound **7d**, compound **9** (10mg, 10%) and starting material (Entry IV and V). Esters are generally labile to hydrolysis; therefore, the low yields obtained here are surprising.



9

Figure IV.5: 3β-Acetoxy-23,24-bisnor-5-en-22-(5-pentanoic acid) amide **9**

In order to improve the yield of the hydrolysis, several methods were investigated (Table IV.7):

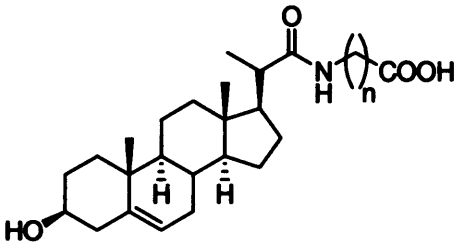
- LiOH / (THF / H_2O)[139];
- KOH / $(CH_2OH)_2$.

Reagent	Compound	Time (h)	Δ (°C)	Yield (%)
LiOH	**8e**	24	50	0
KOH / $(CH_2OH)_2$	**8d**	o/n	60	0

Table IV.7: Second study of the synthesis of the 3β-ol-23, 24-bisnor-5-en-22-(alkyloic acid) amides **8**

Lithium hydroxide is a weaker base than sodium hydroxide and was also investigated. Unfortunately, after 24h, an unidentified compound (not a sterol) was recovered. The use of potassium hydroxide with ethylene glycol did not work either, and only starting material was recovered.

It was decided to revisit the use of sodium hydroxide, which at least led to the desired compound even if the yield was low. A longer reaction time of 48h finally gave a nearly quantitative yield for the whole series (Table IV.8).



Compound	8a	8b	8c	8d	8e	8f	8g
n	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Yield (%)	77	82	85	95	81	86	81

Table IV.8: Yields of the synthesis of the 3β-ol-23,24-bisnor-5-en-22-(alkyloic acid) amides **8**

[139] Evans, D. A.; Ellman, J. A.; Dorow, R. L. Asymmetric Halogenation of Chiral Imide Enolates - A General Approach to the Synthesis of Enantiomerically Pure α-Amino-Acids. *Tetrahedron Lett.* **1987**, *28*, 1123-1126.

Increasing the reaction time further, a decrease of the yield was observed due to decomposition of product (Table IV.9).

Compound	Time (h)	Yield (%)
8g	120	69

Table IV.9: Yield of the synthesis of the 3β-ol-23,24-bisnor-5-en-22-(octanoic acid) amide **8g**

IV.3 Preparation of the 3β-acetoxy-23,24-bisnor-5-en-22-(α-amino acid alkyl) amides (14)

The last series of amide derivatives synthesised were compounds with an amino acid function at the end of the chain. We explained earlier in the introduction that these sterol analogues should mimic the natural product *S*-adenosyl-$_L$-methionine (SAM). Figure III.1 in Chapter III shows that SAM has an α-amino acid function at the end of the chain. Thus, the introduction of such a function on the sterol is expected to give more efficient inhibitors of the 24-SMT than the earlier series.

The introduction of this moiety should be achieved by a coupling reaction between 3-acetoxy-23,24-bisnor-5-cholenic acid **1** and different α-amino acid derivatives with an amine in the side chain (Figure IV.6).

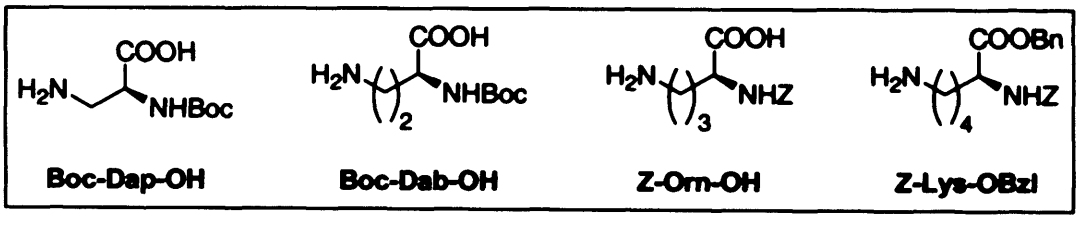
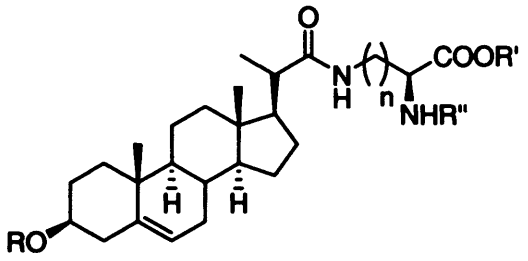


Figure IV.6: α-Amino acids commercially available

In these laboratories, the derivatives with Z-Orn-OH and Z-Lys-OBzl moieties have already been prepared in 55% and 80% yields respectively. The deprotections of the 3 position as well as the amino acid functions were also undertaken (Table IV.10)[126].



n	R	R'	R "	Yields (%)
3	Ac	H	Z	55
3	H	H	Z	30
3	H	H	H	66
4	Ac	Bn	Z	80
4	H	H	Z	36
4	H	H	H	35

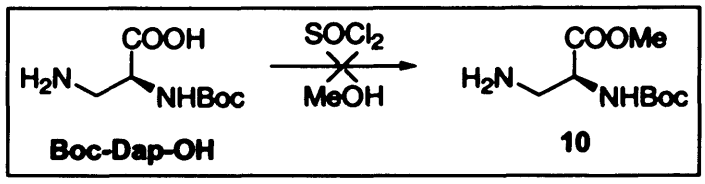
Table IV.10: Z-Orn-OH and Z-Lys-OBzl derivatives

The aim was to complete the series starting with the N-α-Boc-L-2,3-diaminopropionic acid (Boc-Dap-OH) and L-2,4-diaminobutyric acid dihydrochloride (Boc-Dab-OH). First of all, protection of the acid functions were attempted in order to reduce the likelihood of self-condensation during the coupling reaction, and also improve the lipophilicity of the compounds and hence their solubility and capacity to cross cell membranes.

The hydrophobic character of a compound can be a key attribute in determining its interaction with various proteins and tissues in the body. The hydrophobicity of a compound can also have profound effects on cellular permeability and bioavailability. Log D gives information on the solubility properties of a compound and should be around 2-5. Compounds need to have a balance in solubility in aqueous and lipid environments. Solubility in aqueous environment is required for blood plasma levels and transport around the body. Whilst solubility in lipids is required for transport across membranes. Protections of the acid functions would lead in that case to compounds with a Log D close to 6 which may be an acceptable value. These protecting groups can then be removed *in situ* by esterase enzymes.

IV.3.1 Attempted protections of Boc-Dap-OH

The attempted protection of the acid function of Boc-Dap-OH as a methyl ester was first carried out using thionyl chloride in methanol as previously described[129,136] (Scheme IV.5). However, on heating overnight, removal of the Boc group occurred giving compound **11** (Figure IV.7); presumably deprotection occurred due to HCl generated during the reaction.



Scheme IV.5: Protection of Boc-Dap-OH

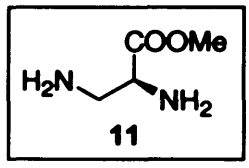
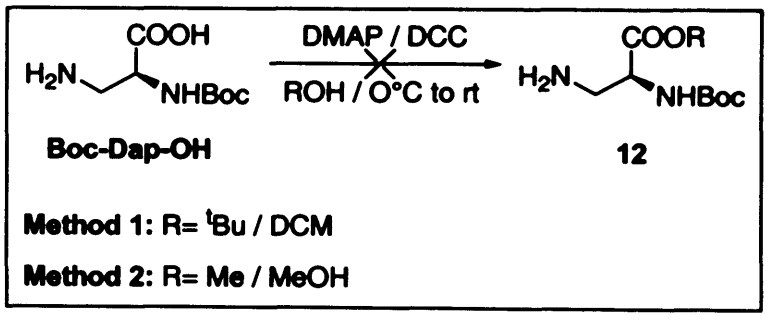


Figure IV.7: Byproduct **11**

A second attempt was tried working at room temperature, but the deprotection of the Boc group on the amine happened again to afford the byproduct **11**.

Another method to transform an acid function into an ester is to perform a coupling reaction between an acid and an alcohol[140] (Scheme IV.6).

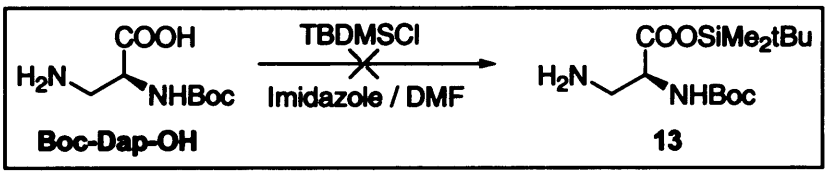


Scheme IV.6: Attempted protection of Boc-Dap-OH

[140] Corey, E. J.; Raju, N. A New General Synthetic Route to Bridged Carboxylic Ortho Esters. *Tetrahedron Lett.* **1983**, *24*, 5571-5574.

In method 1, *tert*-butanol was used as the alcohol and added to DCM. The reaction failed and only starting material was recovered. A problem of solubility of the starting material might be the reason for this failure. In the second method, methanol was used as reagent and solvent, but the reaction did not work and only starting material was recovered. The general procedure for this type of reaction suggests adding the alcohol last, which has not been done and might be the reason for failure.

After those failed attempts, *tert*-butyldimethylsilyl chloride ($tBuMe_2SiCl$) as a protecting group was used (Scheme IV.7)[141]. It has been demonstrated that the *tert*-butyldimethylsilyloxy group is 10^4 times more stable to basic conditions than the trimethylsilyloxy group as ether and this strategy has been applied to acid function protection[142]. Moreover, it is relatively sensitive to acidic conditions; therefore, its deprotection could follow the same procedure as for the Boc group, and TFA would allow the deprotection of both.



Scheme III.7: Attempted protection of Boc-Dap-OH by TBDMS

This reaction was shown to be really slow using pyridine in THF while the use of imidazole in DMF proved to be more effective in the conversion of a variety of alcohols to *tert*-butyldimethylsilyl ethers in high yield under mild conditions. *Tert*-butyldimethylsilyl ethers are also stable in aqueous solution (saponification) and under hydrogenolysis.

This protection was carried out overnight, and a light less polar spot appeared suggesting success of the reaction. Unfortunately, the compound 13 was not recovered after column chromatography, only starting material. The acidity of the silica might have been enough to deprotect compound 13 or little

[141] Corey, E. J.; Venkateswarlu, A. Protection of Hydroxyl Groups as *tert*-Butyldimethylsilyl Derivatives. *J. Am. Chem. Soc.* **1972**, *94*, 6190-6191.

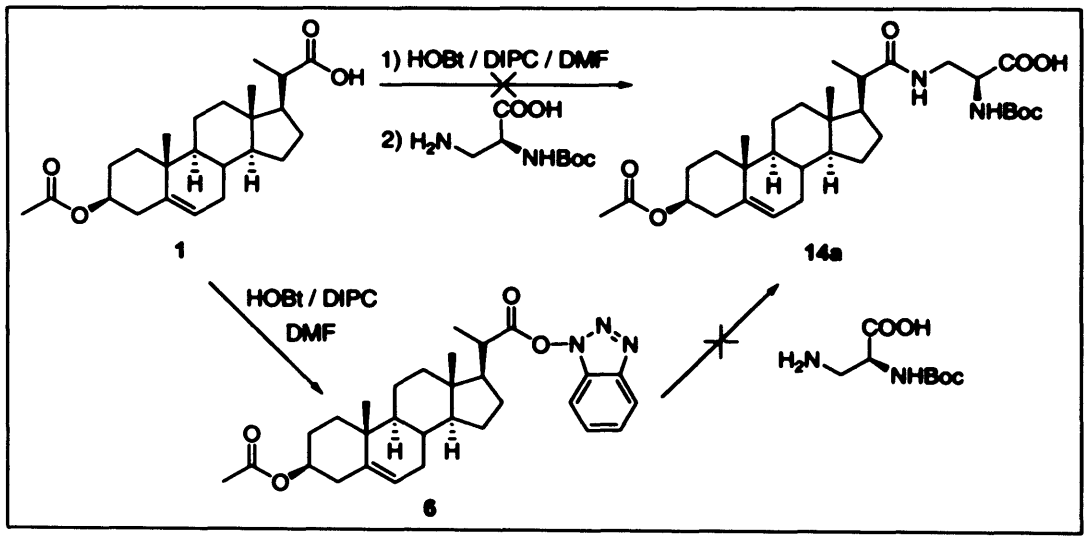
[142] Greene, T. W.; Wuts, P. G. M. Protective Groups in Organic Synthesis, 3rd Edition. *John Wiley & Sons, Inc., UK* **1999**.

product was produced and it was too insignificant to be detected after column chromatography.

Thus the protection of the acid function of the Boc-Dap-OH could not be achieved. Therefore it was decided to continue using Boc-Dap-OH and Boc-Dab-OH without protection of the acid functionality.

IV.3.2 Synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(α-amino acid alkyl) amides (14)

We previously reported in Section IV.1.2 the successful coupling reaction between the carboxylic acid function of 3-acetoxy-23,24-bisnor-5-cholenic acid **1** and the methyl amino esters **3**. It was thought the synthesis of compounds **14** could be achieved by the same method, using DIPC as a base and HOBt as a coupling activating agent. This was attempted with starting material **1** and with the amino acid Boc-Dap-OH (Scheme IV.8).

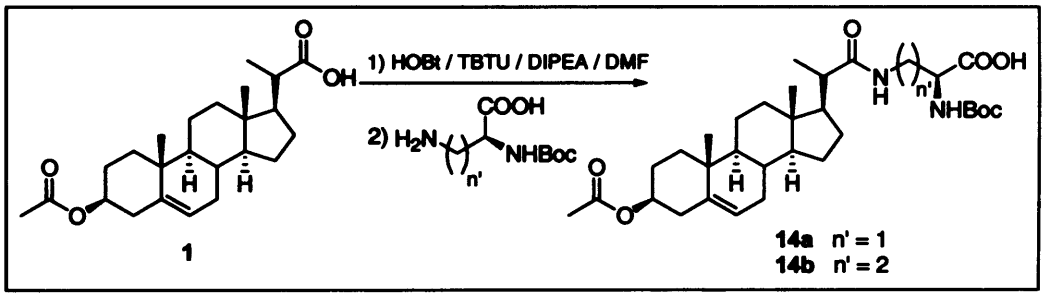


Scheme IV.8: Coupling reaction with Boc-Dap-OH

Unfortunately compound **14a** was not obtained after overnight reaction, and after column chromatography. The analyses by NMR and MS showed only the formation of the intermediate activated ester **6** with a yield of 33% (Scheme

IV.8). Starting material was also recovered after column chromatography. Boc-Dap-OH did not react and was lost in the water layer during the extraction. This failure can be explained by the presence of the Boc-Dap-OH in solution as a zwitterion. Thus, no nucleophilic attack can occur; repeating the reaction in basic conditions might give formation of **14a**.

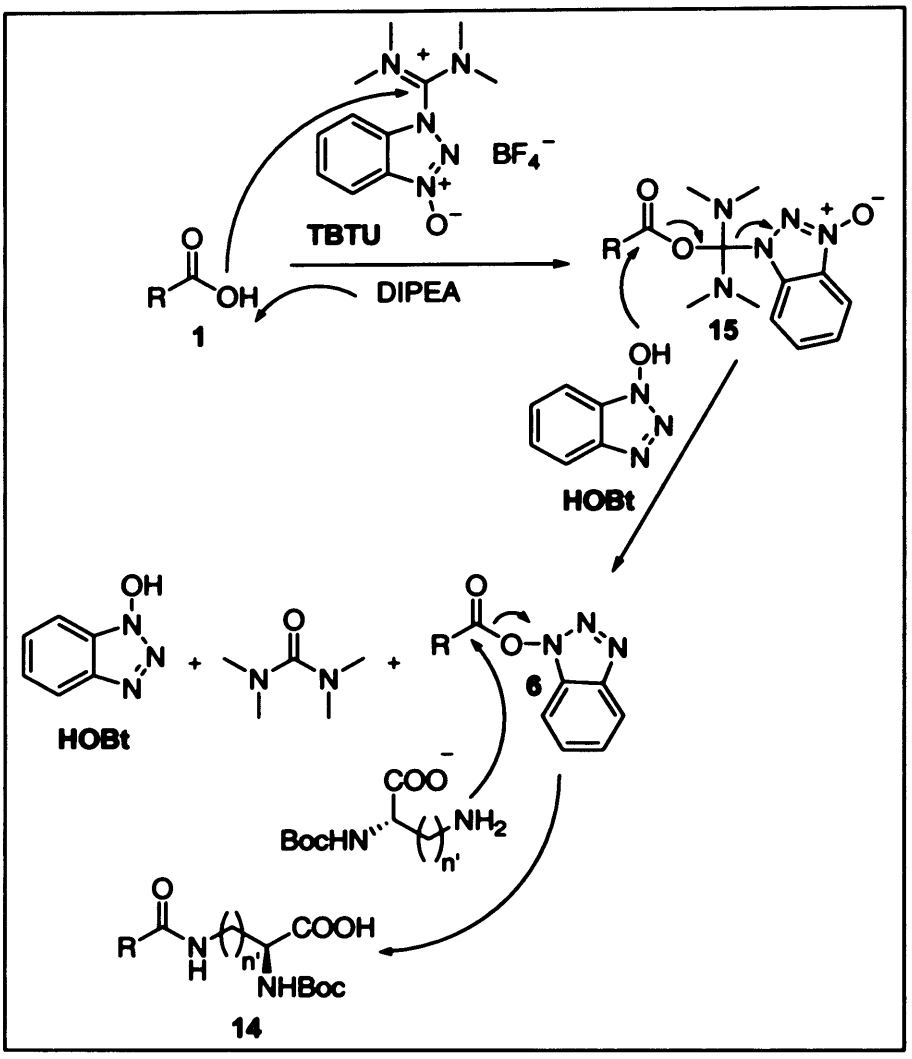
Different activating agents are available in the literature and the use of HOBt with TBTU together is also reported[136]. It has been shown that the use of HOBt and TBTU as activating agents gave a better yield and a faster reaction time for the synthesis of the α-amino acid amide derivatives **14** (Scheme IV.9)[126].



Scheme IV.9: Coupling reaction with HOBt and TBTU

The mechanism can be explained by 3 different successives nucleophilic attacks (Scheme IV.10):

1. The oxygen of the acid function onto TBTU;
2. Then a second with HOBt on the intermediate **15**;
3. And finally, the amino acid onto the intermediate **6**.



Scheme III.10: Mechanism of the coupling reaction with TBTU and HOBt

The main reason for the success of this mechanism is that the formation of the benzotriazole ester **6** is favoured by the formation of the tetramethyl urea. The order of the additions is really important as Boc-Dap-OH was added only when the benzotriazol ester **6** formation was observed by TLC. All TBTU should then be used up and the carboxylate from Boc-Dap-OH can not react with it. This method was successfully applied to both α-amino acids and the results are summarised in Table IV.11:

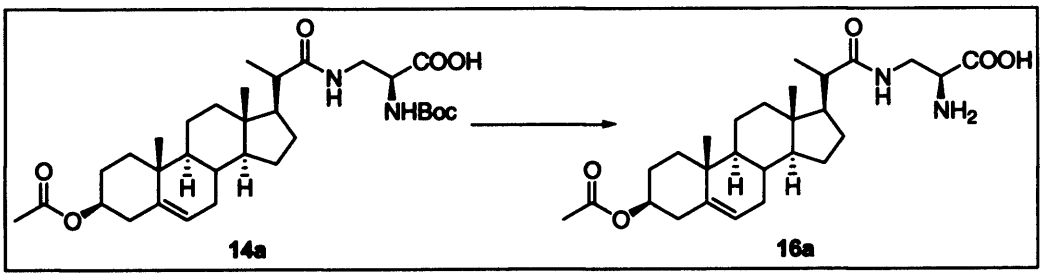
Entry	α-Amino acid	Activating time (h)	Time (h)	Yield (%)
I	Boc-Dap-OH	0.5	o/n	10
II	Boc-Dap-OH	1	24	48
III	Boc-Dap-OH	1	48	55
IV	Boc-Dab-OH	1	o/n	33
V	Boc-Dab-OH	1	48	45

Table IV.11: Study of the coupling reaction with HOBt and TBTU

The two criteria implicated in this reaction are the activating time which leads to the formation of the intermediate **6**, and then how long the reaction was left. Entries **I** and **IV**, the activating time was increased from 30 minutes to an hour and led to a substantial increase of the yield of 23%. This could be explained as all TBTU might not have reacted with starting material **1** after only 30 minutes, while it has after an hour. Then, comparing entries **II** and **III**, 48 hours reaction gave a better yield with 55% while 24 hours led to only 48%.

IV.3.3 Attempted synthesis of 3β-acetoxy-23,24-bisnor-5-en-22-(3-(2-amino)propanoic acid) amide (16a)

There are several ways to remove the Boc group (Scheme IV.11) as reported in the literature[142]. A standard deprotection in acidic conditions involves the use of either hydrochloric acid (HCl) in MeOH or trifluoroacetic acid (TFA) in DCM.



Scheme IV.11: Deprotection of the Boc group

The use of hydrochloric acid gave an unidentified aggregate. Then, the use of TFA led to compound **16a**, as shown by MS. Unfortunately, purification attempted by recrystallisation or extraction did not afford the pure compound **16a**.

IV.4 Summary

Different series of compounds with an amide function at position 23 in the side chain of the sterol were synthesised. The first series was obtained by the key reaction, activation and coupling of the starting material **1** with different methyl amino esters **3**. The second series was obtained by hydrolysis of the ester functions of the compounds of the first series with sodium hydroxide. A third series has been started with amino acid function derivatives. Future work on this series will require the deprotections of the Boc group on the amino group, and the hydroxy group at position 3.

All the series were sent for *in vitro* or *in vivo* testing and the results are discussed in Chapter VI.

CHAPTER V

SYNTHESIS OF THE AMINE DERIVATIVES

Azasterols have been proposed by many authors as lead compounds in the design of inhibitors of the enzyme sterol 24-methyltransferase (24-SMT, Chapter II). The approach is based on the introduction of a positive charge to the side chain of the sterol nucleus to mimic high energy reaction intermediates. Azasterol derivatives (cholestanols) have been reported to be active against ergosterol biosynthesis in Trypanosomatid protozoan *Crithidia fasciculate*[110,111]. Other azasterols, for example the 22,26-azasterol (Figure V.1) showed good *in vitro* activities against both *L. amazonensis* and *T. cruzi*[119,120].

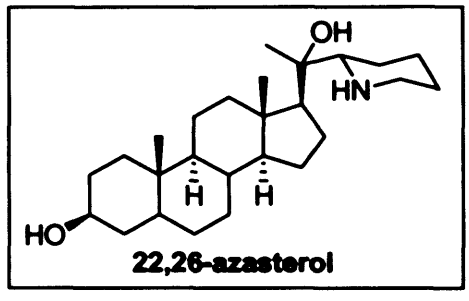


Figure V.1: 24-SMT inhibitor

Some other azasterols with nitrogen at position 23 have also showed some promising data against Leishmania and Trypanosomes[127]. The influence of the position of the positive charge has been studied, showing that azasterols with a positive charge in position 23, 24 and 25 of the side chain are the most active. New targets have therefore been proposed in this project. Different types of side chains for attachment to the sterol nucleus were proposed (Figure V.2). Previously, amine analogues have been reported from this laboratory[126]; the synthetic methodology used for making these amine analogues was initially applied to the carboxylates.

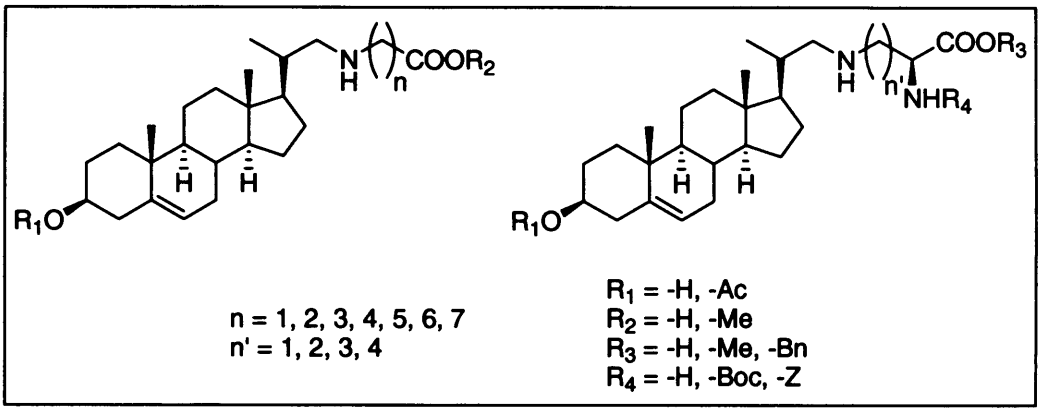


Figure V.2: Series of target derivatives proposed

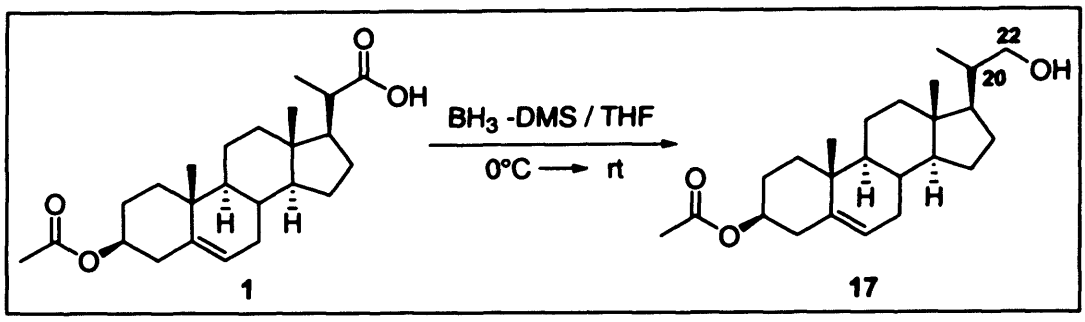
V.1 Attempted synthesis from the tosyl derivative (18)

As presented in the Chapter III (Figure III.8), the azasterol can be obtained by nucleophilic substitution from a tosylate intermediate. The tosylate could be prepared from the alcohol, which could be synthesised by reduction of the carboxylic acid of starting material **1**.

V.1.1 Synthesis of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-ol (17)

The reduction of carboxylic acid using BH_3-DMS has been previously described in the laboratory[126,143] using 1.5 equivalents of borane added at -10°C and then stirred for 8 hours at 0°C in DCM to give a maximum yield of 57%. In order to improve this yield, the conditions were slightly changed. First, looking at the influence of the temperature, the addition of borane was carried out at 0°C, and the resulting solution stirred at room temperature overnight (Scheme V.1). The unexpected result was a near quantitative yield of product (98%) after purification by column chromatography.

[143] Yoon, N. M.; Pak, C. S.; Brown, H. C.; Krishnamurthy, S.; Stocky, T. P. Selective Reductions. XIX. The Rapid Reaction of Carboxylic Acids with Borane-Tetrahydrofuran. A Remarkably Convenient Procedure for the Selective Conversion of Carboxylic Acids to the Corresponding Alcohols in the Presence of Other Functional Groups. *J. Org. Chem.* **1973**, *38*, 2786-2792.



Scheme V.1: Synthesis of the alcohol derivative **17**

The starting material **1** has an acid function, an ester function and a double bond. Boranes, like BH_3-DMS, are particularly good for the reduction of carboxylic acids, but reduction of double bonds also occurs at a similar rate. On the other hand, the reduction of ester functions is more difficult with a slower rate, unless done in refluxing THF[144] or in the presence of certain compounds (LiCl or $AlCl_3$ in diglyme). These slight differences permit selective reduction in the presence of many other groups.

It is remarkable to notice that only the reduction of the acid function occurs in this case, as shown in the ^1H NMR spectrum (Figure V.3). The presence of the double bond signal from ^6H is observed at 5.50ppm, as is the signal of the acetate $^{2'}H$ at 2.17ppm. The signal of ^3H did not move upfield either, which would have occurred if the ester function had been cleaved to give a hydroxyl group.

Sterol NMRs are particularly difficult to analyse as most of the proton signals appear between 1 and 2 ppm. The easiest changes to observe are changes downfield from this region. The main feature of the spectrum of the reduction product **17** is the appearance of a doublet of doublets due to the coupling of the proton at position 20 with the nonequivalent protons of the methylene group at position 22 of the side chain of the sterol (Figure V.3). This is typical of the ABX spin system.

[144] Brown, H. C.; Choi, Y. M.; Narasimhan, S. Selective Reductions .29. A Simple Technique to Achieve and Enhanced Rate of Reduction of Representative Organic-Compounds by Borane-Dimethyl Sulfide. *J. Org. Chem.* **1982**, *47*, 3153-3163.

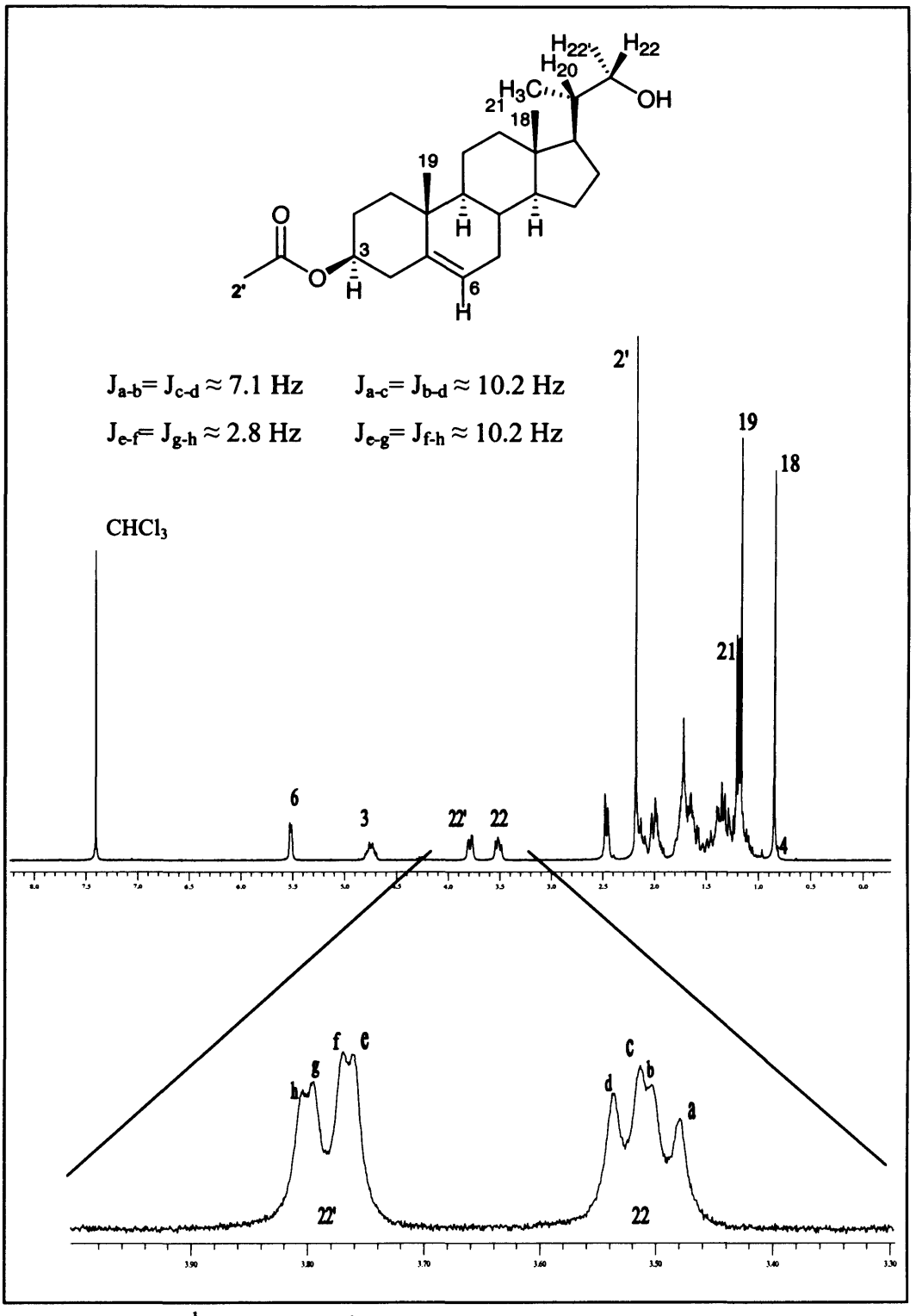


Figure V.3: ^{1}H-NMR of 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-ol **17**

The two signals for H^{22} and $H^{22'}$ at δ 3.51 or δ 3.78 were provisionally assigned by analysis of the coupling constants and the Newman projection (Figure V.4). From the coupling constants, $^2J_{22\text{-}22'} = {}^2J_{22'\text{-}22} \approx 10.2$Hz.

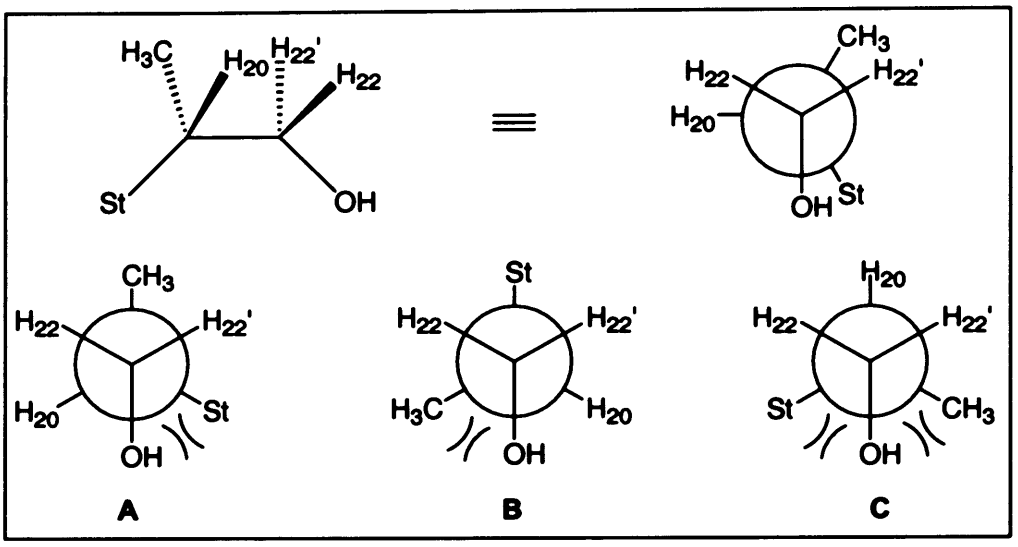


Figure V.4: Stereochemistry C^{20}-C^{22}, Newman projection.

From the Newman projection, the most stable conformation is **B** (Figure V.4). In projection **A** and **C**, there is likely to be clashes between the hydroxyl and sterol, which is a much stronger interaction than between the hydroxyl and methyl groups. So, we can now compare the dihedral angles: H_{22}-H_{20} ~ 180° and $H_{22'}$-H_{20} ~ 60°. From the Karplus equation[145], a 60 degrees angle should give a coupling constant between 2 and 5Hz, and for a 180 degrees angle, between 8 and 15Hz.

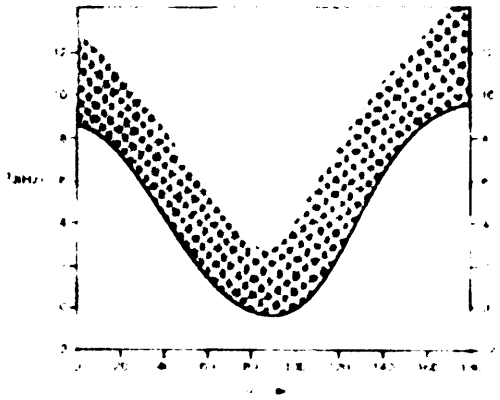


Figure V.5: Coupling constants from Karplus equation[145]

From the analysis of the coupling constants on the ^{1}H-NMR, the assignment is as followed: $J_{22\text{-}20} = J_{a\text{-}b} = J_{c\text{-}d} \approx 7.1$ Hz

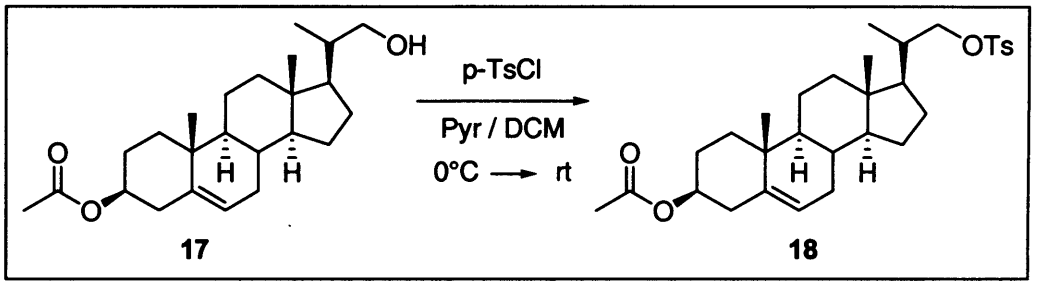
$J_{22'\text{-}20} = J_{e\text{-}f} = J_{g\text{-}h} \approx 2.8$ Hz

[145] http://www.chemistry.ccsu.edu/glagovich/teaching/472/nmr/couplingbasics.html

Therefore the final stereochemistry of the alcohol derivative **17** is tentatively assigned as in Figure V.3.

V.1.2 Synthesis of the 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-(p-toluensulfonyloxy) (18)

The next step was the introduction of a tosyl group[146] from the 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-ol **17** (Scheme V.2). The alcohol intermediate was reacted with p-toluenesulfonyl chloride in a pyridine/DCM (1:1) solution for 24h to afford 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-(p-tolensulfonyloxy) **18**, in a yield of 83%.



Scheme V.2: Synthesis of the 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-(p-tolensulfonyloxy) **18**

Again, in the ^{1}H-NMR spectrum is the appearance of a doublet of doublets, typical of an ABX spin system (Figure V.5). The aromatic protons and the methyl numbered 31, characteristic of a tosyl group, are also seen.

[146] Samano, V.; Robins, M. J. Nucleic-Acid Related-Compounds .70. Synthesis of 2'(and 3')-Deoxy-2'(and 3')-Methyleneadenosines and Bis(Methylene)Furan 4',5'-Didehydro-5'-Deoxy-2'(and 3')-Methyleneadenosines - Inhibitors of *S*-Adenosyl-$_L$-Homocysteine Hydrolase and Ribonucleotide Reductase. *J. Org. Chem.* **1991**, *56*, 7108-7113.

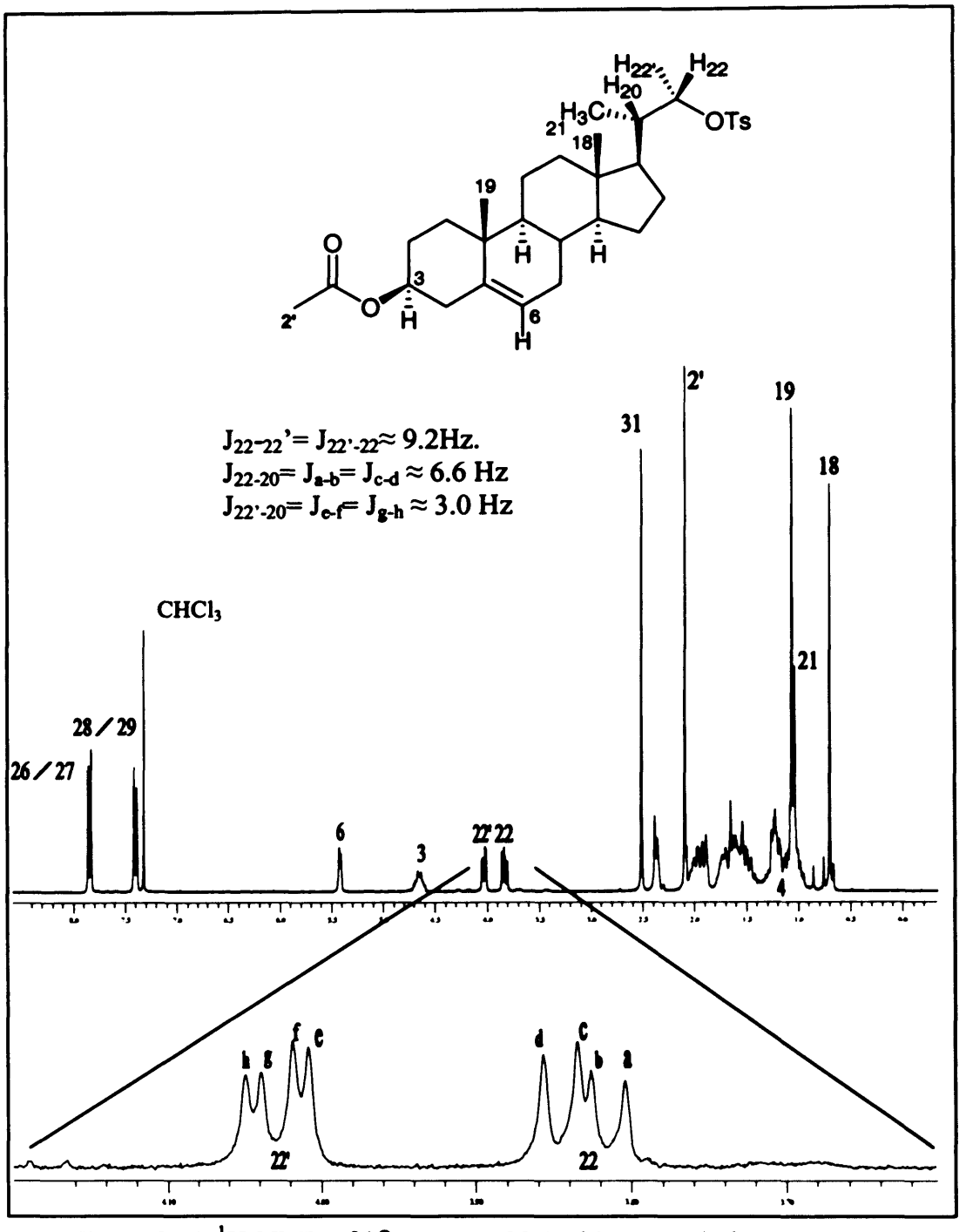


Figure V.6: ^{1}H-NMR of 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-(p-tolensulfonyloxy) **18**

Following the same method of NMR assignment as for the alcohol derivative **17**, the C^{20}-C^{22} stereochemistry of the tosyl derivative **18** is as shown in Figure V.6.

V.1.3 Attempted synthesis of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(alkyloic acid) amines (19) and 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines (20)

The synthesis of the amine derivatives from the tosylate derivative **18** should be achieved by nucleophilic substitution of the tosyl group[147]. This reaction has already been successfully described in the laboratory with different alkyl chains (Figure V.7)[126,127].

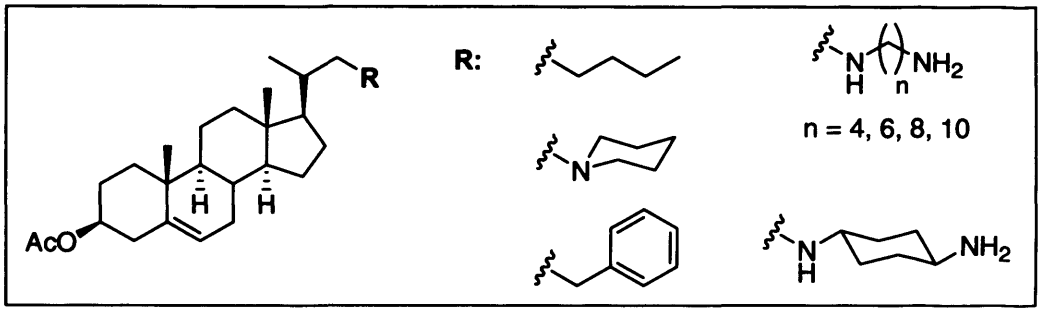


Figure V.7: Alkyl chains previously employed in the laboratory

As discussed before, the aim is to introduce mimics of the SAM moiety as a side chain, in particular in this case an acid or an ester function. The starting point would be the amino acids **2** which are commercially available and the methyl amino esters **3** to be prepared (Figure V.8).

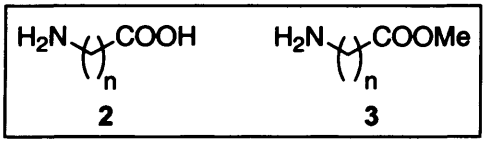


Figure V.8: Alkyl chains to be added as mimic of SAM moieties

The reaction was first tried with the amino acids **2** (Scheme V.3), in order to obtain azasterols with the free acid function at the end of the side chain.

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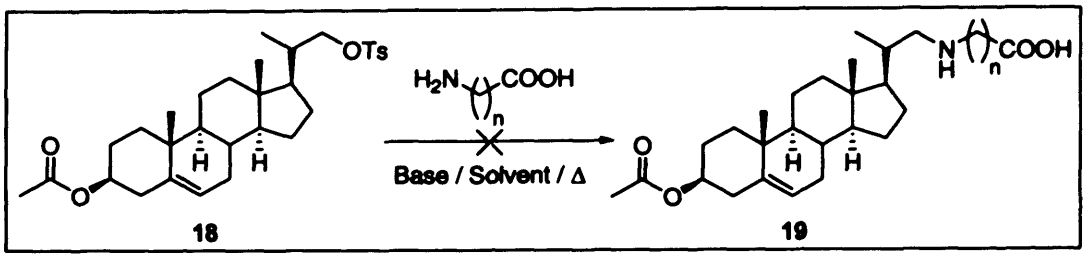
[147] Larock, R. C. Comprehensive Organic Transformations: A Guide to Functional Group Preparations. *VCH Publishers, inc.* **1989**, 967-988



Scheme V.3: Attempted synthesis of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(alkyloic acid) **19**

Different conditions were investigated, using different bases and solvents, but unfortunately, the reaction did not work and the Table V.1 summarises all the failed attempts (Tos: tosylate derivative **18**; Nu: amino acid **2**; B: base; **18**: compound **18**):

Entry	n	Solvent	Base	Eq.Tos.	Eq.B.	Eq.Nu.	Time (h)	T(°C)	Results
I	3	DMF	DIPEA	1	7	10	48	60	**18** (impure)
II	3	DMF	DIPEA	1	6	11	72	60	**17** + **18**
III	5	DMF	DIPEA	1	10	10	66	60	**18** (impure)
IV	7	DMF	K_2CO_3	1	12	5	168	60	**18** (impure)
V	7	CH_3CN	K_2CO_3	1	12	5	72	rt	**18**

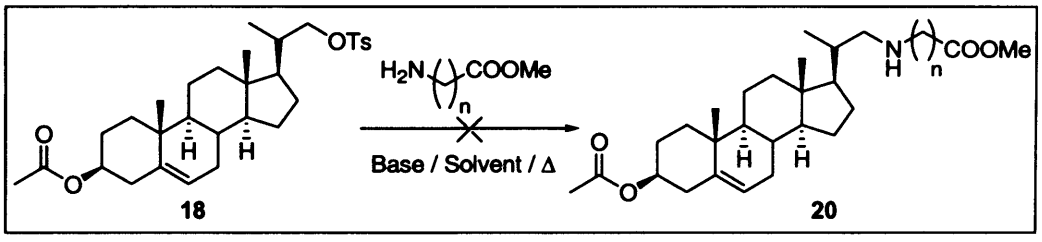
Table V.1: Nucleophilic substitutions with the amino acid **2**

The first attempt (Entry I) used literature conditions with DMF as a solvent and DIPEA as a base[147]. This first try, did not give the desired product, and led to a mixture containing starting material **18**. Subsequently, the conditions were slightly changed. First, the reaction time was increased (Entry II), and then the quantity of base (Entry III). In both cases, starting material was recovered, but also in one case, some alcohol derivative **17** (26%). Use of potassium carbonate as base (Entry IV) did not give the product either, and again, a mixture containing starting material **18** was recovered. Finally, the use

of acetonitrile[148] as a solvent (Entry V) also proved unsuccessful and only pure starting material **18** was recovered.

Potassium carbonate is a stronger base than disopropylethylamine but did not influence the rate of the reaction. Dimethylformamide (DMF) and acetonitrile are both polar aprotic solvents. Polar solvents aid S_N2 reactions when the reactants are uncharged but the transition state has built up a charge[149]. Large anions, such as tosylate, are also better solvated at the transition state. Therefore, both DMF and acetonitrile should favour an S_N2 type reaction, but unfortunately, none of the changes influenced the outcome of the reaction.

At the same time, this reaction was tried with the methyl amino esters **3** (Scheme V.4).



Scheme V.4: Attempted synthesis of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) **20**

As above, different conditions were investigated, and are summarised in the Table V.2 (Tos: tosylate derivative **18**; Nu: methyl amino ester **3**; B: base; **17**: Compound **17**).

[148] De Michelis, C.; Rocheblave, L.; Priem, G.; Chermann, J. C.; Kraus, J. L. New Anti-HIV Derivatives: Synthesis and Antiviral Evaluation. *Bioorg. Med. Chem.* **2000**, *8*, 1253-1262.

[149] Smith, B. M.; March, J. Advanced Organic Chemistry, Reactions, Mechanisms, and Structure. *March's, 5th edition* **2001**.

Entry	n	Solvent	Base	Eq. Tos.	Eq. Nu.	Eq. B.	Time (h)	T (°C)	Results (%)
I	6	DMF	DIPEA	1	3	12	o/n	60	**17** (70)
II	4	DMF	DIPEA	1	8	20	72	60	**17** (60)
III	3	DMF	DIPEA	1	12 1	20 3	48 24	60	**17** (13)
IV	3	DMF	DIPEA	1	12 1	23 3	48 48	60	**17** (40)
V	6	DMF	K_2CO_3/Bu_4NI	1	4	3/1	o/n	100	**17** (60)
VI	6	EtOH/ Dioxane	K_2CO_3	1	1	3	240	60	-
VII	6	CH_3CN	$LiClO_4$	1	2	6	48	100	-

Table V.2: Conditions tried for the nucleophilic substitution with the methyl amino esters **3**

Entry **I** represents the basic conditions normally used in this kind of reactions[147]. Again, this attempt failed and no starting material **18** was recovered. Instead, the major product isolated was 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-ol **17** as a result of hydrolysis of the tosylate **18**. Increasing not only the quantity of the different reagents but also the time of the reaction led to the same result (Entry **II**). Addition of more reagents after two days (Entries **III** and **IV**) also did not lead to isolation of the desired compound. Use of tetrabutylammonium iodide as a phase-transfer catalyst (Entry **V**)[150], with potassium carbonate, did not work and led again to the alcohol intermediate **17**. The use of potassium carbonate or lithium perchlorate as bases, in respectively

[150] Taber, D. F.; You, K. K. Highly Diastereoselective Cyclopentane Construction - Enantioselective Synthesis of the Dendrobatid Alkaloid 251f. *J. Am. Chem. Soc.* **1995**, *117*, 5757-5762.

a mixture of ethanol/dioxane[151] or acetonitrile[152] (Entries **VI** and **VII**), led to an unidentified mixture.

In Entries **I** to **IV**, only the quantity of reagents and the reaction time were changed. None of the modifications led to the derivatives **20**. In Entry **V**, a stronger base, potassium carbonate, and a phase-transfer catalyst, tetrabutylammonium iodide were used at higher temperature. The use of a phase-transfer catalyst such as tetra-alkyl ammonium salt is often used in DMF to accelerate a reaction. An exchange of the tosylate group by iodide should occur, promoting nucleophilic attack. Unfortunately, the intermediate **17** was again obtained. Entry **VI**, using dioxane as a solvent in the presence of potassium carbonate, resulted in the decomposition of the sterol moiety. Finally, lithium perchlorate was used in acetonitrile (Entry **VII**). Perchlorate ions trap the solvent-separated ion pairs to give *in situ* the ion pair $R^+//ClO_4^-$ which would be very reactive[149]. Unfortunately, a complete decomposition of the sterol moiety was observed.

In order to understand why this basic reaction did not work, some molecular modelling was carried out. The energy of the tosyl derivative **18** was minimised using the MM2 force field as implemented in Chem 3D (Figure V.9).

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[151] Ismaiel, A. M.; Dukat, M.; Law, H.; Kamboj, R.; Fan, E. M.; Lee, D. K. H.; Mazzocco, L.; Buekschkens, D.; Teitler, M.; Pierson, M. E.; Glennon, R. A. 2-(1-Naphthyloxy)ethylamines with Enhanced Affinity for Human 5-HT1D Beta (h5-HT1B) Serotonin Receptors. *J. Med. Chem.* **1997**, *40*, 4415-4419.

[152] Nishi, T.; Ishibashi, K.; Takemoto, T.; Nakajima, K.; Fukazawa, T.; Iio, Y.; Itoh, K.; Mukaiyama, O.; Yamaguchi, T. Combined Tachykinin Receptor Antagonist: Synthesis and Stereochemical Structure-Activity Relationships of Novel Morpholine Analogues. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1665-1668.

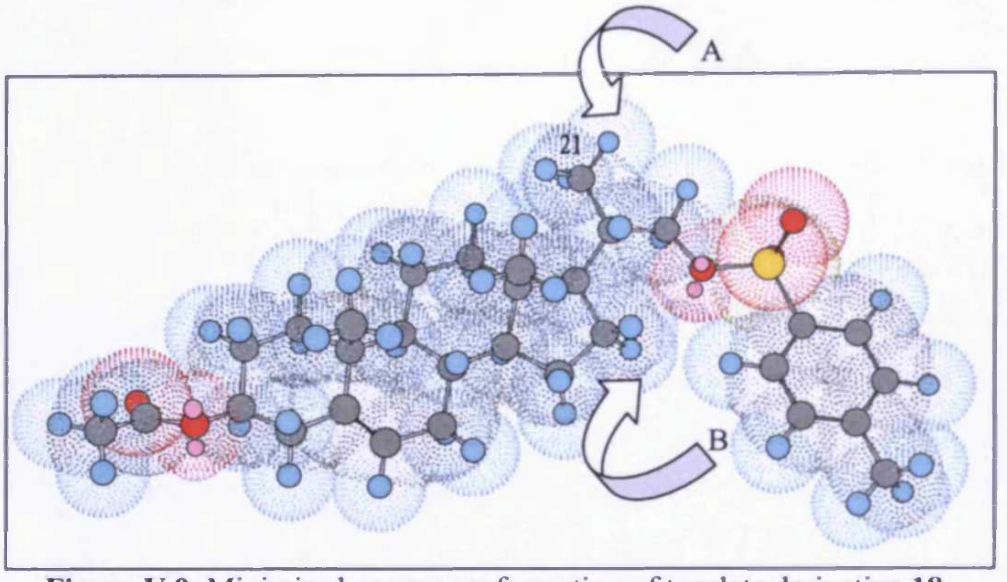
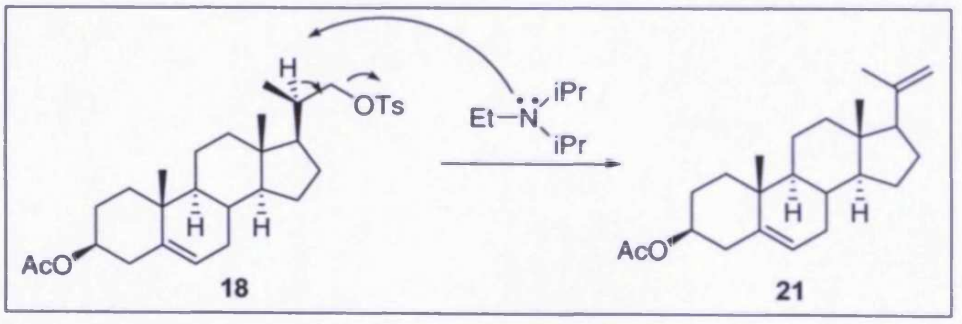


Figure V.9: Minimised energy conformation of tosylate derivative **18**

The nucleophilic substitution could occur by two different approaches **A** and **B**. In approach **A**, the methyl **21** is in a position that prevents the S_N2 reaction by blocking the approach of the nucleophile. In approach **B**, this effect is even more obvious where the entire sterol nucleus interferes with a possible S_N2 mechanism.

In most of the cases reported above, either starting material **18** or compound **17** resulting from its hydrolysis were recovered. One possible side reaction was elimination which would result in the formation of a double bond at position 20 (Scheme V.5), but this was not observed.



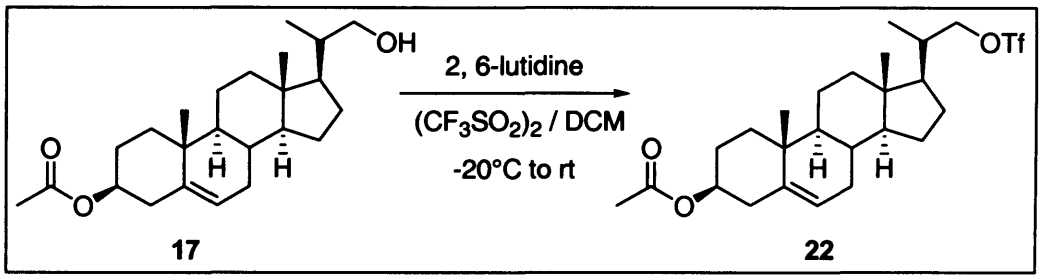
Scheme V.5: Possible side reaction, byproduct **21**

In conclusion, the use of the amino acids **2** or the methyl amino esters **3** as nucleophiles did not give the desired compounds **19** and **20**, under a variety

of conditions. The reason for this may be, as shown in Figure V.9, the steric hindrance caused by both the sterol nucleus and methyl **21**.

V.2 Attempted synthesis from the triflate derivative (22)

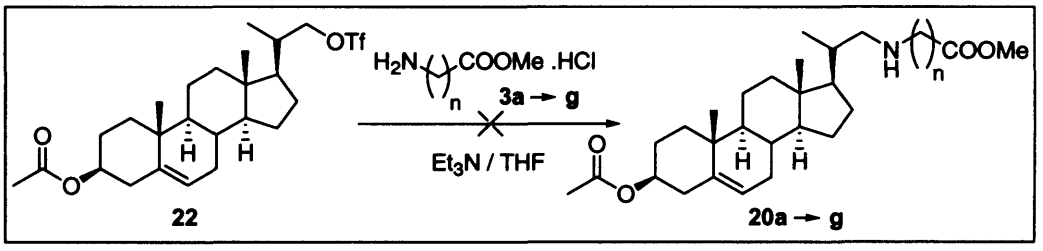
In order to increase the reactivity of the leaving group, it was decided to replace the tosylate with a triflate. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-ol **17** was reacted with 2,6-lutidine in DCM. Triflic anhydride was added slowly at -20°C and the resulting solution stirred for 6h or overnight at room temperature (Scheme V.6).



Scheme V.6: Synthesis of the triflate derivative **22**

After extraction, TLC of the crude showed the disappearance of the alcohol spot and the appearance of a less polar spot, which indicated that the reaction had proceeded. No further purification was attempted and the crude was directly used in the next step.

The synthesis of the amine derivatives from the triflate derivative **22** (Scheme V.7) was to be achieved by a nucleophilic substitution of the triflate group, using the methyl amino esters **3**.



Scheme V.7: Attempted synthesis from the triflate derivative **22**

This reaction was tried twice (n = 3 and 5). Unfortunately, this method, using a better leaving group was unsuccessful. No starting material was recovered, only an unidentified mixture in both cases. The reason might be as before, steric hindrance at position 22 preventing the S_N2 reaction occurring. A decomposition of the sterol moiety under these reaction conditions was also observed.

After this lack of success, the preparation of the amine derivatives was investigated by reduction of the amide function of the compounds **4** prepared in Section IV.1.2.

V.3 Attempted synthesis from the amide derivatives (4)

Preparation of an amine can also be achieved by reduction of an amide group. Several reducing reagents may be used. This reaction was previously studied in the laboratory[126] and four methods were investigated (Table V.3).

Entry	Reagent	Eq. Reag.	Other Reagent	Eq.O. Reag.	Solvent	Time(h)	T (°C)
I	$LiAlH_4$	2	-	-	THF	24	70
II	$NaBH_4$	5	$CoCl_2 \cdot 6H_2O$	1	MeOH	5	rt
III	$NaBH_4$	2	AcOH	2	Dioxane	o/n	Reflux
IV	BH_3-DMS	1	-	-	THF	72	rt

Table V.3: Reducing agent studied[126]

The first reagent used (Entry I) was lithium aluminium hydride[153]. Reduction of an amide is not a rapid reaction with this reagent[154]. The reaction was carried out for 24h at 70°C in THF but resulted only in loss of the acetyl group in position 3. Sodium borohydride by itself does not reduce amides, but

[153] Brown, H. C.; Hess, H. M. Selective Reductions. XIII. Reaction of Δ^2-Cyclopentenones with Representative Complex Hydrides. Aluminium Hydride as a Selective Reagent for the Reduction of the Carbonyl Group in Δ^2-Cyclopentenones. *J. Org. Chem.* **1969**, *34*, 2206-2209.

[154] Micovic, V. M.; Mihailovic, M. L. J. The Reduction of Acid Amides with Lithium Aluminium Hydride. *J. Org. Chem.* **1953**, *18*, 1190-1200.

does so in the presence of other reagents[149,155,156,157]. Therefore in the Entries **II** and **III**, the reduction of the amide **4** was attempted with sodium borohydride in the presence of either cobalt (II) ions or acetic acid. Unfortunately these methods gave only starting material. Finally, in Entry **IV**, borane dimethylsulfoxide was used[158,159]. This reagent has been proved to selectively reduce amides in the presence of ester functions[149]. The mechanism of action of borane is depicted in Scheme V.8[144,160,161,162]. The reduction of secondary amides involves first hydrogen evolution. Two equivalents of borane are then needed for the reduction, and three others to form the amine-borane adduct. In total, six equivalents of hydride are recommended for the reduction of a secondary amide. This method yielded 5% of required product and various by-products with only one equivalent of borane.

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[155] Satoh, T.; Suzuki, S.; Suzuki, Y.; Miyaji, Y.; Imai, Z. Reduction of Organic Compounds with Sodium Borohydride-Transition Metal Salt Systems: Reduction of Organic Nitrile, Nitro and Amide Compounds to Primary Amines. *Tetrahedron Lett.* **1969**, *10*, 4555-4558.

[156] Umino, N.; Iwakuma, T.; Itoh, N. Sodium Acyloxyborohydride as New Reducing Agents. I. Reduction of Carboxamides to the Corresponding Amines. *Tetrahedron Lett.* **1976**, *17*, 763-766.

[157] Cannon, J. G.; Walker, K. A.; Montanari, A.; Long, J. P.; Flynn, J. R. Monomethyl Ether Derivatives of 7,8-Dihydroxy-4-Normal-Propyl- 1,2,3,4,4a,5,6,10b-Octahydrobenzo F Quinoline and 8,9- Dihydroxy-4-Normal-Propyl-1,2,3,4,4a,5,6,10b- Octahydrobenzo F Quinoline as Possible Products of Metabolism by Catechol-O-Methyltransferase. *J. Med. Chem.* **1990**, *33*, 2000-2006.

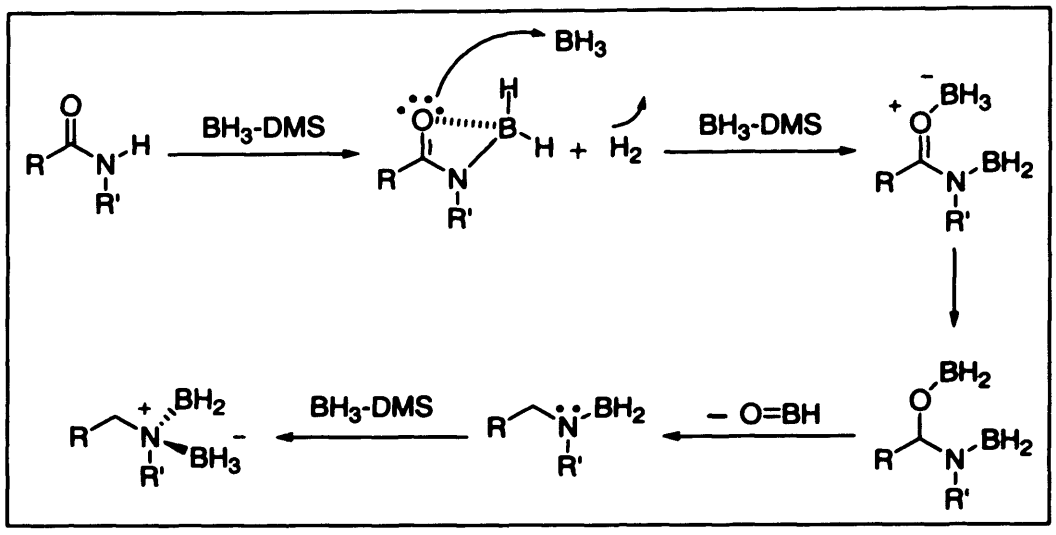
[158] Brown, H. C.; Heim, P. Selective Reductions. XVIII. The Fast Reaction of Primary, Secondary, and Tertiary Amides with Diborane. A Simple, Convenient Procedure for the Conversion of Amides to the Corresponding Amines. *J. Org. Chem.* **1973**, *38*, 912-916.

[159] Russ, P. L.; Caress, E. A. Synthesis of Tertiary Amines by Selective Diborane Reduction. *J. Org. Chem.* **1976**, *41*, 149-151.

[160] Brown, H. C.; Narasimhan, S.; Choi, Y. M. Improved Procedure for Borane-Dimethyl Sulfide Reduction of Primary Amides to Amines. *Synthesis* **1981**, 441-442.

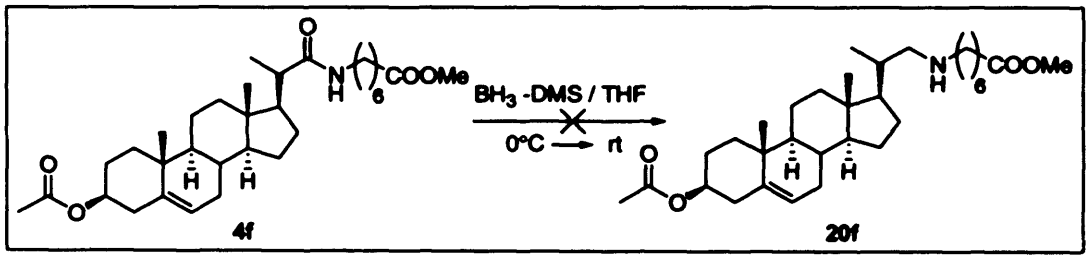
[161] Brown, H. C.; Narasimhan, S.; Choi, Y. M. Improved Procedure for Borane-Dimethyl Sulfide Reduction of Tertiary and Secondary Amides in the Presence of Boron Trifluoride Etherate. *Synthesis* **1981**, 996-998.

[162] de Carvalho Alcântara, A. F.; dos Santos Barroso, H.; Pilo-Veloso, D. Reduçao de Amidas por Boranos. *Quim. Nova* **2000**, *25*, 300-311.



Scheme V.8: Mechanism of amide reduction by borane

Thus the reaction was repeated with 3β-acetoxy-23,24-bisnor-5-en-22-(methyl heptanoate ester) amide **4f** and an excess of borane (20 eq., Scheme V.9). Unfortunately, it was unsuccessful, and a mixture of different products was recovered. The use of too many equivalents of borane may have led to side products due to reduction of esters and alkyne functionalities.



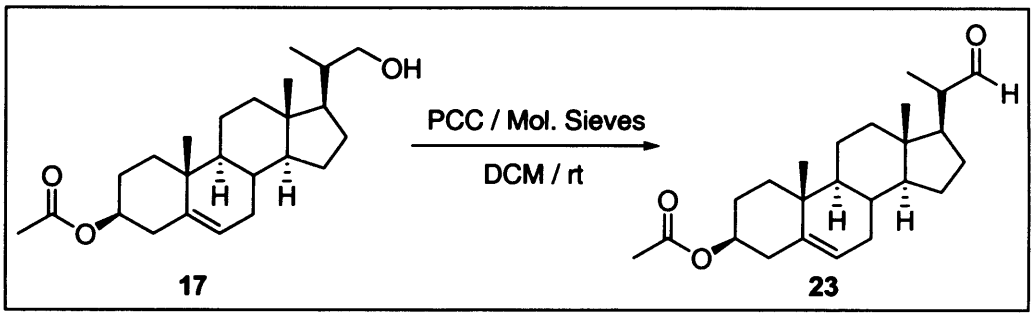
Scheme V.9: Attempted synthesis of the amine derivative **20f**

V.4 Preparation from the aldehyde derivative (23)

Another strategy could have been a reductive amination between an aldehyde derivative and an amine. The aldehyde derivative to be used could be synthesised from the alcohol **17** by simple oxidation, and the amine, the methyl amino esters **3**. Coupling of the amine and aldehyde should give an imine derivative which could be reduced by a suitable reducing agent.

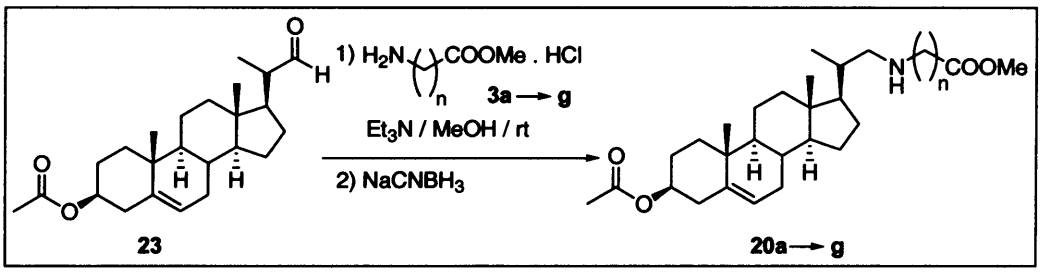
V.4.1 Synthesis of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines (20)

The alcohol derivative **17** was reacted with pyridinium chlorochromate (PCC)[163], with molecular sieves in DCM at room temperature overnight (Scheme V.10). After purification by column chromatography, the aldehyde derivative **23** was obtained in a good yield, 73%.



Scheme V.10: Synthesis of the 3β-acetoxy-23,24-bisnor-5-chol-5-en-22-al **23**

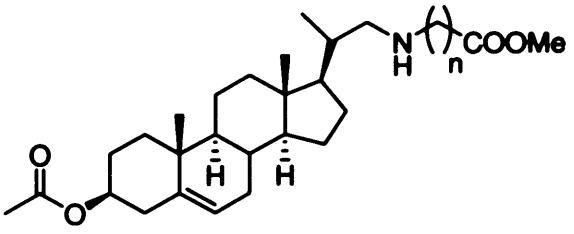
The methyl amino esters **3** were then reacted with triethylamine in methanol at room temperature in order to neutralise HCl (Scheme V.11). After thirty minutes, the aldehyde derivative **23** was added. Finally, thirty minutes later, sodium cyanoborohydride was added, and the solution stirred overnight.



Scheme V.11: Synthesis of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines **20**

[163] Kasmai, H. S.; Mischke, S. G.; Blake, T. J. 18-Crown-6 Complexes of N-Butylammonium and Pyridinium Chlorochromates - Mild and Selective Oxidizing-Agents for Alcohols. *J. Org. Chem.* **1995**, *60*, 2267-2270.

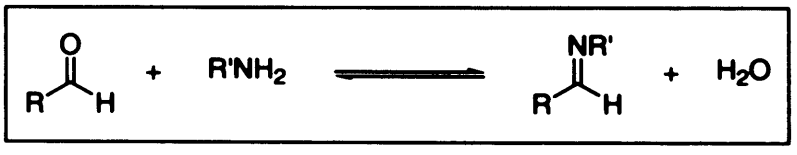
Sodium cyanoborohydride is a good reducing agent for this reaction as it is not strong enough to reduce the starting aldehyde, but can reduce the imine intermediates. Sodium cyanoborohydride was first used as a solid[164]. The appearance of a less polar spot on the TLC suggested that the reaction worked. The products were obtained in reasonable yields, after purification by column chromatography (Table V.4).



Compound	20c	20d	20e	20f	20g
n	3	4	5	6	7
Yield (%)	46	42	67	54	64

Table V.4: Yields of the synthesis of the the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines **20**

In an aim to increase the yields of these reactions, molecular sieves were added in the solution. During the formation of the imine intermediate, water molecules are formed (Scheme V.12), which could be removed by molecular sieves and would displace the equilibrium reaction toward the imine formation.



Scheme V.12: Formation of the imine intermediate

[164] Manescalchi, F.; Nardi, A. R.; Savoia, D. Reductive Amination of 1,4-Dicarbonyl and 1,5-Dicarbonyl Compounds with (S)-Valine Methyl-Ester - Synthesis of (S)-2-Phenylpyrrolidine and (S)-2-Phenylpiperidine. *Tetrahedron Lett.* **1994**, *35*, 2775-2778.

The unexpected result was that the addition of molecular sieves decreased the yields quite significantly (Table V.5). For example, compound **20e** was obtained with only 36% yield compared to 67% previously. The reason for this is unknown.

Compound	**20c**	**20e**	**20f**
Yield (%)	38	36	34

Table V.5: Yields with molecular sieves

An important problem in this one-pot reaction is that the addition of sodium cyanoborohydride requires extreme caution (hygroscopic and toxic). Therefore, different commercially available reducing agents were investigated:

- Sodium cyanoborohydride in a 1M THF solution;
- Sodium triacetoxy borohydride[154,165,166] as a solid.

The use of sodium cyanoborohydride in a 1M THF solution (Table V.6) gave comparable results to the use of sodium cyanoborohydride as a solid, but it is easier and safer to use.

Compound	**20a**	**20b**	**20c**	**20d**	**20e**	**20f**	**20g**
n	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Yield (%)	50	54	56	39	26	51	51

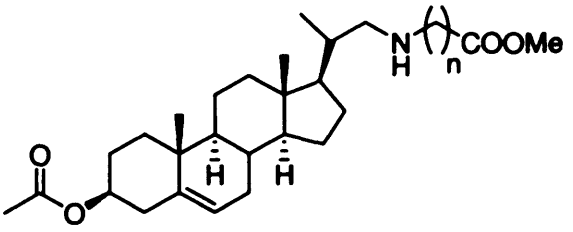
Table V.6: Use of sodium cyanoborohydride in THF solution

An alternative to sodium cyanoborohydride ($NaCNBH_3$) is sodium triacetoxyborohydride ($NaBH(OAc)_3$). It was used as a solid (Table V.7), and

[165] Abdelmagid, A. F.; Maryanoff, C. A.; Carson, K. G. Reductive Amination of Aldehydes and Ketones by Using Sodium Triacetoxyborohydride. *Tetrahedron Lett.* **1990**, *31*, 5595-5598.

[166] AbdelMagid, A. F.; Carson, K. G.; Harris, B. D.; Maryanoff, C. A.; Shah, R. D. Reductive Amination of Aldehydes and Ketones with Sodium Triacetoxyborohydride. Studies on Direct and Indirect Reductive Amination Procedures. *J. Org. Chem.* **1996**, *61*, 3849-3862.

showed better yields for the compounds **20d** and **20g**, and comparable yields for the others.



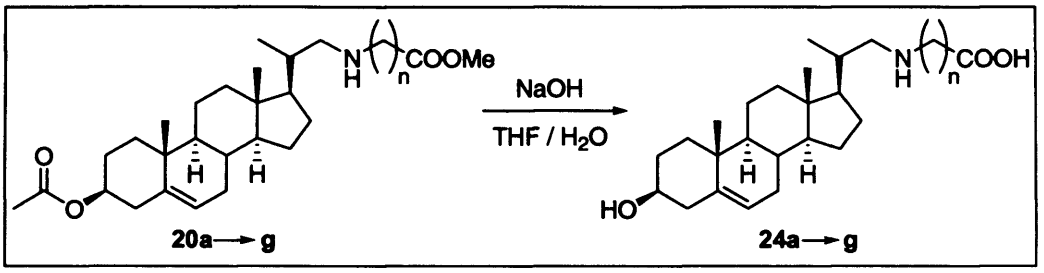
Compound		20c	20d	20e	20f	20g
n		3	4	5	6	7
Yield (%)	$NaBH(OAc)_3$	43	70	52	55	71
	$NaCNBH_3$ (solid)	46	42	67	54	64
	$NaCNBH_3$ (solution)	56	39	26	51	51

Table V.7: Yields of the synthesis of the the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines **20** with different reducing agents

In conclusion, the Table V.7 shows that the use of different reducing agents led to comparable yields. Sodium cyanoborohydride in a 1M solution of THF was chosen for future experiments.

V.4.2 Synthesis of the 3β-ol-23,24-bisnor-5-en-22-(alkyloic acid) amines (24)

The hydrolysis of the ester functions of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines **20** were undertaken using the same procedure as described in Section IV.2 using sodium hydroxide (Scheme V.13). Treatment with sodium hydroxide in a THF / H_2O (1:1) solution for 2 days at room temperature, gave the desired compounds **24** in good yields (Table V.8).



Scheme V.13: Synthesis of the 3β-ol-23,24-bisnor-chol-5-en-22-(alkyloic acid) amines **24**

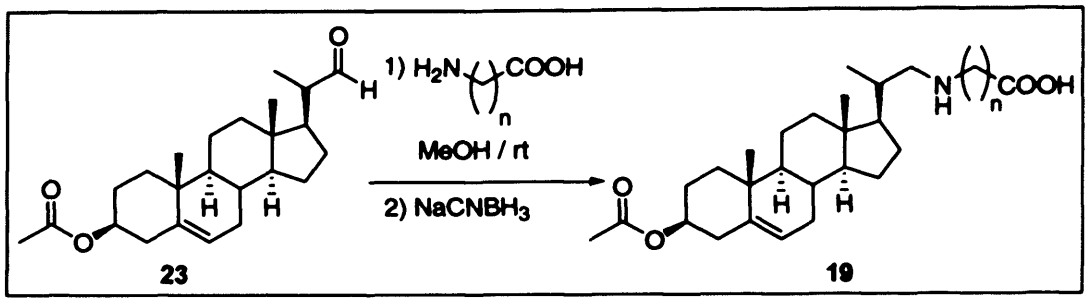
Compounds	**24a**	**24b**	**24c**	**24d**	**24e**	**24f**	**24g**
n	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Yield (%)	84	59	86	83	86	87	100

Table V.8: Yields of the synthesis of the 3β-ol-23,24-bisnor-chol-5-en-22-(alkyloic acid) amines **24**

V.4.3 Synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(alkyloic acid) amines (19)

In vitro experiments (Chapter VI) indicated that some of the amine derivatives (above) were very potent inhibitors of parasite growth. Therefore it was decided to investigate the role of the ester functions at both the 3 position and on the side chain. Analogues with the 3-position still protected as acetate, but with a carboxylic acid function at the end of the side chain were first prepared. The synthesis of only three derivatives was initially undertaken. If any of these compounds showed a good *in vitro* activity, then the synthesis of further examples would be carried out.

The amino acids **2** were directly reacted with the aldehyde derivative **23**, and gave the imine intermediate. After thirty minutes, sodium cyanoborohydride was added, and the solution stirred overnight (Scheme V.14). Compounds with chain length equal to 3, 5 and 7 carbons were prepared (Table V.9)



Scheme V.14: Synthesis of the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(alkyloic acid) amines **19**

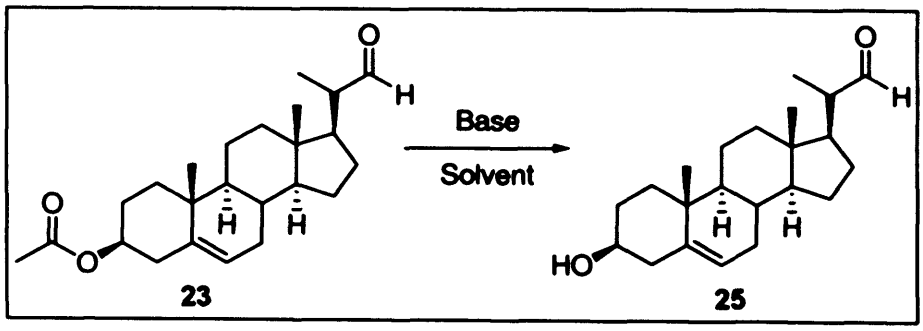
Compounds	19c	19e	19g
n	3	5	7
Yields (%)	63	86	82

Table V.9: Yields of the synthesis of the the 3β-acetoxy-23,24-bisnor-chol-5-en-22-(alkyloic acid) amines **19**

V.4.4 Synthesis of the 3β-ol-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines (26)

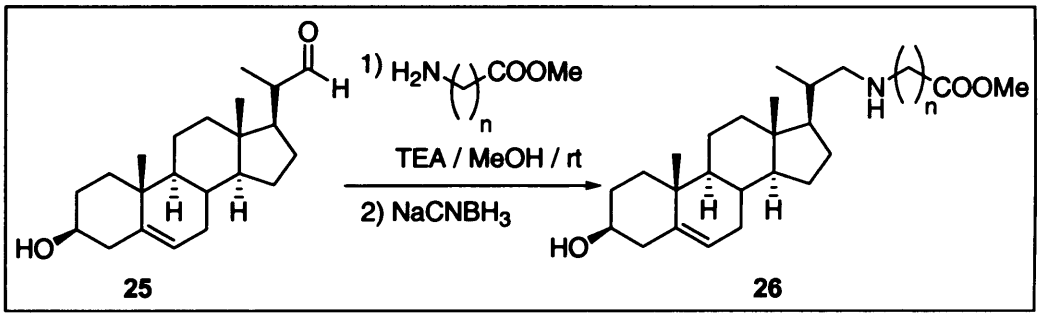
In order to understand the exact role of the 3-position in the sterol moiety, it was also decided to synthesize derivatives with a hydroxyl group at the 3 position and a methyl ester function on the side chain.

The hydrolysis of the aldehyde derivative **23** was investigated using 2 methods. Sodium hydroxide in THF gave only 26% yield whereas potassium carbonate in a mixture methanol/H_2O gave a 94% yield (Scheme V.15).



Scheme V.15: Synthesis of the 3β-ol-23,24-bisnor-chol-5-en-22-al **25**

It was then possible to undertake the reductive amination as described in Section V.4.1. Methyl amino esters **3** were reacted with triethylamine in methanol at room temperature in order to neutralise HCl for thirty minutes (Scheme V.16). The aldehyde derivative **25** was then added to form the imine intermediate. After a further thirty minutes, a solution of sodium cyanoborohydride in THF was added, and the resulting solution stirred overnight. The syntheses were carried out with different chain lengths; after purification by column chromatography, compounds were obtained in good yields (Table V.10).



Scheme V.16: Synthesis of the 3β-ol-23,24-bisnor-chol-5-en-22-(methyl ester alkyloate) amines **26**

Compound	**26a**	**26c**	**26e**	**26g**
n	**1**	**3**	**5**	**7**
Yield (%)	48	61	71	67

Table V.10: Yields of the synthesis of derivatives **26**

The peculiar feature of these compounds with a hydroxyl group at the 3-position is the doubling of most of the signals in ^{13}C-NMR: for example, derivative **26a** has 25 carbons but 38 signals have been identified; Figure V.10 shows the full ^{13}C-NMR spectrum and an expansion where the doubling of carbons 18 and 21 was observed while carbon 19 gave only one signal.

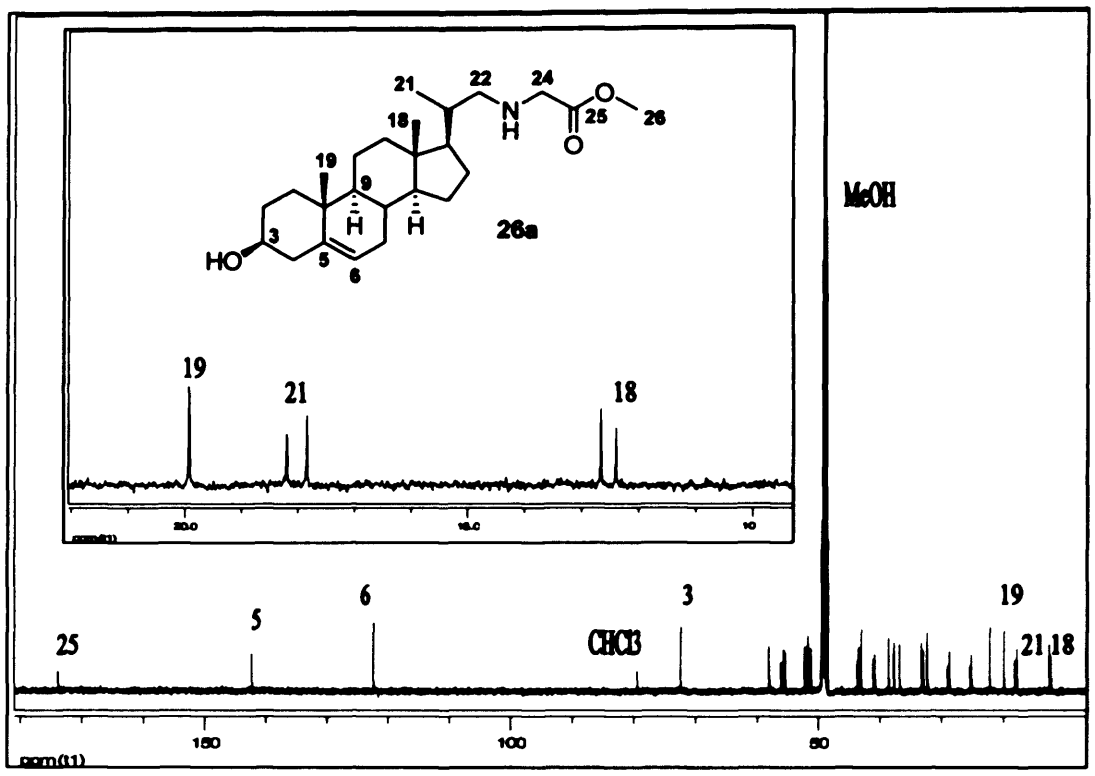
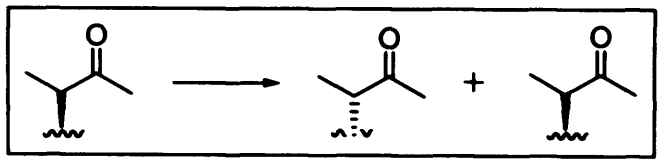


Figure V.10: ^{13}C-NMR of compound **26a**

The doubling up of signals in the ^{13}C-NMR spectrum indicated either two distinct compounds were present probably due to an epimerisation of the aldehyde, or the occurrence of some slow event on the NMR timescale. TLC in a variety of solvents indicated only one compound was present. Mass spectroscopy also only indicated the presence of the desired compound. In the case of epimerisation of the aldehyde (Scheme V.17), the peaks would remain as doublets on heating. This however was not the case.



Scheme V.17: Epimerisation of the aldehyde

To investigate this, ^{13}C-NMR on compound **26a** was carried out at two different temperatures (25°C and 40°C, Figures V.11):

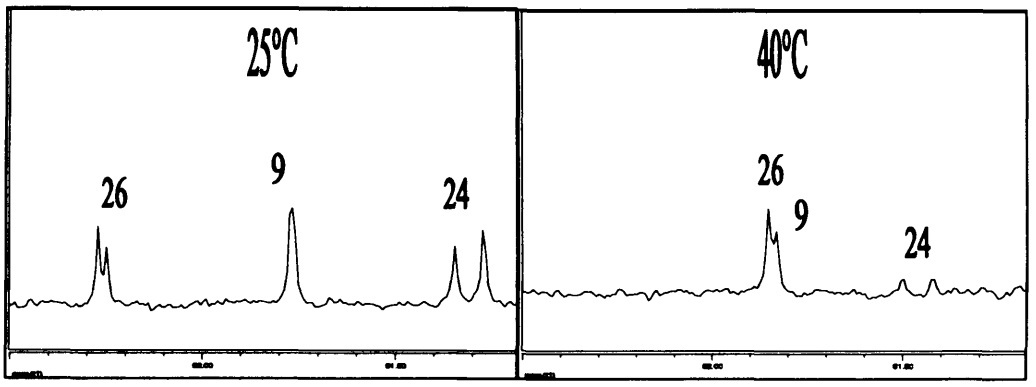


Figure V.11:[13]C-NMR between 51 and 52.5ppm of the 3β-ol-23,24-bisnor-chol-5-en-22-(methyl ethanoate ester) amine **26a** at 25 and 40°C

As a result of these experiments, it was concluded that compound **26a** existed as two distinct conformations and interconversion was slow on the NMR timescale. At 25°C, Figure V.11, carbon 24 and 26 were split into two signals, while carbon 9 was only one. At 40°C, carbon 24 still showed two signals, but carbon 26 had merged into one and moved closer to the signal of carbon 9. The merging of the peaks and displacement up-field of carbon 26 indicated that this compound existed in two different conformations giving two different [13]C NMR.

The same effect was noticed for carbon 25 of compound **26a** (Figure V.12). At 25°C, carbon 25 was split into two signals, while at 40°C carbon 25 merged into one signal and moved slightly downfield.

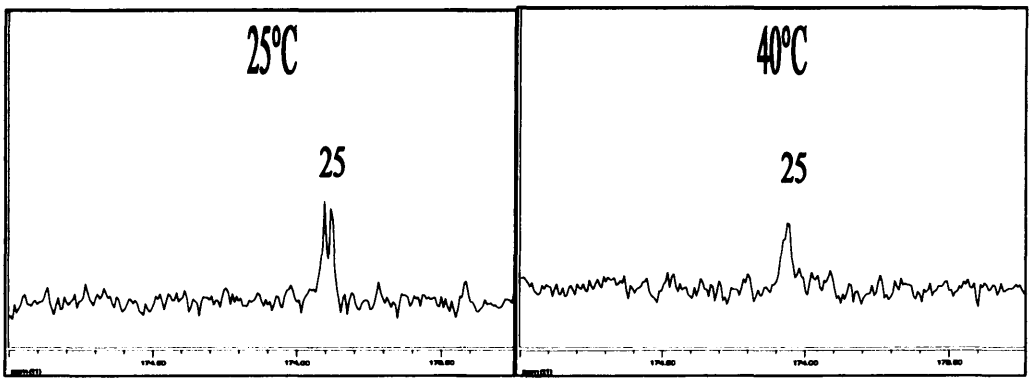
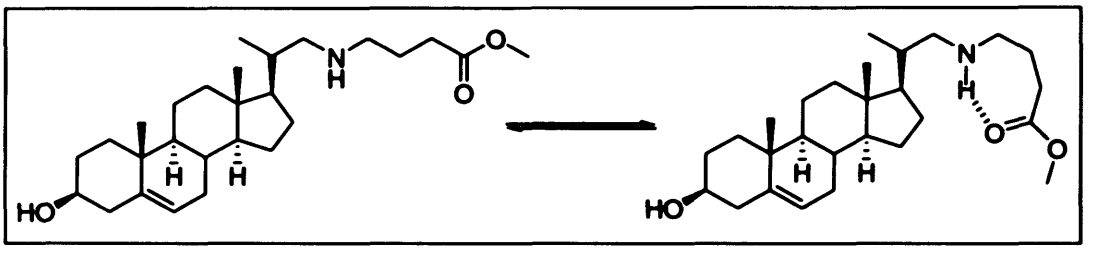


Figure V.12:[13]C-NMR between 173.3 and 175.0 ppm of the 3β-ol-23,24-bisnor-chol-5-en-22-(methyl ethanoate ester) amine **26a** at 25 and 40°C

This NMR experiment showed us that the unexpected number of carbon signals for this series was not due to by-products but instead was due to this series of compounds having two different conformations. As an example, compound **26c**, with a chain length of 3, shows that an interaction between the carbonyl and the amino function in the side chain could lead to a 7 member ring conformation (Scheme V.18). The same phenomenon can occur in all series of compounds. Therefore, it is possible that in solution a mixture of compounds with a free rotation and others with a blocked conformation exists.



Scheme V.18: Equilibrium between the two conformations of 3β-ol-23,24-bisnor-chol-5-en-22-(methyl butanoate ester) amine **26c**

The displacement up-field (^{26}C) or downfield (^{25}C) can be explained by a change of environment in the whole structure of the compound. As an example, the chemical shift of carbon 9 in the amide derivative **4a** is 49.9ppm while in the amine derivative **20a** it is at 50.4 (Figure V.13). A similar effect was seen for carbon 26 in which the signal moved from 52.4ppm to 52.2ppm. Although the only change in the structure was the presence of an oxo group at the 22-position for compound **4a**, all chemical shifts of the sterol nucleus and the side chain were affected.

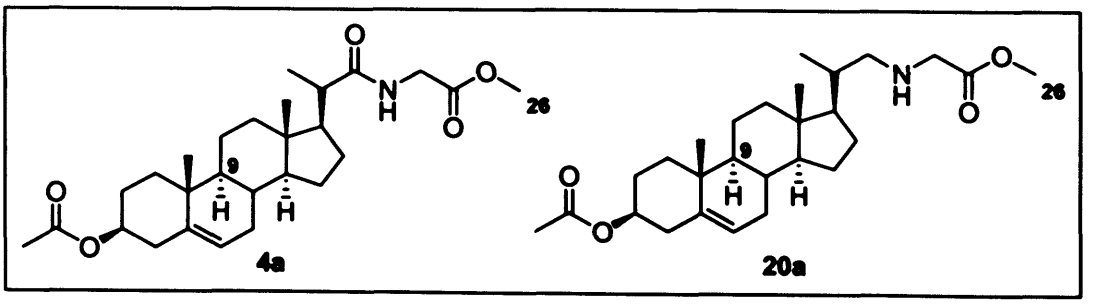


Figure V.13: Derivatives **4a** and **20a**

V.4.5 Synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(3-(α–amino acid alkyl) amines

After the completion of this series, the synthesis of compounds with an amino acid function (Figure V.2) at the end of the chain length was undertaken. As mentioned in the Chapter IV (Section IV.3), compounds bearing an amino acid function in the side chain (Figure IV.6) would be relevant as they should mimic SAM (Figure V.14).

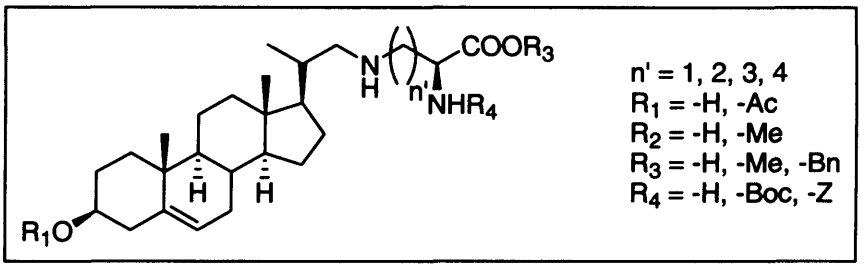


Figure V.14: Target molecules

The synthesis of these compounds was previously attempted in this laboratory by Silvia Lorente[126] starting with the tosylate derivative **18** but was unsuccessful, with recovery of only starting material. Thus, the reductive amination as developed here, seemed to be a good alternative. The reactions were first carried out with Boc-Dap-OH and Boc-Dab-OH (Figure V.15).

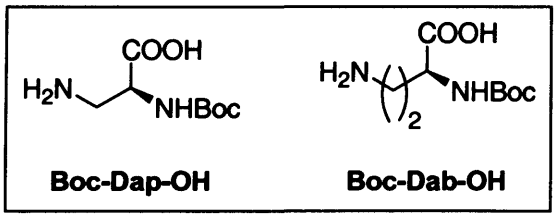
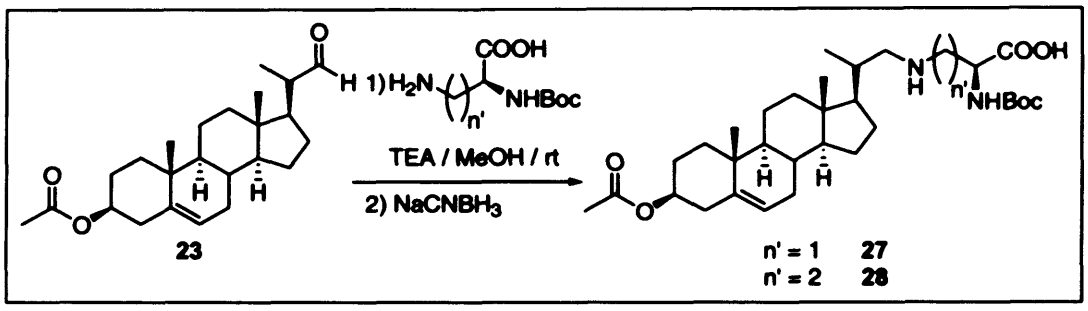


Figure V.15: Boc-Dap-OH and Boc-Dab-OH

Boc-Dap-OH and Boc-Dab-OH were reacted with the aldehyde derivative **23** (Scheme V.19). After thirty minutes, sodium cyanoborohydride was added and the solution stirred overnight at room temperature. The amine

derivatives **27** and **28** were obtained after extraction and purification by column chromatography in yields of 77 and 80% respectively.



Scheme V.19: Synthesis of amine derivatives **27** and **28**

Other commercially available amino acids such as Z-Orn-OH and Z-Lys-OBzl (Figure V.16) should also have given access to further amino acid substituted analogues.

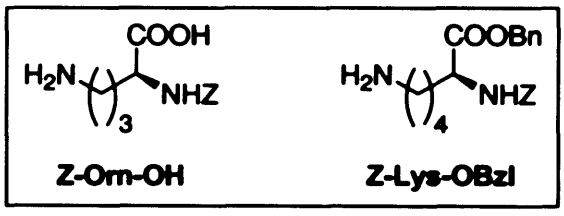
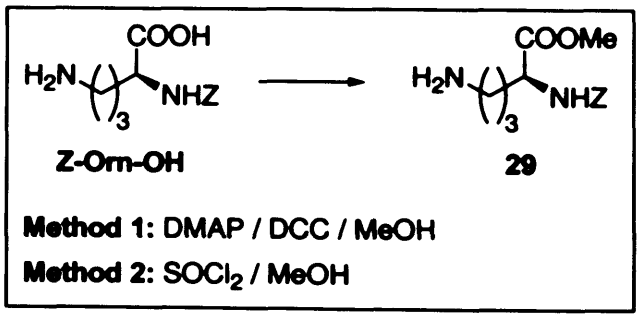


Figure V.16: Z-Orn-OH and Z-Lys-OBzl

In Chapter IV, Section IV.3.1, the attempted protection of Boc-Dap-OH and Boc-Dab-OH was reported in an attempt to improve the lipophilicity of the final compounds. Z-Orn-OMe is not commercially available. Therefore, we wanted here to protect the acid function of Z-Orn-OH as a methyl ester (Scheme V.20).



Scheme V.20: Synthesis of Z-Orn-OMe **29**

The first method attempted was a coupling reaction using DCC and DMAP, but unfortunately only starting material was recovered. As was found to be the case with Boc-Dap-OH (Section IV.3.1), the alcohol should be added last in this type of reaction, which was not the case here as methanol was used as solvent and reagent.

The second method used was protection of the amino acid **2**, with thionyl chloride in methanol. A less polar spot was identified by TLC and the crude was purified by column chromatography using triethylamine (1%). A product was recovered in 38% yield but was not identified as Z-Orn-OMe after analysis by ^{1}H NMR. In fact, the byprodyct **30** was obtained (Figure V.17).

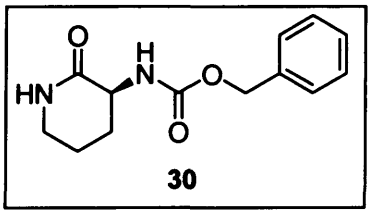
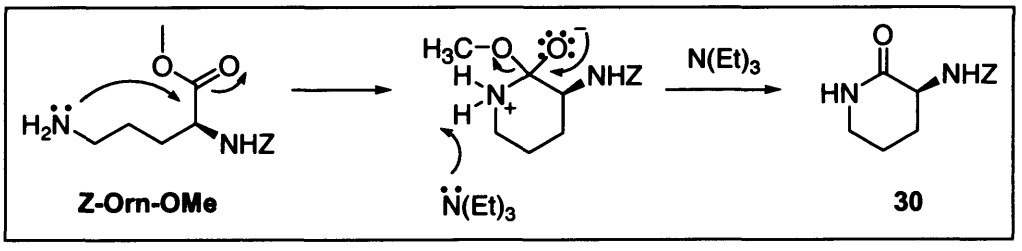


Figure V.17: byproduct **30**

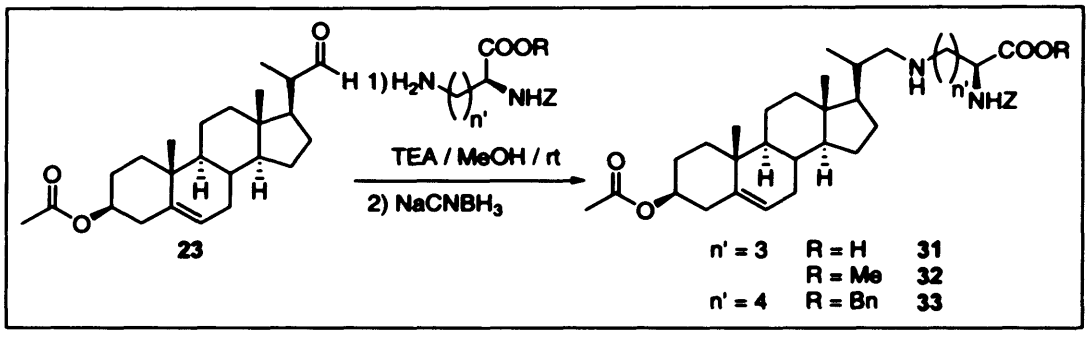
The mechanism of action can be explained as follow (Scheme V.21): an intermolecular reaction occurred with attack of the amine function onto the ester leading to a 6 member ring intermediate which after rearrangement in the presence of triethylamine, gave the byproduct **30**.



Scheme V.21: Mechanism of cyclisation of Z-Orn-OMe

The reaction was then repeated, and the purification by column chromatography carried out without triethylamine affording the desired product **29** in 75% yield.

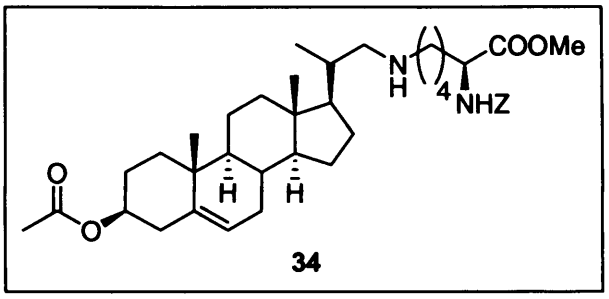
Subsequently, the reductive aminations with the α-amino acids without a carboxylate protective group and the 3β-acetoxy-23,24-bisnor-5-en-22-al **23** were carried out (Scheme V.22). The amine derivative **31** was obtained after purification by column chromatography in 57% yield.



Scheme V.22: Synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(α-amino acid alkyl) amines **31**, **32** and **33**

The reductive amination leading to compound **32** was unsuccessfull, and starting material **23** (15%) and alcohol derivative **17** (2%) were recovered. It is possible that TEA in a slight excess (1.5eq.) in the solution, reacted with Z-Orn-OMe as shown Scheme V.21. Therefore, the reductive amination could not occur. The slow addition of 1 equivalent of triethylamine to a diluted solution of Z-Orn-OMe.HCl in methanol should allow the reductive amination to happen. Unfortunately, due to the lack of time, this reaction was not attempted.

Finally, for the lysine derivative, two close spots were identified by TLC. Two compounds were obtained after purification by column chromatography: the expected compound **33** was obtained in 23% yield. The byproduct **34** (Figure V.18) was also identified in 9% yield resulting from a transesterification with methanol of **33**.



34

Figure V.18: Byproduct **34**

Figure V.19 is the ^{1}H-NMR spectrum of derivatives **33** and **34**. The typical proton signals from the sterol nucleus are present in both NMRs: 3, 4, 6, 18, 19, 21, 22, 23, 24 and 2'. Proton 28 (between the amino and carboxylate functionality) and proton 31 (from the benzyl group) were visible in both spectra at approximately the same chemical shift. There are only three differences:

- At 7.40ppm, the integration shows the presence of 10 protons for compound **33** and only 5 for compound **34**; clearly, a phenyl group was lost;

- The signal of the methylene group 37 at 5.22ppm was not present; therefore the whole benzyl group next to carbon 36 was not present in compound **34**;

- Appearance at 3.79ppm of a singlet which integrates for 3 protons, typical of methyl ester signals: in conclusion, a transesterification by methanol took place.

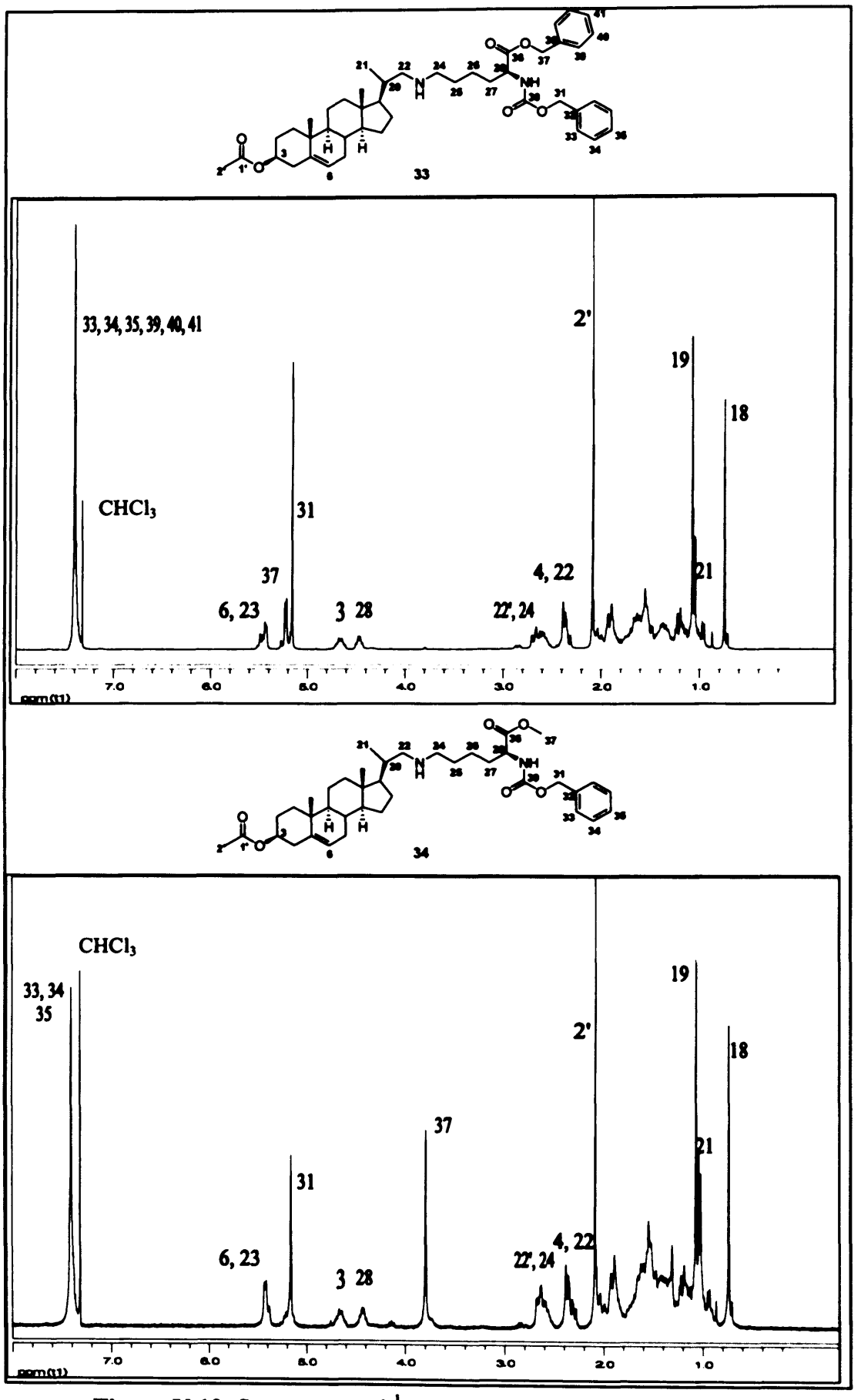
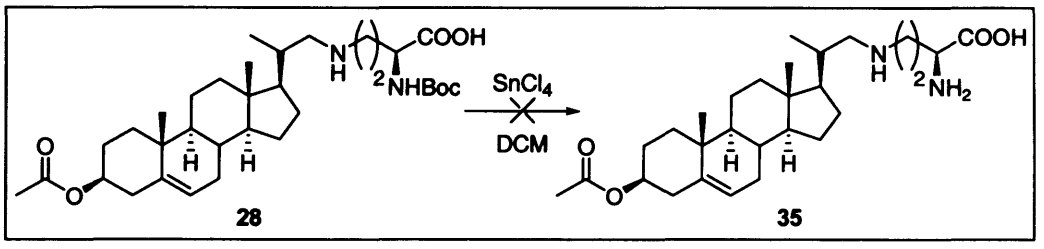


Figure V.19: Structures and ^{1}H NMR of compounds **33** and **34**

V.4.6 Attempted deprotections of the 3β-acetoxy-23,24-bisnor-5-en-22-(α-amino acid) amines

Previously (Section IV.3.3), deprotection of the Boc group failed using TFA. In the literature[142], other reagents have been shown to be efficient in removal of Boc groups. Tin (IV) chloride was reported by Miel and Rault[167] for the total and clean deprotection of *N,N'*-bis-Boc guanidine (Scheme V.23).

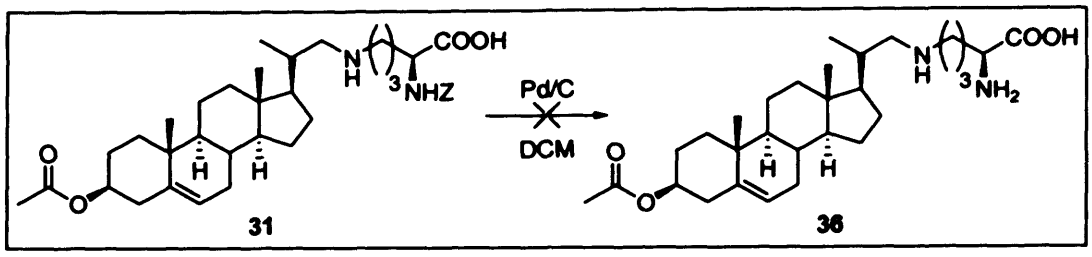


Scheme V.23: Attempted Boc deprotection of compound **28**

After stirring tin (IV) chloride and 3β-acetoxy-23,24-bisnor-5-en-22-(4-(2-Boc-amino)butanoic acid) amine **28** in DCM for 2h, TLC showed the consumption of starting material and the appearance of a lower spot. Tin (IV) chloride in excess and the solvent were removed under vaccum. Analysis by ^{1}H NMR showed the presence of compound **35** with disappearance of the Boc group. Mass spectrum (ES+) confirmed the presence of compound **35** with the correct molecular weight at 475.2 corresponding to $(M+H)^+$. Mass spectrum (ES^-) also showed the presence of tin (IV) chloride in the crude. Therefore, recrystallisation in cold ethanol was carried out, but unfortunately compound **35** could not be obtained pure. Other purification methods such as reverse phase column chromatography might yield pure product.

The deprotection of the Z group of amine **31** by hydrogenation[142] was also investigated (Scheme V.24).

[167] Miel, H.; Rault, S. Total Deprotection of N,N'-Bis(tert-Butoxycarbonyl)guanidines Using $SnCl_4$. *Tetrahedron Lett.* **1997**, *38*, 7865-7866.



Scheme V.24: Attempted deprotection of compound **31**

The first attempt with Pd/C 10% was successful. After 5 hours, TLC showed loss of the starting material and the appearance of a spot with lower R_f. The reaction was stopped, but after filtration on celite with DCM, nothing was recovered. The second attempt was carried out in exactly the same conditions, but the filtration was undertaken with methanol. An inseparable mixture of the desired product **36** and the compound with the carbon-carbon double bond reduced **37** (Figure V.19) was obtained.

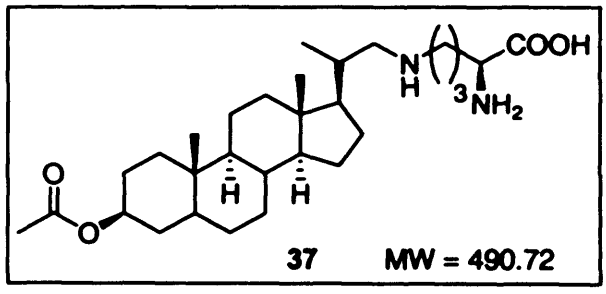


Figure V.19: byproduct **37**

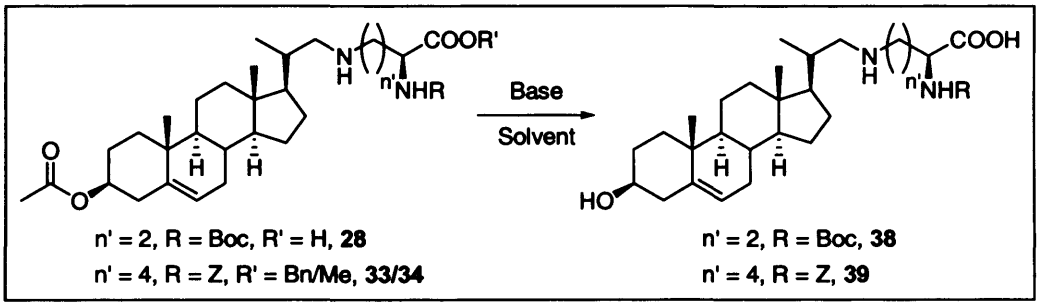
Analysis of the ^1H NMR showed a complete disappearance of the benzyl group, but also that the integration of proton 6 (double bond) did not correspond to one when compared to proton 3 for example. Moreover, mass spectrum (ES+) showed two peaks: peak at 489.4 showed the presence of compound **36**, while peak 491.4 the presence of compound **37**. Therefore, a partial reduction of the double bond in the sterol nucleus occurred. No purification of the mixture was attempted.

A third attempt was undertaken using Pd/C 1%, but after 24h, only starting material **31** was recovered. Finally, the hydrogenation was carried out with Pd/C 5%. After 7 hours, a less polar spot was identified by TLC. The

crude was filtered on celite with methanol, and analysis of the crude by NMR showed the presence of both starting material and compound **36**. Purification was attempted by differential solubilisation in methanol, as the starting material **31** was found to have low solubility in methanol, but compound **36** could not be obtained pure. Purification of the crude using reverse phase column chromatography might afford product **36**.

V.4.7 Synthesis of the 3β-ol-23,24-bisnor-5-en-22-(α–amino acid alkyl) amines

Different series of compounds can be obtained by differentially deprotecting the acid and amine functions on the side chain, and hydroxyl at the 3 position. We decided to carry out the hydrolysis of the acetate at the 3-position to obtain the free hydroxyl compounds (Scheme V.25).



Scheme V.25: Hydrolysis of compound **28** and mixture **33/34**

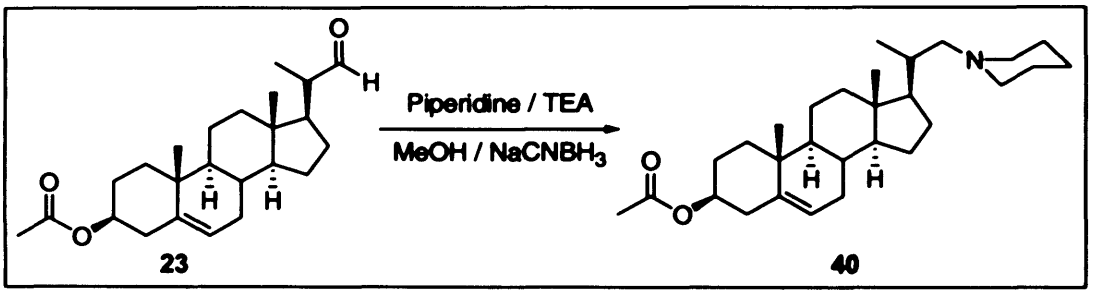
Compound **28** was reacted with potassium carbonate in a mixture MeOH / H_2O for 48h at room temperature. Compound **38** was obtained in 70% yield.

As described in Section V.4.5, the reductive amination with the lysine derivative led to two compounds **33** and **34**. This reaction was undertaken again and the crude directly used in the next step. The hydrolysis of the mixture was carried out with sodium hydroxide in THF for 48h at room temperature, and led to compound **39** in a reasonable 23% yield (the yield was calculated for the two steps).

^{13}C NMR analysis of both compounds **38** and **39** resulted again in the appearance of doubled signals for most of the carbon like derivatives **26** (Section V.4.4).

V.4.8 Synthesis of the 3β-acetoxy-23,24-bisnor-5-en-22-(piperidyl) amine (40)

As part of the scale-up synthesis of 3β-acetoxy-23,24-bisnor-5-en-22-(piperidyl) amine **40**[126], a reductive amination with piperidine and the aldehyde derivative **23** was carried out (Scheme V.26). Sodium cyanoborohydride was then added and the resulting solution stirred overnight. After extraction and purification by column chromatography, compound **40** was obtained in 38% yield.

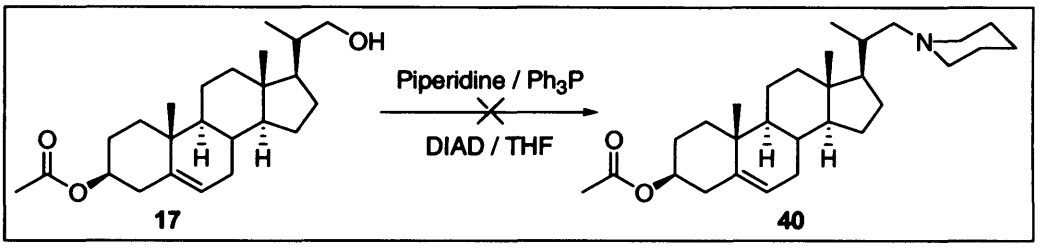


Scheme V.26: Synthesis of the piperidyl derivative **40**

This compound had already been obtained in this laboratory[126] by a nucleophilic substitution on the tosyl derivative **18** with 51% yield. In order to improve the yield, we attempted the reductive amination previously described but this resulted in a lower yield. The reductive amination was then revisited. In the absence of TEA, compound **40** was obtained in only 24% yield. The reductive amination was also carried out in acidic conditions by adding acetic acid in the solution and compound **40** was obtained in 53% yield.

A Mitsunobu reaction was also attempted in an approach to increase yield. The alcohol derivative **17**, piperidine and triphenylphosphine were dissolved in THF at room temperature. DIAD was then added and the solution

stirred for 72h. Only starting material was recovered (98%). A second attempt was carried out with addition of DIAD at 0°C and then stirring at room temperature. This time again, only starting material was recovered (99%). Looking closer at the mechanism of action of this reaction[168], it appeared that DIAD reacts first with triphenylphosphine. The addition of DIAD last probably led to the non-production of compound **40**.



Scheme V.26: Attempted synthesis of the piperidyl derivative **40**

V.5 Summary

Different series of compounds with an amine function at position 23 in the side chain of the sterol were synthesised. The first series of compounds **20** were finally obtained by a reductive amination with the methyl amino esters **3** and the aldehyde derivative **23**. The fully deprotected compounds **24** were obtained by hydrolysis with sodium hydroxide. Series of compounds **19** and **26** were then obtained respectively by reductive amination with the amino acid **2** and aldehyde **25** which was previously obtained by hydrolysis of aldehyde **23**. The final series synthesised were compounds with a α-amino acid function at the end of the chain length. Some deprotections were attempted leading to different derivatives, but some others will be the concern of future work.

All series have been sent for *in vitro* or *in vivo* testing and results are discussed in chapter VI.

[168] Clayden, J.; Greeves, N.; Warren, S.; Wothers, P. Organic Chemistry. *Oxford University Press* **2001**.

CHAPTER VI

BIOLOGICAL EVALUATION

Compounds synthesised in Chapters IV and V were evaluated against four different parasites: *Leishmania donovani* (Leishmaniasis), *Trypanosoma cruzi* (Chagas' disease), *Trypanosoma brucei rhodesiense* (HAT) and *Plasmodium falciparum* (3D7 or K1 strains). Four compounds were also tested *in vivo* against the rodent model of *T.b. rhodesiense*. The biological assays (*in vitro* and *in vivo*) were carried out by the group of Dr. Simon Croft at the 'London School of Hygiene and Tropical Medicine' (London), according to procedures reported in the Appendix 1.

The parasites which give rise to these diseases were discussed in Chapter I. They are essentially parasites of the blood stream, although some

have adapted to an intracellular existence[169]. They have different morphological forms which change during the life cycle of the parasite. *Leishmania spp* and *T. cruzi* synthesize ergosterol and other sterols in all their different life cycle stages[169]. On the other hand, only the procyclic form (vector form) of *T. brucei* is thought to synthesize ergosterol[170]. The blood stream form of *T. brucei* does not synthesize sterols *de novo* and obtains cholesterol acquired from exogenous sources[70]. Similarly sterol biosynthesis is not predicted to occur in *P. falciparum*[171].

The compounds synthesized (Chapter IV and V) were expected to inhibit ergosterol biosynthesis by inhibition of the sterol 24-methyltransferase (24-SMT) enzyme. This should lead to growth inhibition of the parasites and eventually death. Biological evaluation of the compounds was carried out with the clinically relevant forms of the parasites (Table VI.1). Biological evaluation of both *T.b. rhodesiense* and *P. falciparum* were carried out as part of routine screening, although no *de novo* ergosterol biosynthesis is thought to occur in the human stage of these organisms.

Parasite	***L. donovani***	***T. cruzi***	***T.b. rho.***	***P. falc.***
Life cycle stage	Intracellular amastigote	Intracellular amastigote	Blood stream form	Red blood cell stage

Table VI.1: Clinically relevant forms of the parasites used for the *in vitro* screening of the compounds

The biological results are reported below. *In vitro* and *in vivo* assays of the derivatives synthesized will be discussed first in Section VI.1. Then,

[169] Goad, L. J.; Holz, G. G.; Beach, D. H. Sterols of Leishmania Species - Implications for Biosynthesis. *Mol. Biochem. Parasitol.* **1984**, *10*, 161-170.

[170] Buckner, F. S.; Nguyen, L. N.; Joubert, B. M.; Matsuda, S. P. T. Cloning and Heterologous Expression of the *Trypanosoma brucei* Lanosterol Synthase Gene. *Mol. Biochem. Parasitol.* **2000**, *110*, 399-403.

[171] http://www.genome.ad.jp/dbget-bin/get_pathway?org_name=pfa&mapno=00100

enzyme assays on both recombinant *T.b. brucei* and *L. major* enzymes are presented in Section VI.2.

VI.1 Biological assays

Table VI.2 shows controls used in assays against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and two *P. falciparum* strains (3D7, and K1 which is chloroquine resistant) and their respective ED_{50} values.

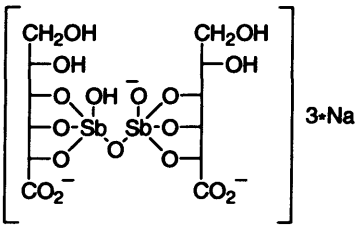
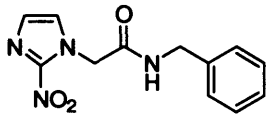
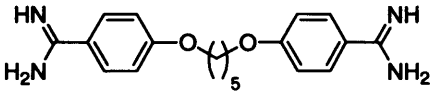
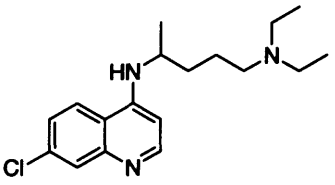
Parasite	Drug	ED_{50} (μM)
L. donovani	 **Sodium stibogluconate (Pentostam)**	> 10
T. cruzi	 **Benznidazole**	~ 1
T.b. rhodesiense	 **Pentamidine**	~ 0.001
***P. falciparum* (3D7)** ***P. falciparum* (K1)**	 **Chloroquine**	~ 0.003 ~ 0.1

Table VI.2: Structures and ED_{50} values of control drugs against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and *P. falciparum* (3D7 and K1)

Results of the biological screening are shown as ED_{50} values (μM), which is the minimum effective dose of inhibitor required to kill 50% of the parasites in culture. Toxicity against macrophages was also evaluated as TD_{50},

which indicates the minimum effective dose of inhibitor required to kill 50% of uninfected cells and it is a measure of the cytotoxicity towards mammalian cells.

VI.1.1 *In vitro* assays of the amide derivatives

Two series of 3β-acetoxy and one series of 3β-hydroxy amide derivatives synthesized Chapter IV were screened *in vitro* against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*. The data are presented into two tables, one for the 3β-acetoxy derivatives (Section VI.1.1.1) and another one for the 3β-hydroxy derivatives (Section VI.1.1.2). The screening of all derivatives has not been completed yet.

VI.1.1.1 3β-Acetoxy amide derivatives

Amide derivatives bearing an acetate group at the position 3 were first evaluated (Table VI.3). The Series I are compounds with a terminal methyl ester function in the side chain. Two Series I (I^a and I^b) have been synthesized as series I^a was contaminated by diisopropylurea (DIPU, Section IV.1.2). Neither series showed toxicity which means that DIPU itself was not toxic for mammalian cells.

The derivatives I^a proved to be totally inactive against both *L. donovani* and *P. falciparum* with ED_{50} values over 50μM (Table VI.3). On the other hand, this series was active against both *T. cruzi* in the micromolar range and *T.b. rhodesiense* in the nanomolar range. The best derivative was in both cases compounds **4d** with IC_{50} values of 3.6 and 0.10μM respectively. This shows the importance of the chain length where in this particular case n = 4 seems to be the best.

Series I^b was also screened. Against *T.b. rhodesiense*, the same trend as for series I^a was observed as even though a global loss of activity appeared (30 fold less for compound **4d**). That would suggest that DIPU act synergistically with the inhibitors by also interacting with the parasite metabolism. The most

potent compound was **4g**, which showed a similar activity as with the series I[a] and an IC_{50} value of 0.61μM. This series was also evaluated against both strain of *P. falciparum*. Compound **4d** showed a good activity against the 3D7 strain and an IC_{50} value of 0.74μM, while compound **4f**, with a longer side chain, was evaluated as the more potent against K1 strain with an IC_{50} value of 1.3μM.

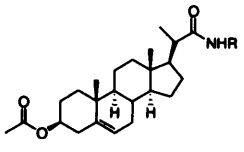
Series		WSP	ED_{50} (μM) *L. dono.*	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)	ED_{50} (μM) *P. falc.* (K1)	TD_{50} (μM) Toxicity
	Control drugs	-	> 10	~ 1	~0.001	~0.003	~0.1	-
I	**4a**, R=$(CH_2)CO_2Me$	**991**[a]	-	>65	>65	>65	-	>652
		1307[b]	-	-	24.0	60.0	9.6	>652
	4b, R=$(CH_2)_2CO_2Me$	**990**[a]	-	13.0	5.3	>63	-	>633
		1308[b]	-	-	6.0	17.4	19.1	597.3
	4c, R=$(CH_2)_3CO_2Me$	**844**[a]	>61	5.7	0.12	>61	-	>615
		1309[b]	-	-	9.1	16.2	11.6	>615
	4d, R=$(CH_2)_4CO_2Me$	**845**[a]	>59	3.6	0.10	>59	-	>597
		1310[b]	-	-	3.0	0.74	8.43	>597
	4e, R=$(CH_2)_5CO_2Me$	**846**[a]	>58	3.7	0.17	>58	-	>581
		1311[b]	-	-	3.4	21.3	19.8	>581
	4f, R=$(CH_2)_6CO_2Me$	**847**[a]	>56	10.2	3.2	>56	-	>566
		1312[b]	-	-	7.9	14.4	1.3	>566
	4g, R=$(CH_2)_7CO_2Me$	**848**[a]	>55	25.6	0.24	>55	-	>551
		1313[b]	-	-	0.61	21.5	2.9	>551
II	**14a**, R=Boc-Dap-OH	**954**	>52	8.7	2.7	-	-	-
	14b, R=Boc-Dab-OH	**989**	>50	26.8	17.5	-	-	-

Table VI.3: Biological evaluation of the 3β-acetoxy amide derivatives (Series I and II)

Figure VI.1 shows the biological evaluation of the 3β-acetoxy amide derivatives (Series I) against *T. cruzi*, *T.b. rhodesiense*.and *P. falciparum*. This

shows the importance of chain lengths for activity. For *T. cruzi*, the shortest and longest chain length led to a poor activity with the best result for compound **4d** (n = 4). For *T.b. rhodesiense*, series I^a and I^b led to a similar trend, where the longer the chain is, the better the activity. Finally, different trends were obtained against *P. falciparum* strains with compound **4d** being the most potent against 3D7 strain and compound **4f** against K1 strain. The high activity of compound **4g** suggested that the synthesis of derivatives with a long chain length might be the concern of future work.

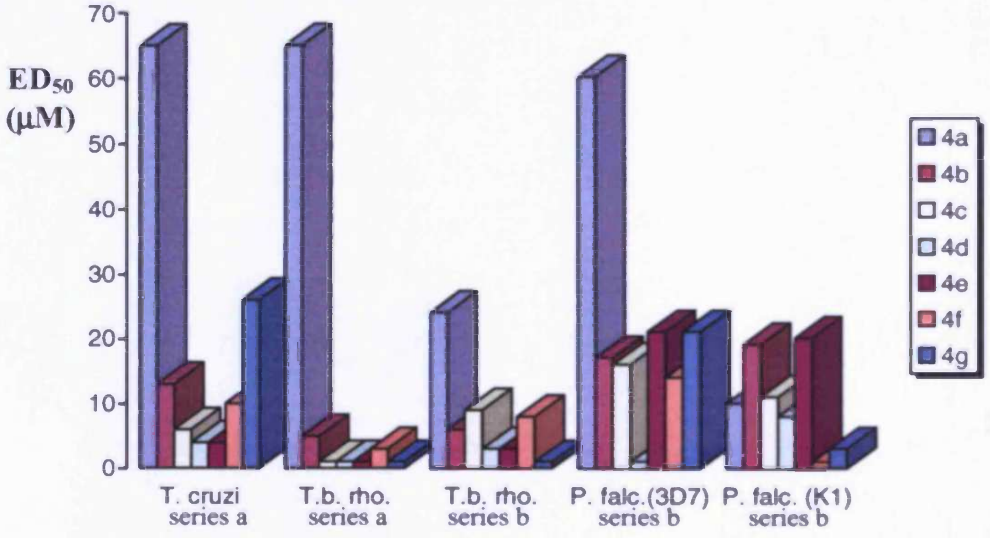


Figure VI.1: Graphical representation of the biological evaluation of the 3β-acetoxy amide derivatives (Series I) against *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*

Finally, derivatives bearing a terminal amino acid function in the side chain were screened (Series II, Table VI.3). Both compounds **14a** and **14b** were inactive against *L. donovani*. Better results were observed against both *T. cruzi* and *T.b. rhodesiense* with compound **14a** having IC_{50} values of 8.7 and 2.7µM respectively. This series II, which is supposed to mimic the amino acid function of the SAM moiety, was found to be less active, especially compound **14b**. The presence of a bulky group (Boc) on the amino function in the side chain might interfere with the binding toward the enzyme.

None of the compounds showed significant toxicity to mammalians cells. Therefore, compounds **4** showed a good selectivity against *T.b. rhodesiense* (for example, 6000 fold for compound **4d**). Both compounds **4c** and **4g** were sent for *in vivo* screening (Section VI.1.3).

VI.1.1.2 3β-Hydroxy amide derivatives

Analogues with a free hydroxyl group at position 3 of the sterol nucleus were expected to be more active as the 3-OH is thought to have an important interaction with the sterol binding site. The data in Table VI.4 showed no improvement of activities compared to previous derivatives. Series III was shown to be inactive against both *L. donovani* and *P. falciparum*. This series showed better activities against *T. cruzi* and *T.b. rhodesiense* as for the 3β-acetoxy amide derivatives. Again, the derivatives with the shortest chain were inactive, while derivatives with the longer chain were more potent. Compound **8g** was found to be the more potent of the series with IC_{50} values of 6.5 and 0.43μM against *T. cruzi* and *T.b. rhodesiense* respectively.

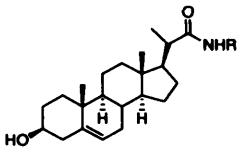
Series		WSP	ED_{50} (μM) *L. Dono.*	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)	TD_{50} (μM) Toxicity
	Control drugs	-	> 10	~ 1	~0.001	~0.003	-
III	**8a**, R= $(CH_2)CO_2H$	**995**	-	>74	>74	>74	>743
	8b, R= $(CH_2)_2CO_2H$	**994**	-	>71	>71	>71	615.4
	8c, R= $(CH_2)_3CO_2H$	**849**	29.2	39.8	49.8	45.6	616.3
	8d, R= $(CH_2)_4CO_2H$	**850**	>67	32.8	7.8	>67	560.5
	8e, R= $(CH_2)_5CO_2H$	**851**	>65	11.4	10.4	>65	302.8
	8f, R= $(CH_2)_6CO_2H$	**852**	>63	34.5	2.3	>63	252.1
	8g, R= $(CH_2)_7CO_2H$	**853**	25.0	6.5	0.43	>61	377.5

Table VI.4: Biological evaluation of the 3β-hydroxy amide derivatives (Series III)

Figure VI.2 shows the biological evaluation of the 3β-hydroxy amide derivatives (Series III) against *T. cruzi* and *T.b. rhodesiense*. Compound **8g** with the longest chain (n = 7) showed the best activities. These results suggest again that the synthesis of derivatives with longer chains may be a future direction for the project.

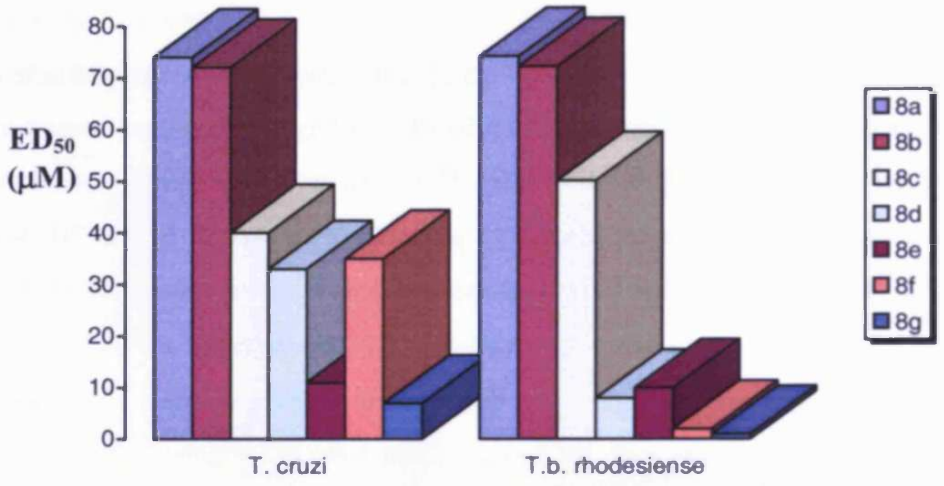


Figure VI.2: Graphical representation of the biological evaluation of the 3β-hydroxy amide derivatives (Series III) against *T. cruzi* and *T.b. rhodesiense*

All series showed some relative toxicity toward mammalian cells. Nevertheless, the selectivity of the most active derivatives is still good (900 fold for example for compound **8g** compared to *T.b. rhodesiense).*

VI.1.2 *In vitro* assays of the amine derivatives

Three series of 3β-acetoxy and three series of 3β-hydroxy amine derivatives (Chapter V) were screened *in vitro* against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*. The data are presented in four tables, two tables for the 3β-acetoxy derivatives (Section VI.1.2.1) and two tables for the 3β-hydroxy derivatives (Section VI.1.2.2). The screening of all derivatives has not been completed yet.

VI.1.2.1 3β-Acetoxy amine derivatives

Generally, the 3β-acetoxy amine derivatives showed better activities than the amide analogues previously reported (Section VI.1.1.1). The series I have a terminal ester function in the side chain (Table VI.5). The amide analogues were not active against *L. donovani* and *P. falciparum*. The amine derivatives showed some potency against both: no particular trend was observed against *L. donovani* as the most active derivative was compound **20c** with an IC_{50} value of 3.2μM, which is of interest if compared to the drug in use, Pentostam (> 10μM); compounds **20f** and **20g** also showed good activities with IC_{50} values of 9.5 and 14.8μM respectively, while the compound the least active in this series against *L. donovani* was compound **20d** (>61μM); on the other hand, a trend was observed against *P. falciparum* as the derivatives with the longest chain showed the best activities; compound **20g** was found the most active and had an IC_{50} value of 2.6μM.

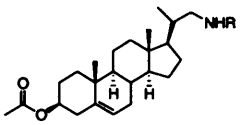
Series		WSP	ED_{50} (μM) *L. Dono.*	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)	TD_{50} (μM) Toxicity
	Control drugs	-	> 10	~ 1	~0.001	~0.003	-
IV	**20a**, R=$(CH_2)CO_2Me$	**993**	-	5.6	7.8	>67	94.5
	20b, R=$(CH_2)_2CO_2Me$	**992**	22.8	28.9	0.30	>65	17.4
	20c, R=$(CH_2)_3CO_2Me$	**879**	3.2	24.7	0.012	15.4	19.2
	20d, R=$(CH_2)_4CO_2Me$	**880**	>61	11.7	0.032	8.6	26.1
	20e, R=$(CH_2)_5CO_2Me$	**881**	21.9	20.5	0.033	7.3	15.5
	20f, R=$(CH_2)_6CO_2Me$	**882**	9.5	53.5	0.025	3.5	11.2
	20g, R=$(CH_2)_7CO_2Me$	**883**	14.8	28.7	0.098	2.6	10.0

Table VI.5: Biological evaluation of the 3β-acetoxy amine derivatives (Series IV)

Surprisingly, series IV was found less active against *T. cruzi* than the amide analogues (Series I[a], Table VI.3). The best derivative was found to be

the compound with the shortest side chain, compound **20a** with an IC_{50} value of 5.6μM. Very potent activities were detected against *T.b. rhodesiense* with IC_{50} values in the nanomolar range for the whole series, except compound **20a** with the shortest side chain (7.8μM). A slight trend was also observed, as the derivatives with medium chain length were the most active (**20c**, **20d**, **20e** and **20f**: IC_{50} ~ 12 to 33nM). The amine derivatives of this series were more toxic than the amide analogues. Therefore, the activities against *L. donovani*, *T. cruzi* and *P. falciparum* are not significant whereas against *T.b. rhodesiense*, a good selectivity was found (for example, 1600 fold for compound **20c**).

Figure VI.3 shows the biological evaluation of the 3β-acetoxy amine derivatives (Series IV) against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*. As previously mentioned, no particular trend was observed against *L. donovani* with compound **20c** being the most active. Compound **20d** was found to be the most active against *T. cruzi*, while the derivatives with the longest side chains were found the more potent against both *T.b. rhodesiense* and *P. falciparum*. Both compounds **20c** and **20g** were sent for *in vivo* screening (Section VI.1.3).

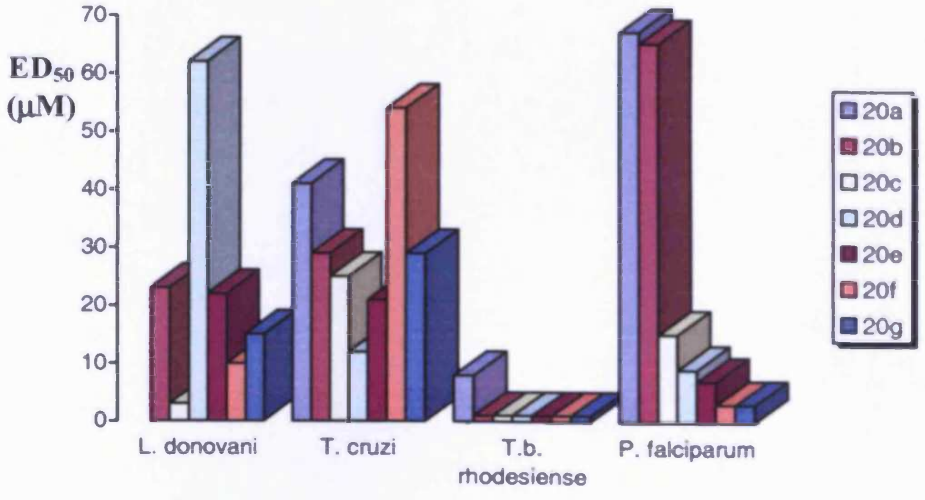


Figure VI.3: Graphical representation of the biological evaluation of the 3β-acetoxy amine derivatives (Series IV) against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*

Two other series with a 3β-acetoxy group were evaluated. Series V has a terminal acid function in the side chain whereas series VI has a terminal amino acid function.

Series V were inactive against both *L. donovani* and *P. falciparum*. Against *T. cruzi*, similar IC_{50} values as in series IV were obtained with compound **19c** being the most active with an activity of 12.6μM. A loss of activity was also observed against *T.b. rhodesiense* for compounds **19c** and **19e**, whilst derivative **19g** with the longest side chain showed a poor activity of 9.1μM. These derivatives are less toxic than in series IV except for compound **19g**.

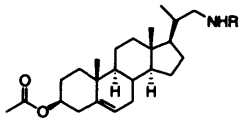
Series		WSP	ED_{50} (μM) *L. dono.*	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)	ED_{50} (μM) *P. falc.* (K1)	TD_{50} (μM) Toxicity
	Control drugs	-	> 10	~ 1	~0.001	~0.003	~0.1	-
V	**19c**, R= $(CH_2)_3CO_2H$	**999**	>65	12.6	54.6	>65	-	504.7
	19e, R= $(CH_2)_5CO_2H$	**997**	>61	>61	55.2	>61	-	432.6
	19g, R= $(CH_2)_7CO_2H$	**998**	-	21.0	9.1	>58	-	58.7
VI	**27**, R= Boc-Dap-OH	**955**	>53	4.6	>53	-	-	-
	28, R= Boc-Dab-OH	**956**	>52	13.2	>52	-	-	-
	31, R= Z-Orn-OH	**957**	>48	18.5	17.4	-	-	-
	34, R= Z-Lys-OMe	**1326**	-	-	0.75	0.80	0.22	5.3
	33, R= Z-Lys-OBzl	**1327**	-	-	0.62	0.84	0.07	29.8

Table VI.6: Biological evaluation of the 3β-acetoxy amine derivatives (Series V and VI)

Series VI proved to be totally inactive against *L. donovani*. Compounds **27** and **28** were inactive against *T.b. rhodesiense* although their amide analogues **14a** and **14b** were active (2.7 and 17.5μM respectively, Table VI.3). Derivatives with longer side chain and different functionalitites proved to be more potent, with IC_{50} values in the sub-micromolar range for compounds **34**

and **33**. Against *T. cruzi*, the compounds showed some activity, especially compound **27** with the shortest side chain (IC_{50}= 4.6μM). This trend was also observed with the amide analogues (derivatives **14a** and **14b**). Finally, compounds **34** and **33** were also active against both *P. falciparum* strains with IC_{50} values in the sub-micromolar range. Some toxicology results are still awaited, but a good selectivity for compound **33** was determined, (426 fold against *P. falciparum* (K1) for example) while selectivity was less significant for compound **34**.

Figure VI.4 shows the biological evaluation of the 3β-acetoxy amine derivatives (Series V and VI) against *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*. Against *T. cruzi*, compounds **19c** (Series V) and **27** (Series VI) were the most active, while against *T.b. rhodesiense* and *P. falciparum*, derivatives with the longest side chain, compound **19g** in series V and compounds **34** and **33** in series VI were the most active.

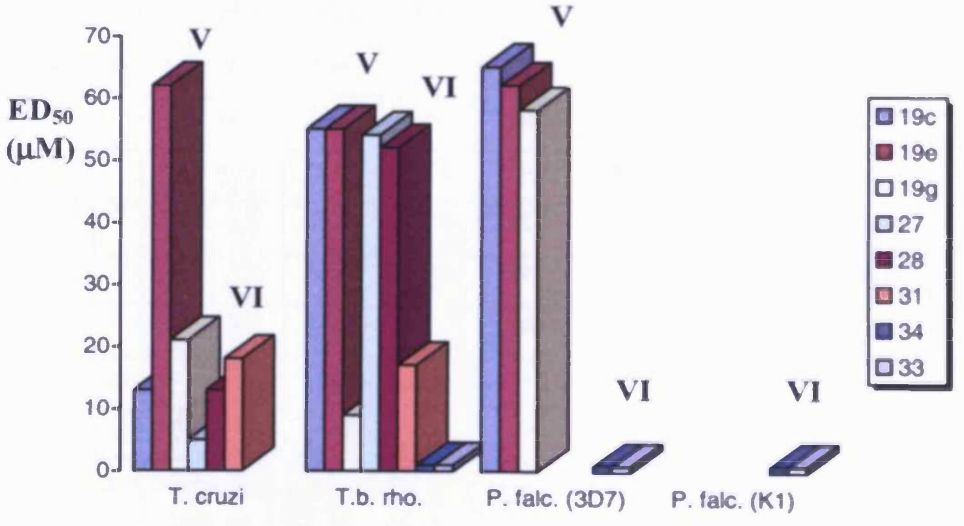


Figure VI.4: Graphical representation of the biological evaluation of the 3β-acetoxy amine derivatives (Series V and VI) against *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*

VI.1.2.2 3β-Hydroxy amine derivatives

As previously discussed, derivatives with a free hydroxyl group at position 3 of the sterol nucleus were expected to be more active. Table VI.7 shows the biological evaluation of derivatives with a terminal acid function in the side chain. This series VII was found difficult to solubilise and no NMR experiment could be run on compound **24b** which was therefore not sent for screening.

Series VII was generally found less active than the 3β-acetoxy derivatives. No activities were detected against both *L. donovani* and *P. falciparum* as was the case for the amide analogues. A slight activity was observed against *T. cruzi* for the compounds with a longer side chain. Against *T.b. rhodesiense*, no particular trend was observed, with derivative **24g** being the most active with an IC_{50} of 6.6μM. A loss of activity was already observed for derivatives without an ester in the side chain (compounds **19** and **20**). The loss of the second ester functionality at position 3 resulted in a total loss of activity which might be due to poor solubility. This series did not show any significant toxicity except for compound **24f**.

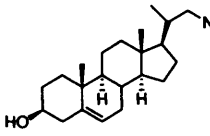
Series		WSP	ED_{50} (μM) *L. dono.*	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)	TD_{50} (μM) Toxicity
	Control drugs	-	> 10	~ 1	~0.001	~0.003	-
VII	**24a**, R= $(CH_2)CO_2H$	**996**	>77	>77	>77	>77	>770
	24b, R= $(CH_2)_2CO_2H$	-	-	-	-	-	-
	24c, R= $(CH_2)_3CO_2H$	**884**	>71	47.3	14.4	56.5	450.6
	24d, R= $(CH_2)_4CO_2H$	**885**	>69	66.2	30.1	>69	>695
	24e, R= $(CH_2)_5CO_2H$	**886**	54.5	56.3	>67	>67	>673
	24f, R= $(CH_2)_6CO_2H$	**887**	>65	44.9	47.6	>65	72.0
	24g, R= $(CH_2)_7CO_2H$	**888**	26.4	38.4	6.6	>63	>633

Table VI.7: Biological evaluation of the 3β-hydroxy amine derivatives (Series VII)

Figure VI.5 shows the biological evaluation of the 3β-hydroxy amine derivatives (Series VII) against *L. donovani*, *T. cruzi* and *T.b. rhodesiense*. This series of compounds was not found particularly active and the best results were obtained with the derivative **24g** having the longest side chain.

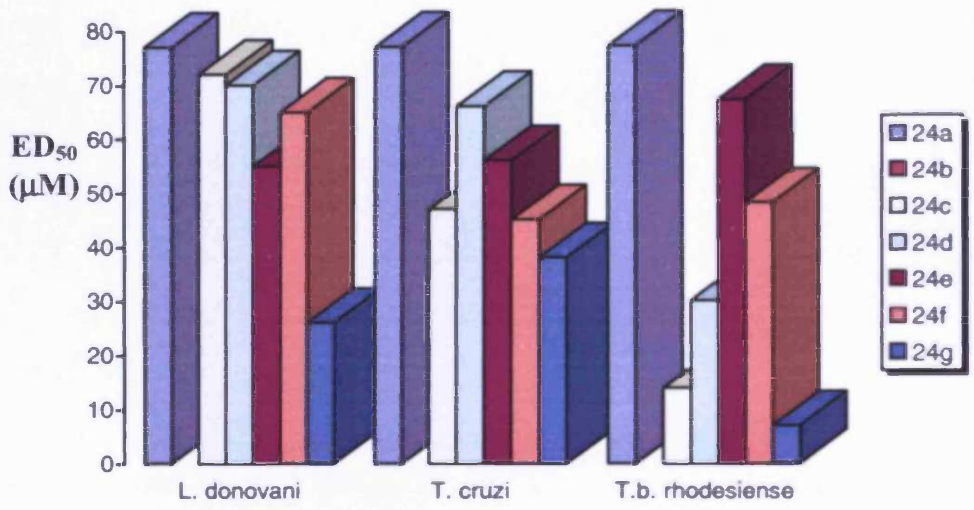


Figure VI.5: Graphical representation of the biological evaluation of the 3β-hydroxy amine derivatives (Series VII) against *L. donovani*, *T. cruzi* and *T.b. rhodesiense*

Two other series with a 3β-hydroxy group were evaluated (Table VI.8). Series VIII has a terminal methyl ester functionality in the side chain whereas series IX has a terminal amino acid function.

Series VIII showed some potency against both *T.b. rhodesiense* and *P. falciparum* in the sub-micromolar range. Compound **26g**, with the longest side chain, showed the best activities with IC_{50} values of 0.31, 0.35 and 0.06μM against *T.b. rhodesiense*, *P. falciparum* (3D7) and *P. falciparum* (K1) respectively. These compounds have some toxicity which affects their selectivity. Compound **26g** showed a selectivity of 20, 18 and 103 fold against *T.b. rhodesiense*, *P. falciparum* (3D7) and *P. falciparum* (K1) respectively, while compounds **26a** and **26c** showed poor selectivities.

Series IX was shown inactive against *T.b. rhodesiense* and slighty active against *P. falciparum* with the best IC_{50} for compound **39** against *P. falciparum* (K1) and 6.3μM. No toxicity was detected.

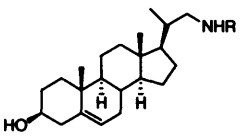
Series		WSP	ED_{50} (μM) *L. dono.*	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)	ED_{50} (μM) *P. falc.* (K1)	TD_{50} (μM) Toxicity
	Control drugs	-	> 10	~ 1	~0.001	~0.003	~0.1	-
VIII	**26a**, R= $(CH_2)CO_2Me$	**1336**	-	-	4.9	40.5	22.2	45.0
	26c, R=$(CH_2)_3CO_2Me$	**1337**	-	-	5.0	7.5	1.9	1.5
	26e, R=$(CH_2)_5CO_2Me$	**1338**	-	-	<0.27	1.3	0.50	6.1
	26g, R=$(CH_2)_7CO_2Me$	**1339**	-	-	0.31	0.35	0.06	6.2
IX	**38**, R= Boc-Dab-OH	**1340**	-	-	>56	36.6	38.1	>563
	39, R= Z-Lys-OH	**1341**	-	-	>50	16.7	6.3	>504

Table VI.8: Biological evaluation of the 3β-hydroxy amine derivatives (Series VIII and IX)

Figure VI.6 shows the biological evaluation of the 3β-hydroxy amine derivatives (Series VIII and IX) against *T.b. rhodesiense* and *P. falciparum*. Series VIII was found quite potent against both strains and the length of the side chain is once again highlighted as the derivative with the longest chain length showed the greatest activity. On the other hand, series IX was poorly active.

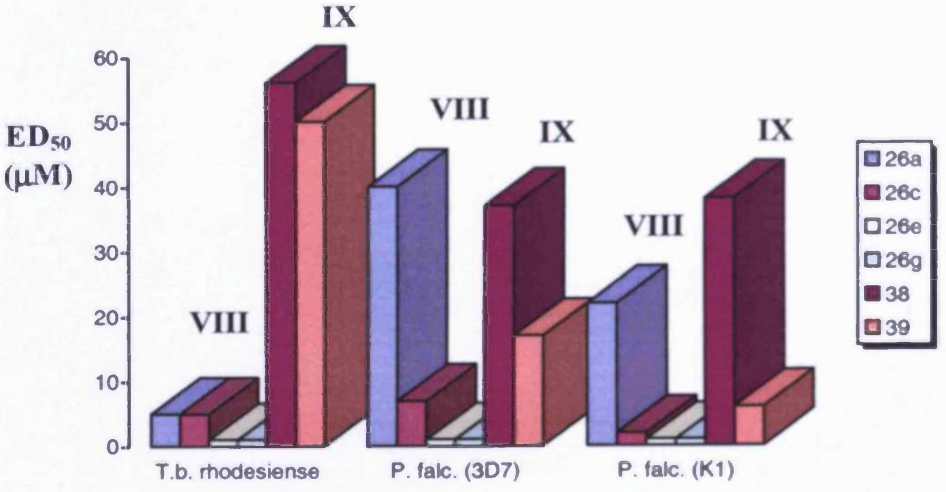


Figure VI.6: Graphical representation of the biological evaluation of the 3β-hydroxy amine derivatives (Series II and III) against *T.b. rhodesiense* and *P. falciparum*

VI.1.3 *In vivo* assays

Two amide and two amine derivatives were chosen for *in vivo* evaluation against the blood stream form of *T.b. rhodesiense*. Mice infected were treated with the compounds **4c**, **4g**, **20c** and **20g** at a dose of 25mg/kg for 4 days. Another group of mice received a single dose of pentamidine (10mg/kg) as a control.

Untreated mice died 7 days post-infection while mice receiving pentamidine survived 30 days post-infection (Table VI.9). Mice treated with the four selected inhibitors also died after 7 days post-infection. Thus, these compounds proved to be totally ineffective in curing infected animal models, and therefore, no further *in vivo* studies have been carried out. Considering the potency of the amine derivatives *in vitro*, the lack of activity may be due to pharmacokinetic factors.

Group	Raw Data (Mean Survival Time in days)					Statistics		
	m1	m2	m3	m4	m5	MST	SD	St Err
Control	7.00	7.00	7.00	7.00	7.00	7.00	0.00	0.00
Pentamidine	›30	›30	›30	›30	›30	›30	0.00	0.00
4c	7.00	7.00	7.00	7.00	7.00	7.00	0.00	0.00
4g	7.00	7.00	7.00	7.00	7.00	7.00	0.00	0.00
20c	7.00	7.00	7.00	7.00	7.00	7.00	0.00	0.00
20g	7.00	7.00	7.00	7.00	7.00	7.00	0.00	0.00

Table VI.9: Biological *in vivo* evaluation of compounds **4c, 4g, 20c** and **20g**

VI.1.4 Conclusion

Data collected from the *in vitro* screening of the compounds against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and *P. falciparum* showed some promising lead compounds. Some IC_{50} values were in the micromolar range, which data given by other inhibitors against the 24-SMT enzyme isolated from fungi compares well to literature data for 24-SMT inhibitors against yeast (Section II.3.2). But other compounds showed activity in the nanomolar range. The following SAR trends were apparent:

Amide derivatives / Amine derivatives: the amide and amine derivatives showed good activities against the different parasites. Amine derivatives were generally more potent than the corresponding amide analogues. Amide derivatives **4** were shown to be inactive against both *L. donovani* and *P. falciparum* (Table VI.3) whereas their amine analogues **20** (Table VI.5) showed IC_{50} values in the micromolar range (Table VI.10). Series **20** was shown to be slightly less active against *T. cruzi*, but on the other hand, showed greater activity against *T.b. rhodesiense* leading to IC_{50} values in the nanomolar range (Table VI.10).

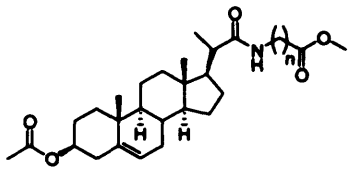
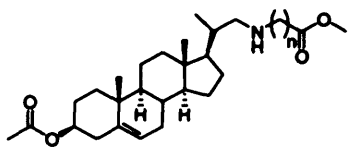
Compounds		n	ED_{50} (μM) *L. dono.*	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)
	**4c**	**3**	>61	5.7	9.1	>61
	4f	**6**	>56	10.2	7.9	>56
	**20c**	**3**	3.2	24.7	0.012	15.4
	20f	**6**	9.5	53.5	0.025	3.5

Table VI.10: Biological evaluation of the 3β-acetoxy amide and amine derivatives **4c, 4f, 20c** and **20f** against *L. donovani*, *T. cruzi*, *T.b. rhodesiense* and *P. falciparum*

We proposed (Chapter III) to prepare compounds acting by inhibition of 24-SMT (Figure VI.7). It was shown that a positive charge in the side chain might be of importance for activity. Thus, the better activities shown by the amine derivatives could be due to a protonation of nitrogen at position 23 in the side chain under physiological condition, a feature which is absent in the amide derivatives.

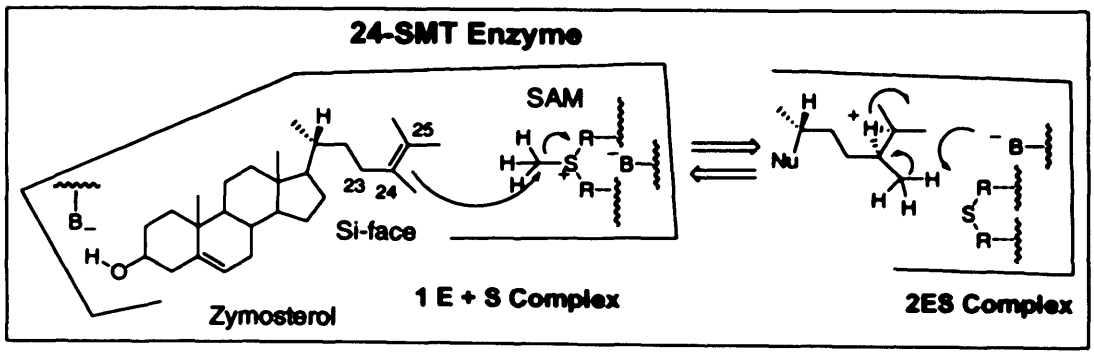


Figure VI.7: Proposed methenylation mechanism by the 24-SMT enzyme

Side chain length: the length of the side chain may play an important role in the interaction with the enzyme as the positions of the functionalities of the inhibitor have to interact with particular residue in the active site of the

enzyme. The amide derivatives **4g** and **8g** with the longer side chain were shown to be the most potent of their series against *T.b. rhodesiense* with IC_{50} values of 0.61 and 0.43μM respectively (Tables VI.11). A similar pattern was observed for the derivatives **26**, where compound **26g**, having the longest side chain of the series, showed the best activity and an IC_{50} value of 0.31μM against *T.b. rhodesiense* (Table VI.11).

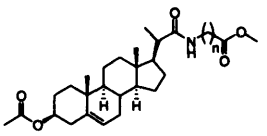
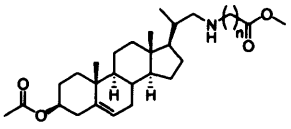
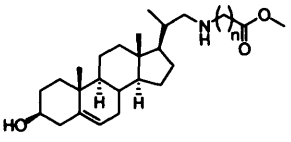
	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**
n	1	2	3	4	5	6	7
IC_{50} (μM)	24.0	6.0	9.15	3.01	3.45	7.91	0.61
	**8a**	**8b**	**8c**	**8d**	**8e**	**8f**	**8g**
n	1	2	3	4	5	6	7
IC_{50} (μM)	>74	>71	49.8	7.8	10.5	2.3	0.43
	**26a**	-	**26c**	-	**26e**	-	**26g**
n	1	2	3	4	5	6	7
IC_{50} (μM)	4.9	-	5.0	-	<0.27	-	0.31

Table VI.11: IC_{50} values of derivatives **4, 8** and **26** against *T.b. rhodesiense*

Compounds **34** and **33** with a terminal amino acid functionality in the side chain also showed much greater activities than derivatives with a shorter side chain (Table VI.6), although in this case, the increase of activity may be due to the presence of different functionalities. Nevertheless, the length of the side chain plays an important role in the inhibition of the different parasite strains. Thus, the synthesis of derivatives with a longer side chain (n = 8, 9, 10) is a future direction for the project.

Functionality in the side chain: the type of interaction with the active site of the enzyme is also important. A strong interaction will allow a good binding with the enzyme and might interfere with its specific activity. Here, the methyl ester, acid and amino acid functionalities in the side chain were studied. Compound **20c** with a methyl ester in the side chain was shown to be potent against both *L. donovani* and *T.b. rhodesiense* with IC_{50} values of 3.2μM and 12nM respectively (Table VI.12). Compound **19c** with an acid function in the side chain was shown to be totally inactive against the same strains of parasite.

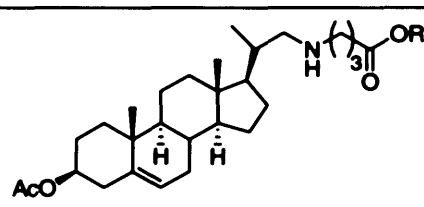
		**ED_{50} (μM)** *L. dono.*	**ED_{50} (μM)** *T.b. rho.*
Compound	**R**		
20c	Me	3.2	0.012
19c	H	>65	54.6

Table VI.12: Biological evaluation of derivatives **20c** and **19c** against *L. donovani* and *T.b. rhodesiense*

In the series with an hydroxyl group at the 3 position (Table VI.13), compound **24g** with an acid function in the side chain was shown to be poorly active against *T.b. rhodesiense* and *P. falciparum* with IC_{50} of 6.6 and >63μM respectively, while compound **26g** with a methyl ester in the side chain was shown to be quite potent with IC_{50} values of 0.31 and 0.35μM. Thus, in both cases, the presence of a methyl ester functionality increased the activities of the compounds.

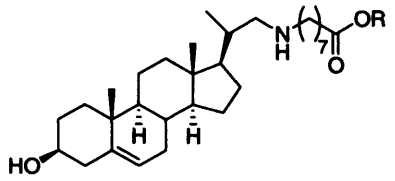
		ED_{50} (μM) *T.b. rho.*	ED_{50} (μM) *P. falc.* (3D7)
Compound	**R**		
24g	H	6.6	>63
26g	Me	0.31	0.35

Table VI.13: Biological evaluation of derivatives **24g** and **26g** against *T.b. rhodesiense* and *P. falciparum*

Position 3 of the sterol nucleus: the presence of a hydroxyl group at position 3 in the sterol nucleus is thought to undergo an important binding interaction in the enzyme active site (Figure VI.7). We wanted to investigate further its exact role. Compound **20c** bearing an acetate group at position 3 of the sterol nucleus, as previously reported showed an activity of 12nM against *T.b. rhodesiense* (Table VI.14). The derivative **26c** which has a hydroxyl group at position 3 was poorly active with an IC_{50} value of 5.0μM. The same pattern was observed with the amine derivatives having an amino acid function in the side chain. Compounds **34** and **33** were active against *T.b. rhodesiense* with IC_{50} values of 0.75 and 0.62μM while compound **39** was totally inactive (Table VI.14). Globally, the removal of the acetate functionality at position 3 of the sterol nucleus did not improve the activities of the compounds.

Problems of solubility were observed during the synthesis of the compounds. The log D values of compounds are assumed to be optimal around 2 to 5 (Section IV.3). The derivatives in table VI.14 all show reasonable log D values. Nevertheless, the poor solubility of the derivatives with a hydroxyl group at position 3, like compounds **24c** and **26c**, might be the reason for their poor activities. Hence, the acetate derivatives may be important for cell permeability and even have some effect on the mode of action.

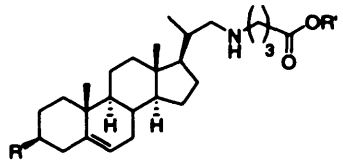
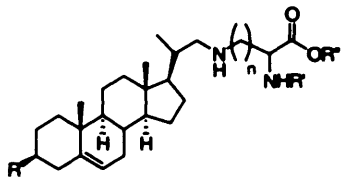
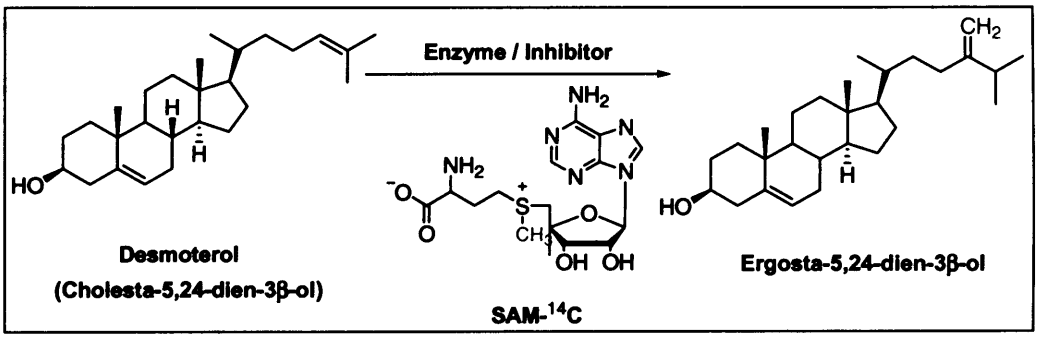
Compound		n	R	R'	R"	Log D	ED_{50} (μM) *T.b. rho.*
	**20c**	3	OAc	Me	-	4.54	0.012
	19c	3	OAc	H	-	4.04	54.6
	26c	3	OH	Me	-	3.69	5.0
	24c	3	OH	H	-	3.18	14.4
	**28**	2	OAc	Boc	H	5.42	>52
	38	2	H	Boc	H	4.57	>56
	34/33	4	OAc	Z	Me/Bn	6.15/7.53	0.75/0.62
	39	4	H	Z	H	5.18	>50

Table VI.14: Log D and IC_{50} values against *T.b. rhodesiense* of different amine derivatives

Parasite strains: sterol metabolism differs from one organism to another and between different stages of the life cycle (intracellular or extracellular forms). Therefore, the effectiveness of a drug depends mainly on the parasite metabolism. We were interested in the inhibition of the 24-SMT enzyme. In Table VI.3, amide derivatives **4** showed different inhibition patterns. They were not active against both *L. donovani* and *P. falciparum*, while compound **4d** was found the most active against *T. cruzi*, and compound **4g** against *T.b. rhodesiense*. The amine derivatives **20** also showed different results as compound **20c** proved to be the most active against both *L. donovani* and *T.b. rhodesiense*, while compounds **20d** and **20g** were the most active against *T. cruzi* and *P. falciparum* respectively. This shows that the drugs act differently considering the organism targeted. *T.b. rhodesiense* and *P. falciparum* do not synthesise ergosterol. Therefore, the activities, against these two strains, of some derivatives, should be due to another mode of action. The compounds might interfere with the membrane functionalities causing its rupture, or they may inhibit another enzyme which is vital for the survival of the parasite.

VI.2 Enzyme assays

The *L. major* and *T.b. brucei* 24-SMT enzymes were over expressed at Instituto de Parasitología y Biomedicina "López-Neyra" (Granada, Spain, Chapter VIII and X). The enzymatic assays were carried out with saturated desmosterol and SAM-^{14}C solutions, and different concentrations of inhibitors (Scheme VI.1). The enzymes were over expressed in *E. coli*. However due to difficulties with purification, cell-free extracts were used for assays.



Scheme VI.1: Reaction of methenylation of desmosterol carried out with the *L. major* or *T.b. brucei* 24-SMT enzymes

VI.2.1 Assays against the recombinant *T.b. brucei* 24-SMT enzyme

The assays were carried out on a selected range of compounds due to the lack of time. Nevertheless, different series were evaluated. The first compound evaluated was WSP 310. This derivative was already synthesized in this laboratory[119] and was found to be active against the *L. major* 24-SMT enzyme with an IC_{50} of 28nM. We wanted to investigate if there was a similar pattern with the *T.b. brucei* 24-SMT. WSP 310 is a piperidine sterol derivative. This compound has been evaluated and showed *in vitro* activity against *T. cruzi* (7.4 μM), *L. donovani* (8.9 μM) and the *T.b. rhodesiense* (3.3 μM). The activity found here against the *T.b. brucei* 24-SMT enzyme was 1.76 μM (Table VI.15). This result is in agreement with the *in vitro* assays, and suggests that the mode of action of WSP 310 is by inhibition of the 24-SMT enzyme.

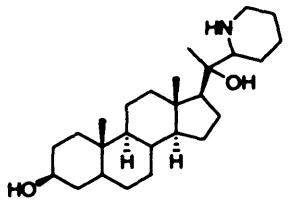
Compound	WSP	IC_{50} (μM)
	310	1.76

Table VI.15: IC_{50} value of the WSP 310 derivative against therecombinant *T.b. brucei* 24-SMT enzyme

Amide and amine derivatives showed some potency against *T.b. rhodesiense* (Section VI.1). We wanted to investigate if these inhibitors were acting as 24-SMT inhibitors as has been demonstrated for WSP 310. Enzyme assays were carried out against the recombinant *T.b. brucei* 24-SMT (Chapter VIII). Table VI.16 summarises the assays. The results showed clearly that there was no inhibition of the *T.b. brucei* 24-SMT enzyme with IC_{50} values over 100μM.

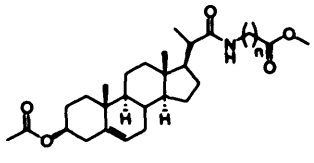
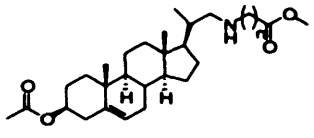
Structure	n	1	2	3	4	5	6	7
	**Compound**	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**
	IC_{50} (μM)	-	-	›100	›100	›100	-	-
	**Compound**	**20a**	**20b**	**20c**	**20d**	**20e**	**20f**	**20g**
	IC_{50} (μM)	-	-	›100	-	›100	›100	›100

Table VI.16: Enzyme assays against the recombinant *T.b. brucei* 24-SMT enzyme with amide and amine derivatives **4** and **20**

These results are not in agreement with the inhibitors acting by inhibition of the 24-SMT. Therefore an explanation would be that the derivatives that showed *in vitro* potency, do not work by inhibition of 24-SMT, but have a different mode of action, unknown to date. The *in vitro* screening was carried out against the *T.b. rhodesiense* whilst enzyme assays were carried out against the recombinant *T.b. brucei* 24-SMT enzyme. However, there is

unlikely to be a big difference in 24-SMT from *T.b. brucei* and *T.b. rhodesiense* that would explain this result.

VI.2.2 Assays against the recombinant *L. major* 24-SMT enzyme

Enzyme assays have also been carried out against the recombinant *L. major* enzyme, in similar conditions to those described above, with cell-free extracts from *E. coli* over expressing *L. major* 24-SMT enzyme. Amine derivatives **24** were evaluated first (Table VI.17). Derivatives with the longest side chain were not active. On the other hand, compound **24c** was active with an IC_{50} of 4μM. Due to lack of time derivatives **24a** and **24b** were not evaluated. It would be interesting to investigate these compounds with the shortest side chain which may lead to greater activity.

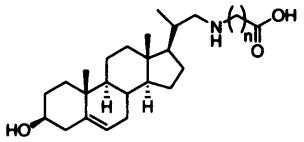
Structure	**n**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	**Compound**	**24a**	**24b**	**24c**	**24d**	**24e**	**24f**	**24g**
	IC_{50} (μM)	-	-	4	48.9	›100	›100	›100

Table VI.17: Enzyme assays against the recombinant *L. major* 24-SMT enzyme with the amine derivatives **24**

These derivatives were not active *in vitro* against *L. donovani*. In Section VI.1.4, solubility problems were reported with derivatives with a free hydroxyl group at position 3. The solubility was even more a problem with derivatives bearing an acid function in the side chain. Therefore, these derivatives might not be able to cross cell membranes because of this poor solubility. The derivatives **20** that have an acetate group at position 3 and a methyl ester function in the side chain were quite potent against *L. donovani* with compound **20c** having an IC_{50} value of 3.2μM (Table VI.5). These derivatives might act as prodrugs, which *in situ*, after cleavage of the ester protecting groups, would be compounds of structure **24**.

Enzyme assays were also carried out on a wide range of compounds synthesised in the laboratory by S. L. Orenes and F. Magaraci (denoted with *)[119,126], and by the author. The design of these compounds was either chosen for their analogy to SAM or to mimic the high energy intermediate. Table VI.18 showed enzymatic assays carried out against the *L. major* 24-SMT enzyme and IC_{50} values of *in vitro* assays against *L. donovani* (intracellular amastigote), with pyridine and piperidine sterol derivatives. None of them showed any activity against the *L. major* 24-SMT enzyme. *In vitro* activities against *L. donovani* were already evaluated. Compound WSP 690 showed the most potent 24-SMT inhibitor with an IC_{50} of 2.3μM. Therefore, some activity against the *L. major* enzyme was expected. This result shows that *L. donovani* 24-SMT is significantly different from the *L. major* or more likely the 24-SMT enzyme might not be the target of this drug.

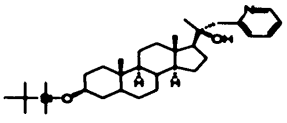
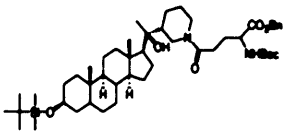
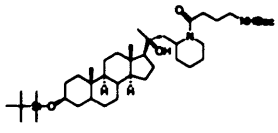
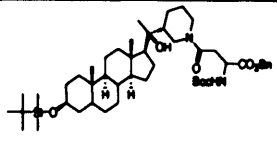
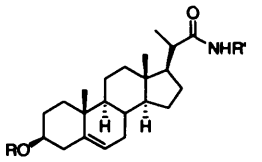
Compound	WSP	ED_{50} (μM) *L. donovani*	IC_{50} (μM) *L. major* enzyme
	489*	26.8	› 100
	690*	2.3	
	750*	› 42	
	774*	› 36	

Table VI.18: Pyridine and piperidine biological evaluation against the recombinant *L. major* 24-SMT enzyme

Then, a wide range of amides derivatives were evaluated (Table VI.19 and VI.20). Again, none of them showed any potency against the *L. major* 24-SMT enzyme with the best activity for WSP 702 and an IC_{50} of 93µM. *In vitro* biological evaluation against *L. donovani* did not show great activities either, with the best results for compounds WSP 701 and WSP 696 with IC_{50} values of 16.1 and 10.6µM respectively (Table VI.19).



R	R'	WSP	*L. donovani* ED_{50} (µM)	*L. major* IC_{50} (µM)
H	Benzyl	563*	› 70	› 100
H	Butyl	565*	› 75	› 100
H	Piperidyl	567*	› 72	› 100
H	$(CH_2)_4NH_2$	702*	› 72	93.4
H	$(CH_2)_6NH_2$	700*	38.9	› 100
H	$(CH_2)_8NH_2$	699*	44.4	› 100
H	$(CH_2)_{10}NH_2$	701*	16.1	› 100
H	$(CH_2)_2CO_2H$	994	-	› 100
H	CH_2CO_2H	995	-	› 100
Ac	Benzyl	568*	› 62	› 100
Ac	Butyl	564*	› 68	› 100
Ac	Piperidyl	566*	› 66	› 100
Ac	$(CH_2)_4NH_2$	697*	› 65	› 100
Ac	$(CH_2)_6NH_2$	695*	35.6	› 100
Ac	$(CH_2)_{10}NH_2$	696*	10.6	› 100
Ac	$(CH_2)_6$NH-steroid	692*	› 35	› 100
Ac	*Trans*-1,4-$C_6H_{10}NH_2$	698*	14.3	› 100

Table VI.19: Biological evaluation of amide derivatives against the recombinant *L. major* 24-SMT enzyme

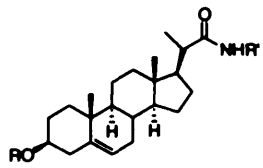
		WSP	*L. donovani* ED_{50} (μM)	*L. major* IC_{50} (μM)
R	**R'**			
Ac	Z-Lys-OBzl	765	› 40	› 100
Ac	Z-Orn-OH	767	› 47	› 100
Ac	Boc-Dap-OH	954	› 52	› 100
Ac	Boc-Dab-OH	989	› 50	› 100
Ac	$(CH_2)_2CO_2Me$	990	-	› 100
Ac	CH_2CO_2Me	991	-	› 100

Table VI.20: Biological evaluation of amide derivatives against the recombinant *L. major* 24-SMT enzyme

Amine derivatives were finally evaluated (Table VI.21 and VI.22). Once again, no enzyme activity was detected against the recombinant *L. major* 24-SMT enzyme, which is in agreement with the poor *in vitro* activities found against *L. donovani*.

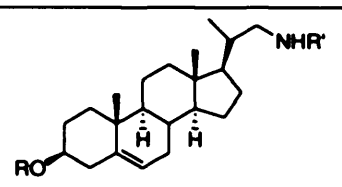
		WSP	*L. donovani* ED_{50} (μM)	*L. major* IC_{50} (μM)
R	**R'**			
H	CH_2CO_2H	996	› 77	›100
Ac	$(CH_2)_{10}NH_2$	633	46.0	›100
Ac	$(CH_2)_6NHCHO$	636	34.6	›100
Ac	Boc-Dap-OH	955	› 53	›100
Ac	Boc-Dab-OH	956	› 52	›100
Ac	Z-Orn-OH	957	› 48	›100
Ac	$(CH_2)_2CO_2Me$	992	22.8	›100

Table VI.21: Biological evaluation of amine derivatives against the recombinant *L. major* 24-SMT enzyme

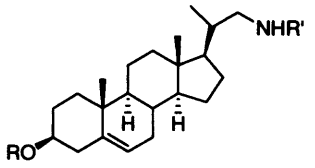
		WSP	*L. donovani* ED_{50} (µM)	*L. major* IC_{50} (µM)
R	**R'**			
H	CH_2CO_2H	996	› 77	›100
Ac	$(CH_2)_{10}NH_2$	633*	46.0	›100
Ac	$(CH_2)_6NHCHO$	636*	34.6	›100
Ac	Boc-Dap-OH	955	› 53	›100
Ac	Boc-Dab-OH	956	› 52	›100
Ac	Z-Orn-OH	957	› 48	›100
Ac	$(CH_2)_2CO_2Me$	992	22.8	›100
Ac	CH_2CO_2Me	993	-	›100
Ac	$(CH_2)_5CO_2H$	997	› 61	›100
Ac	$(CH_2)_7CO_2H$	998	-	›100
Ac	$(CH_2)_3CO_2H$	999	› 65	›100

Table VI.22: Biological evaluation of amine derivatives against the recombinant *L. major* 24-SMT enzyme

VI.2.3 Conclusion

Enzymatic assays were first carried out against the recombinant *T.b. brucei* 24-SMT enzyme. The derivative WSP 310 showed a good correlation between enzymatic and *in vitro* assays. This suggests that compound WSP 310 act by inhibition of the 24-SMT enzyme. On the other hand, amide and amine derivatives tested were not active against the enzyme while some were potent *in vitro* against *L. donovani*. Therefore, these inhibitors might have a different mode of action, unknown to date or the differences in the *Leishmania* strains might interfere in the uptake of the drug.

L. major 24-SMT enzyme assays have also been carried out with a wide range of compounds. Derivative **24c** was active against the enzyme while not against *L. donovani*. The poor solubility of these compounds might be the

reason for their lack of *in vitro* activity. The analogues **20** with the functionalities protected showed greater *in vitro* activities which suggest that they may act as prodrugs. The activity could be due to the compounds acting against other biological targets, by disorganisation of the membrane functions causing its rupture or might inhibit other enzymes. The other derivatives evaluated showed poor activities against both the *L. major* 24-SMT enzyme and *in vitro* against *L. donovani*. These analogues (Table VI.21 and VI.22) are not potent inhibitors of *L. major* 24-SMT and do not have good activity against the parasite *in vitro*.

CHAPTER VII

PROTEOMIC APPROACH

In order to investigate the mode of action of the compounds, a different approach of the project was undertaken in collaboration with Alwen Jones, a 4th year Pharmacy undergraduate student.

Most cellular functions are carried out by proteins. Proteins catalyze reactions, allow passage of molecules into and out of cells, transmit signals and control growth and differentiation[172]. It can therefore be assumed that these synthesized compounds are binding and interacting with proteins because they have a very potent and selective activity that indicates a specific response and not a general one. However, the determination of the function of any protein is really difficult, as they are not always transcribed and translated from DNA[172], or, because the post-translation modification of a single type of peptide can lead to the formation of several different functional proteins[173]. Furthermore,

[172] Huang, X.; Tan, E. L. P.; Chen, G. Y. J.; Yao, S. Q. Enzyme-Targeting Small-Molecule Probes for Proteomics Applications. *Applied Genomics and Proteomics* **2003**, *2*, 225-238.

[173] Banks, R. E.; Dunn, M. J.; Hochstrasser, D. F.; Sanchez, J. C.; Blackstock, W.; Pappin, D. J.; Selby, P. J. Proteomics: New Perspectives, New Biomedical Opportunities. *Lancet* **2000**, *356*, 1749-1756.

not all proteins are expressed at all stages of the parasites cycle[174]; one protein may have different roles at different stages of the life cycle, and the metabolic functions of many proteins are still not well understood[175].

In order to overcome these problems, a chemical proteomic approach can be applied. Chemical proteomics is a technique which uses chemical probes to purify and identify proteins and to determine protein interaction and activity[176]. A chemical probe is made of three basic elements (Figure VII.1).

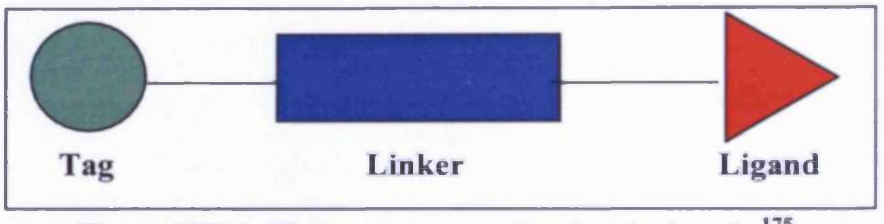


Figure VII.1: The components of a chemical probe[175]

1. The tag: its role is to aid the identification, quantification and purification of the probe bound protein. Three different tags are commonly used:
    - Isotope tag: used to directly visualize the probe-protein complex[176] and to measure protein abundance[177];
    - Fluorescent tag: used in the same way as isotope tag, but also to locate where the protein is in an intact cell[178];

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[174] de Walque, S.; Kiel, J.; Veenhuis, M.; Opperdoes, F. R.; Michels, P. A. M. Cloning and Analysis of PTS-1 Receptor in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **1999**, *104*, 107-119.

[175] Fang, J.; Beattie, D. S. Alternative Oxidase Present in Procyclic *Trypanosoma brucei* May Act to Lower the Mitochondrial Production of Superoxide. *Arch. Biochem. Biophys.* **2003**, *414*, 294-302.

[176] Jeffery, D. A.; Bogyo, M. Chemical Proteomics and its Application to Drug Discovery. *Curr. Opin. Biotechnol.* **2003**, *14*, 87-95.

[177] Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. Chemical Strategies for Functional Proteomics. *Mol Cell Proteomics* **2002**, *1*, 781-790.

[178] Greenbaum, D.; Baruch, A.; Hayrapetian, L.; Darula, Z.; Burlingame, A.; Medzihradszky, K. F.; Bogyo, M. Chemical Approaches for Functionally Probing the Proteome. *Mol Cell Proteomics* **2002**, *1*, 60-68.

- Biotin tag: used to purify proteins from a complex mixture[176]; the proteins bound to the biotin-probe and can be isolated using a streptavidin column;
- Solid support.

2. The linker: the linkers' primary role is to provide distance between the ligand and the tag. This should prevent the tag from interfering with the ligand-protein interaction[176]. The linker can also be used to modify the hydrophilic properties of the probe. Two type of linkers are usually used:
   - Alkyl linkers;
   - Polyethylene glycol (PEG) linkers.

3. The ligand: attached to the linker, is the component that has a high affinity for the specific protein to be analysed. It can be for example a known inhibitor of the target protein of an enzyme. Attachment can be covalent or non-covalent.

In Chapter VI, the amine derivative **20e** (Figure VII.2) was evaluated. It has been demonstrated that this compound was not active against the *Trypanosoma brucei brucei* 24-SMT enzyme, but showed high potency *in vitro* against *Trypanosoma brucei rhodesiense* (IC_{50} = 33nM).

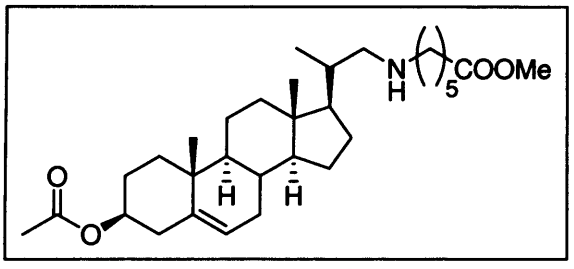


Figure VII.2: Amine derivative **20e**

Therefore, we wanted to determine the role of these series of compounds by using a proteomic probe where the ligand would be the amine

derivative **20e**, while the tag could be biotin and the linkers, either hexamethylenediamine or 6-aminocaproic acid (Figure VII.3).

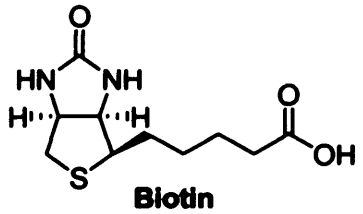
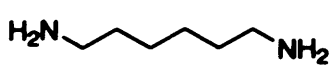
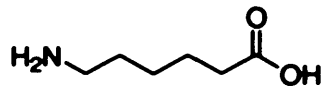
Tag	Linkers
 Biotin	 Hexamethylenediamine  6-aminocaproic acid

Figure VII.3: Tag and linkers proposed for the proteomic probe

The use of biotin as a tag will allow the purification of the protein on a streptavidin column[177]. Hexamethylenediamine and 6-aminocaproic acid will be the choice of linkers as they are believed to be long enough to minimize steric hindrance. Different points of attachment of the sterol to the linker will be used as the part of the molecule binding to protein(s) is not known (Figure VII.4).

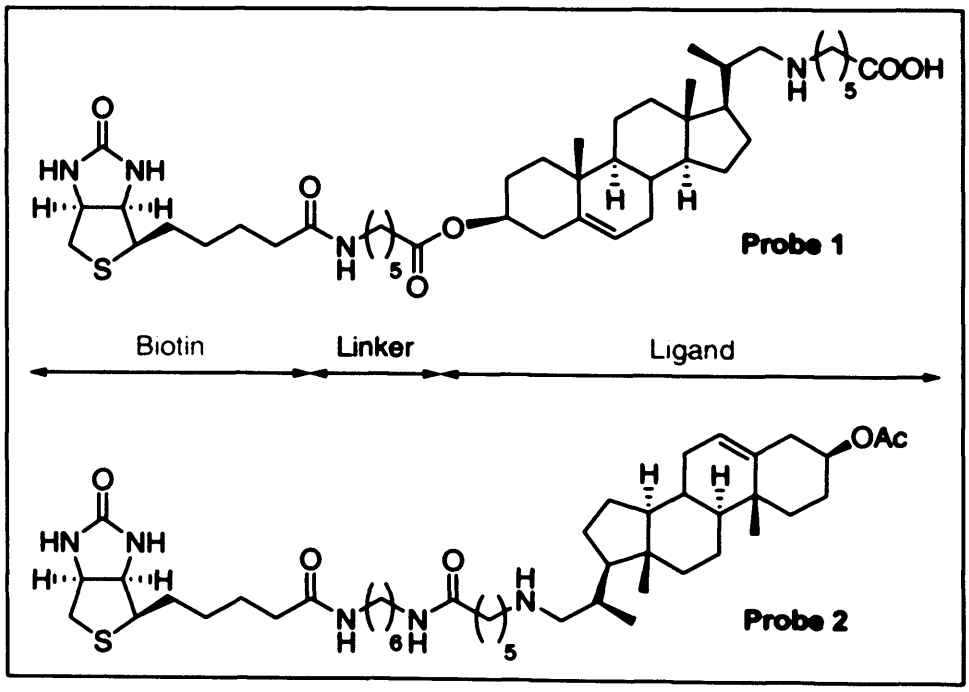
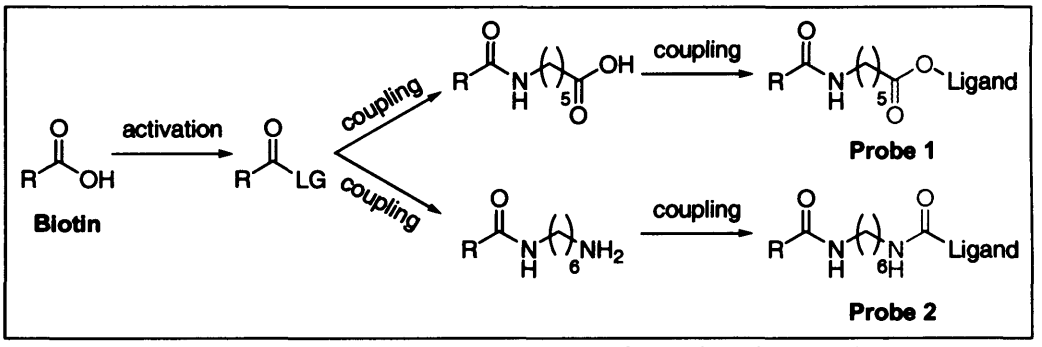


Figure VII.4: Final target probe structures

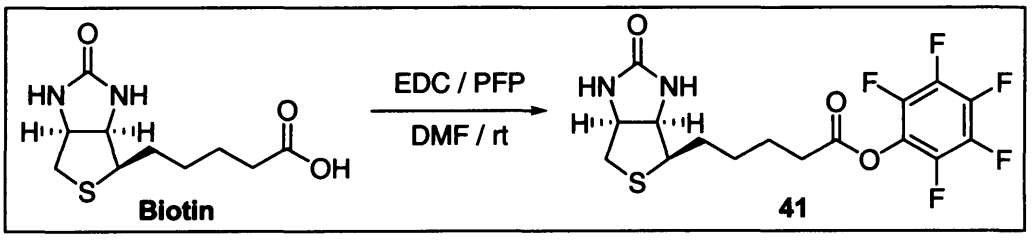
The synthetic approach to the chemical probes will be activation of the carboxylic acid of biotin before coupling with the amino group of a linker (Scheme VII.1). A second coupling with the ligand should lead to the synthesis of probes 1 and 2 (Figure VII.4). Probe 1 would be obtained through an ester bond from an alcohol functionality, while probe 2 through an amide bond from a carboxylate functionality.



Scheme VII.1: Synthetic approach of the chemical probes

VII.1 Synthesis of 9-(6-hexanoic acid) amide biotin (42)

An activated ester was first synthesized by coupling biotin to pentafluorophenol (PFP)[133,135] using the coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)[134] (Scheme VII.2).



Scheme VII.2: Synthesis of the 9-(pentafluorophenyl) ester biotin **41**

After purification by column chromatography compound **41** was obtained in 37% yield. Analysis of the ^{19}F NMR confirmed the presence of compound **41** and the removal of excess PFP (Figure VII.5):

- PFP showed only two peaks at -163.8 and -168.4 using ^{19}F NMR spectroscopy: presumably, as the electronegativities of fluorine and oxygen are close, the four fluorines on carbon 2 and 3 are equivalent and showed only one peak; the second peak is for the fluorine on carbon 1;
- In compound **41**, three peaks were observed corresponding to the fluorines on carbon 11, 12 and 13.

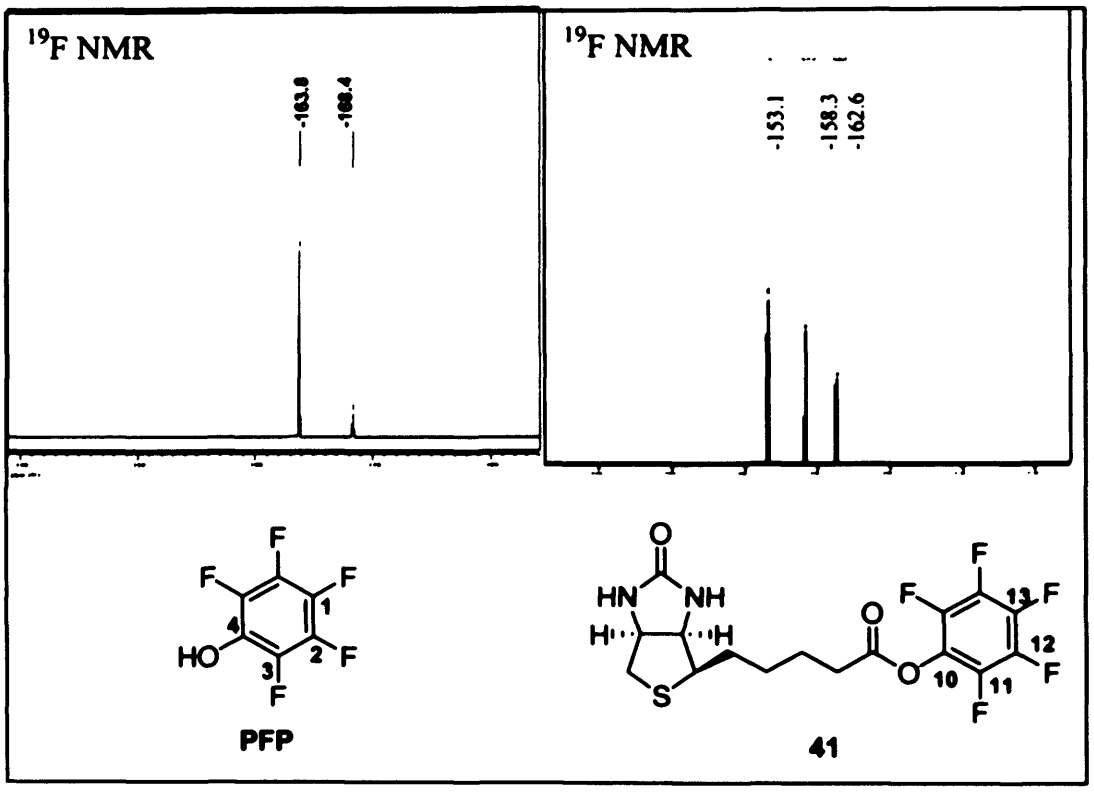


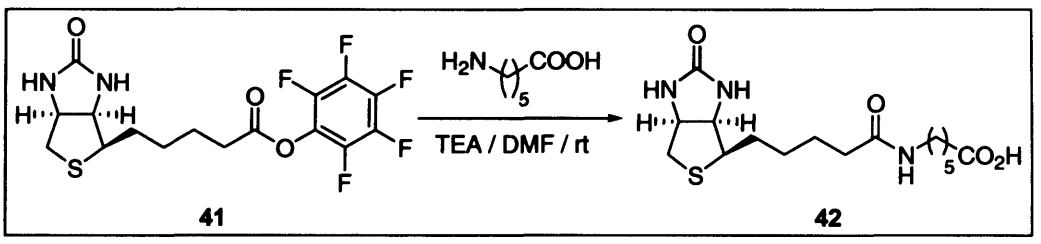
Figure VII.5: ^{19}F NMR of PFP and compound **41**

Due to the low yield (37%) the reaction was repeated using DCC[126] as coupling agent. After extraction and purification by column chromatography ^{1}H NMR and ^{19}F NMR were analysed. Both the ^{1}H NMR and ^{19}F NMR spectrum confirmed the presence of compound **41**. However ^{1}H NMR also showed the presence of DMF and the byproduct dicyclohexylurea (DCU). By comparing peak heights of the compound **41**, DCU and DMF from the ^{1}H NMR spectrum it was calculated that the solid contained 1.052g (61%) of **41**,

1.437g of **DCU** and 0.094g of **DMF**. Fortunately DCU is chemically inert therefore its presence should not have any effect on following reactions.

The second step was to synthesize the biotin-linker. This was achieved by displacing the PFP moiety of the ester with a linker. Two different linkers were used, 6-aminocaproic acid and hexamethylenediamine. The former would provide an acid group for coupling and the latter an amino group.

PFP-Biotin **41** was reacted with 6-aminocaproic acid under basic conditions (Scheme VII.3).

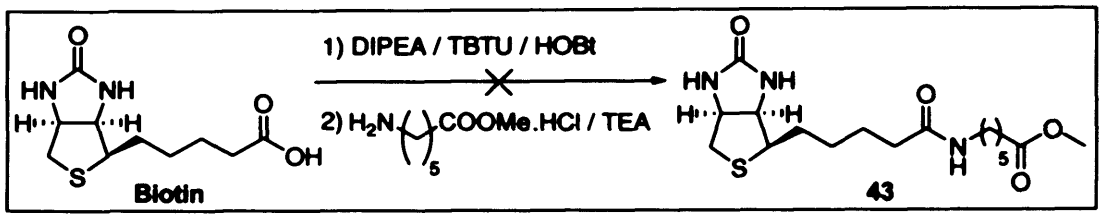


Scheme VII.3: Synthesis of 9-(6-hexanoic acid) amide biotin **42**

After extraction with $CHCl_3$, ^{1}H-NMR of the organic layer did not correspond to the expected compound. The aqueous layer was reduced under vacuum and analysis by MS showed the expected peak of compound **42**, and also 6-aminocaproic acid. Believing purification by column chromatography would be difficult to run either with normal phase silica or reverse phase silica, solubility testing was carried out. It was discovered that 9-(6-hexanoic acid) amide biotin **42** was not soluble in water but 6-aminocaproic acid and PFP were. The presence of compound **42** in the aqueous layer after extraction might be explained by the formation as a salt with triethylamine which was used in large excess (7 eq.). It was therefore decided not to use the reverse phase column chromatography but to neutralize the solution with a hydrochloric acid solution in order to get the acid derivative **42**. The crude was washed with water and filtered. A white powder was recovered which was confirmed to be pure product **42** by MS and ^{1}H NMR, with a yield of 52%.

In an alternative approach, methyl-6-aminohexanoate ester monohydrochloric acid **3e** was used instead of 6-aminocaproic acid. The methyl group should protect the acid group and makes the product less polar.

The resulting compound **43** would then be hydrolysed resulting in the formation of compound **42**. The synthesis was attempted in similar conditions as in Section IV.3.2 using HOBt and TBTU as activating agents (Scheme VII.4).

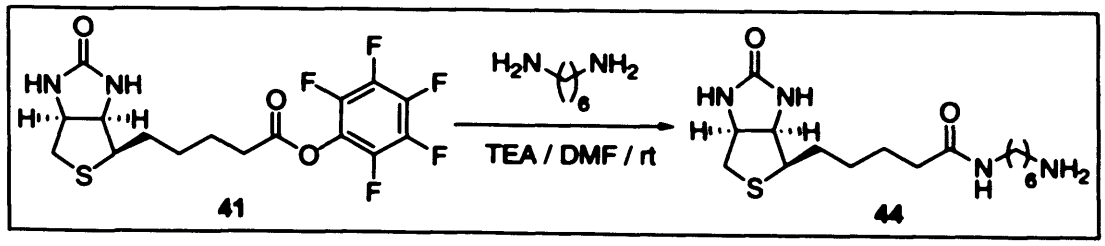


Scheme VII.4: Attempted synthesis of compound **43**

After extraction, ^{1}H NMR and MS were run but did not show the presence of compound **43**. Only the intermediate biotin-HOBt was identified in the mixture with biotin itself, which suggests that the substitution was the problem. We proposed in Section IV.3.2 that basic conditions probably favoured the reaction happening. The slight excess of triethylamine used in this case, was not enough to influence the outcome of the reaction.

VII.2 Synthesis of 9-(6-aminohexyl) amide biotin (44)

As previously described, the ligand could be attached to the linker though different points of attachment. Therefore, hexamethylenediamine will be used as linker in probe 2. 9-(Pentafluorophenyl) ester biotin **41** was coupled under basic conditions with hexamethylenediamine (Scheme VII.5).



Scheme VII.5: synthesis of 9-(6-aminohexyl) amide biotin **44**

Triethylamine was first added dropwise to a solution of hexamethylenediamine in DMF. Then, this solution was slowly added to the solubilised 9-(pentafluorophenyl) ester biotin **41** in order to minimise the production of the dialkylated derivative 1,6-dibiotin-1,6-diamino hexane **45** (Figure VII.6).

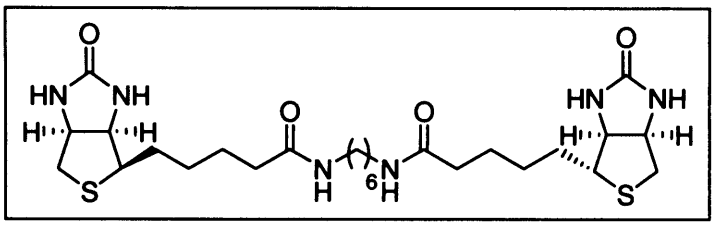


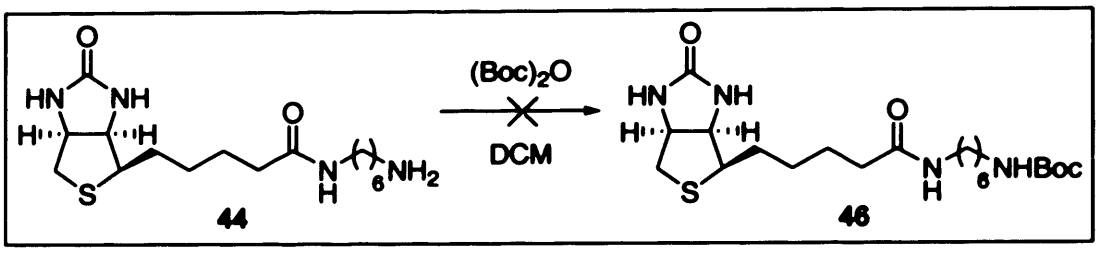
Figure VII.6: 1,6-dibiotin-1,6-diamino hexane **45**

The following up of the reaction by TLC showed the consumption of starting material. This time again, after extraction, 9-(6-aminohexyl) amide biotin **44** was identified by MS in the aqueous layer. Purification by column chromatography was carried out to yield only 4% of compound **44**.

Due to this low yield, the reaction was repeated. This second attempt did not improve the yield as compound **44** was not recovered after column chromatography. 9-(6-Aminohexyl) amide biotin **44** is a polar compound. Thus, the purification by column chromatography might not be possible even using NH_4OH 1%, which should neutralize the acidity of silica.

Hence, due to previous purification problems there was no attempt this time to purify the crude product. After extraction with $CHCl_3$, solvents from the water layer were removed under reduced pressure to yield 429mg (163%) as a white solid, MS in ES^+ showing the presence of 9-(6-aminohexyl) amide biotin **44**. It was decided that the terminal amino group of **44** could be protected with a Boc group. The resulting compound would be less polar resulting in it eluting from the column sooner.

The terminal amino group of 9-(6-aminohexyl) amide biotin **44** was Boc protected[179]. The crude was directly used in this step using $(Boc)_2O$ in DCM (Scheme VII.6).



Scheme VII.6: Attempted synthesis of 6-(*tert*-butanoate ester amine) hexyl amide biotin **46**

A less polar spot was observed by TLC. After extraction with ether, the ether was removed by reduced pressure to recover a white solid. MS of this solid in ES^+ showed the presence of the product plus Na^+(465.2m/z) and 1,6-ditert-butanoate ester-1,6-diamino hexane (diprotected hexamethylenediamine) plus Na^+ (339.1m/z). Diprotected hexamethylenediamine was present due to the presence of hexamethylenediamine from the unpurified starting material **44** being Boc protected twice. Purification by column chromatography was achieved until a ratio of $CHCl_3$:MeOH (80:20) was reached. However analysis of the column fractions by TLC showed no product **46**.

As compound **46** was not being produced from the un-purified crude mixture **44**, it was decided that a different approach to make it would be better. It was decided to Boc protect one terminal amino group of hexamethylenediamine[179,180], purify it and then react it with the 9-(pentafluorophenyl) ester biotin **41** (Scheme VII.7).

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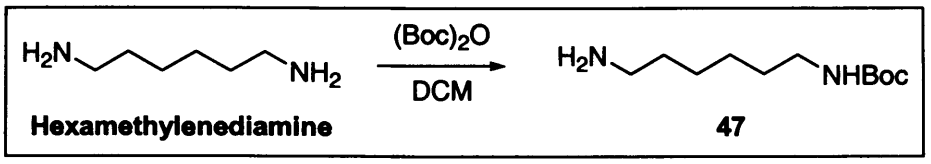
[179] Tilley, J. W.; Levitan, P.; Kierstead, R. W.; Cohen, M. Antihypertensive (2-Aminoethyl)thiourea Derivatives. 1. *J. Med. Chem.* **1980**, *23*, 1387-1392.

[180] Sabatino, G.; Chinol, M.; Paganelli, G.; Papi, S.; Chelli, M.; Leone, G.; Papini, A. M.; De Luca, A.; Ginanneschi, M. A New Biotin Derivative-DOTA Conjugate as a Candidate for Pretargeted Diagnosis and Therapy of Tumors. *J. Med. Chem.* **2003**, *46*, 3170-3173.



Scheme VII.7: Synthesis of 6-(aminohexyl)*tert*-butanoate ester amine **47**

The reaction was carried out by adding the solubilised Boc anhydride in DCM drop wise to a solution of hexamethylenediamine in DCM, in order to minimize the production of diprotected hexamethylenediamine **48** (Figure VII.7).

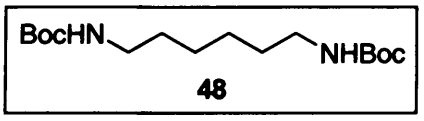


Figure VII.7: 1,6-di-*tert*-butanoate ester-1,6-diamino hexane **48**

Reaction progress could not be monitored by TLC as a fine solid in the reaction mixture kept blocking the capillary tube. MS (ES^+) analysis of this solid showed the presence of the product (217.1m/z) and hexamethylenediamine (117m/z). The presence of hexamethylenediamine confirmed that the reaction had not gone to completion. Nevertheless, the reaction was stopped and after extraction, MS of the organic layer confirmed that the excess hexamethylenediamine had been removed. Unfortunately the MS showed the presence of diprotected hexamethylenediamine **48** (339.2m/z) in addition to the product. After purification by column chromatography, 88mg of diprotected compound **48** (18%) and 14mg of monoprotected compound **47** (4%) were recovered. This relatively high yield of diprotected hexamethylenediamine **48** compared to monoprotected hexamethylenediamine **47** may be due to the extraction. By comparing the MS prior to extraction to the MS post extraction (Figure VII.8), it was observed that the diprotected hexamethylenediamine became concentrated in the organic phase. Some of the monoprotected compound **47** being more polar was probably lost in the aqueous layer.

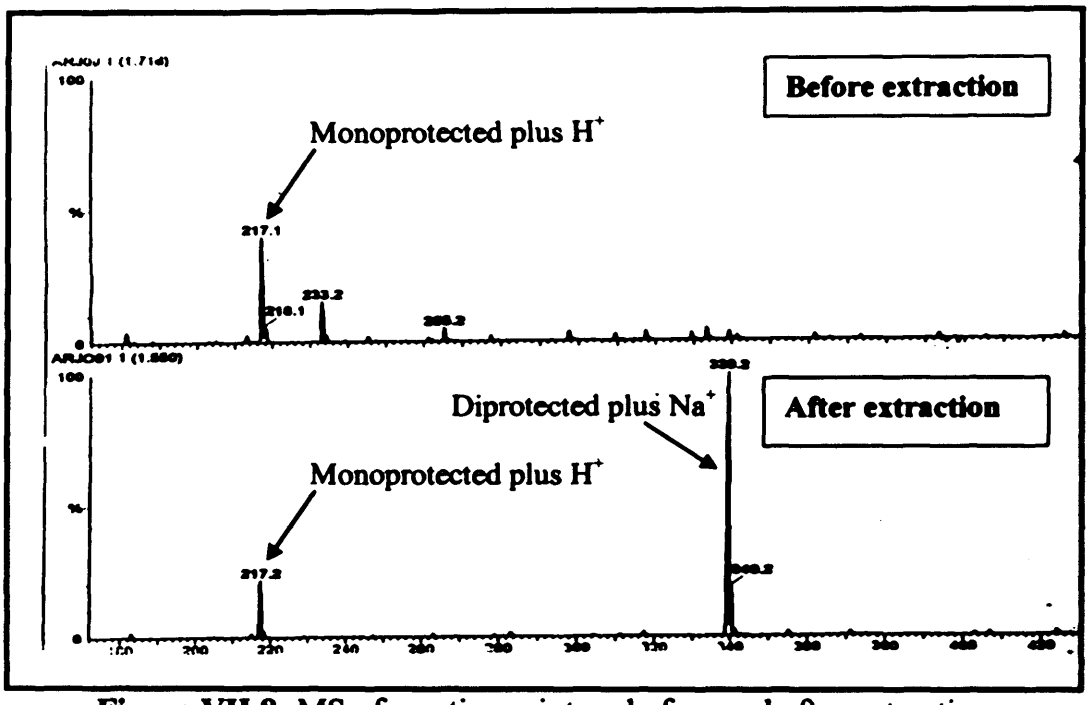


Figure VII.8: MS of reaction mixture before and after extraction

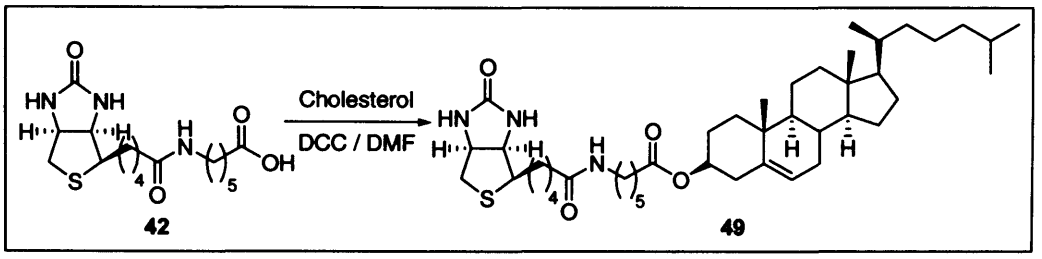
Due to the low yield of monoprotected hexamethylenediamine **47**, the reaction was repeated. As the extraction was believed to be the cause of the low product yield, this time no extraction was performed. $(Boc)_2O$ in excess and the solvent DCM were removed under reduced pressure from the crude. After purification by column chromatography, compound **47** was obtained in a greater yield, 45%.

Unfortunately, due to lack of time the monoprotected hexamethylenediamine **47** was not coupled to 9-(pentafluorophenyl) ester biotin **41**.

VII.3 Attempted synthesis of 6-(cholesteryl hexanoate ester)-amide biotin (49)

The final step to the probe's synthesis, involved the coupling of the ligand with the biotin-linker **42**. This was first attempted with cholesterol in

order to establish a methodology. Biotin-6-aminocaproic acid **42** was coupled to cholesterol using the coupling agent DCC in DMF (Scheme VII.8)[133,181].



Scheme VII.8: Attempted synthesis of 6-(cholesteryl hexanoate ester)-amide biotin **49**

By TLC, a new less polar spot was identified and was believed to be the product. MS was carried out in both (ES^+) and (ES^-) before extraction. In (ES^-) a peak at 356.1m/z was observed which represented the starting material **42**. This confirmed that the reaction had not gone to completion. As the product contains three amide groups a peak representing the product cation was expected in (ES^+). However no such peak was observed. The product might be difficult to ionize and therefore there were no ions for the MS to detect.

After extraction with $CHCl_3$, ^1H NMR of the crude showed in particular the presence of a biotin derivative that might be the compound **49**. Unfortunately, after purification by column chromatography, no product was recovered. This may be due to two reasons:

1. The hydroxyl group of the cholesterol may be sterically hindered;
2. Compound **42** has a hindered conformation. Molecular dynamics was run on compound **42** (Chem 3D). The carboxylic terminal end of the molecule was shown to bend towards the biotin moiety and a sulphur-hydrogen interaction was formed keeping the molecule in this conformation (Figure VII.9). The sulphur-hydrogen distance measures 2.433Å which is close enough to form a strong interaction.

[181] Kremsky, J. N.; Pluskal, M.; Casey, S.; PerryOkeefe, H.; Kates, S. A.; Sinha, N. D. Biotin and Fluorescein Labeling of Biomolecules by Active Esters of 1-Phenylpyrazolin-5-ones. *Tetrahedron Lett.* **1996**, *37*, 4313-4316.

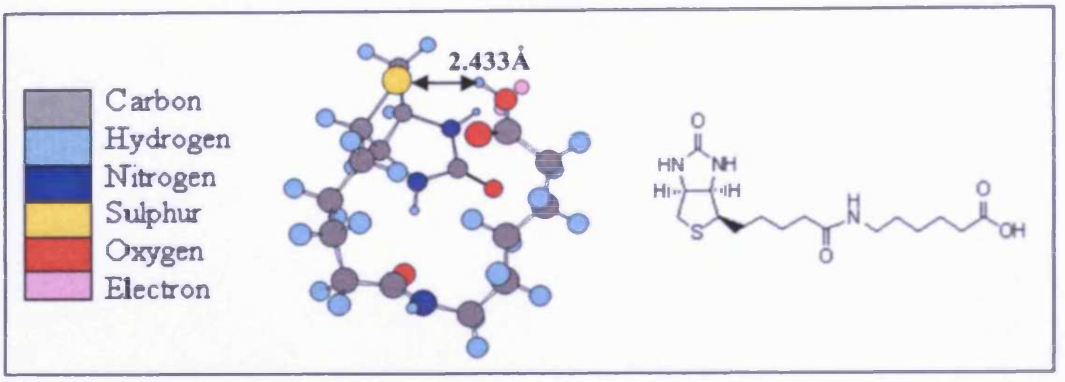


Figure VII.9: The 3D bent and 2D linear conformation of 9-(6-hexanoic acid) amide biotin **42**

This interaction may render the molecule un-reactive and/or prevent the relatively bulky DCC gaining access to the terminal hydrogen. Using the same software, molecular dynamics of 9-(5-pentanoic acid) amide biotin and 9-(4-butanoic acid) amide biotin (Figure VII.10) did not show any sulphur-hydrogen interaction and their conformation remained linear. They might be an alternative to the probe synthesis.

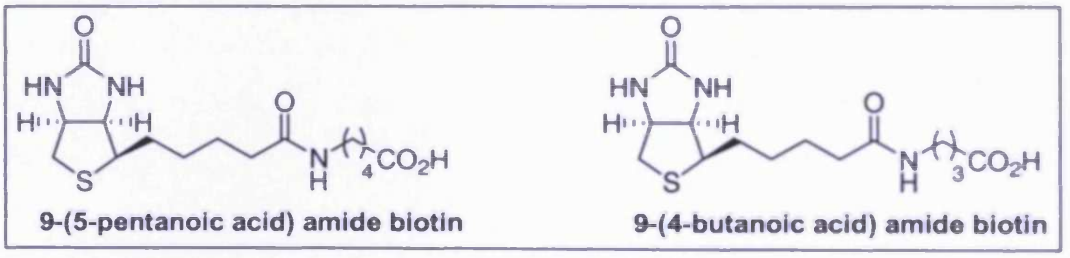


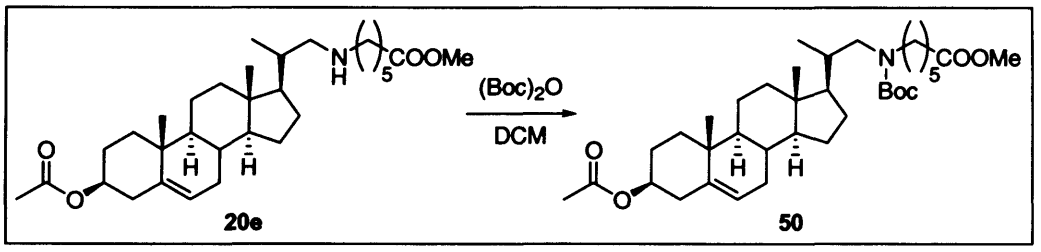
Figure VII.10: Molecular structure of 9-(5-pentanoic acid) amide biotin and 9-(4-butanoic acid) amide biotin

VII.4 Attempted synthesis of 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl hexanoate)-(tert-butyl ester) amine (50)

The ligand will be attached to compound **42** though an ester bond via the alcohol functionality at position 3. Therefore it was decided to protect the secondary nitrogen of 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl hexanoate) amine **20e** for two reasons:

- The ligand would be compound **24e** which has been shown to be poorly lipophilic; this can be improved by adding a Boc group on the secondary nitrogen;
- If the unprotected ligand was reacted with compound **42**, the amine could potentially react with the terminal carboxylic group leading to the formation of a byproduct.

$(Boc)_2O$ was added drop wise in a solution with compound **20e** in DCM and stirred overnight (Scheme VII.9).



Scheme VII.9: Attempted synthesis of 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl hexanoate)-(tertbutyl ester) amine **50**

Analysis by TLC showed the presence of a new spot close to the solvent front believed to be compound **50**. MS of the crude confirmed the presence of the product (623.9m/z). After purification by column chromatography no compound **50** was recovered. ^1H NMR showed the presence of an unidentified compound bearing the Boc group. The hydrolysis of the Boc group from compound **50** could have been due to the Boc group being sterically crowded. The crowding may have made the Boc group more sensitive to the acidity of the silica column.

Unfortunately, due to lack of time the final synthesis of the ligand was not completed, neither was the final synthesis of the probes.

VII.5 Summary

The synthesis of two types of probes has been started. Biotin has been coupled with 6-aminocaproic acid leading to compound **42**, but unfortunately the last step, the coupling with cholesterol has not been worked out.

The coupling with hexamethylenediamine, did not work. The intermediate **44** could not be isolated, and the investigation of other routes did not give the desired probe. This will be the subject of future work.

CHAPTER VIII

MOLECULAR BIOLOGY AND ENZYME ASSAYS WITH 24-SMT

VIII.1 Aims and objectives

This chapter describes some research undertaken by the author at Instituto de Parasitología y Biomedicina "López-Neyra" (Granada, Spain). The aim of this work was to investigate further the role of azasterols as inhibitors of the 24-SMT enzyme. It was discovered that azasterols are active against the blood stream form of *Trypanosoma brucei* (*in vitro* assays, Chapter VI). It has been proposed in the literature that ergosterol biosynthesis does not occur in the blood stream form of *T. brucei*, and the organism scavenges cholesterol from human host[68,70]. This is contrary to the vector form, where ergosterol biosynthesis has been found and the enzyme 24-SMT is expressed. We wanted to investigate if the *T.b. brucei* 24-SMT is expressed in the blood stream form and if it is inhibited by azasterols, to establish a possible mode of action. The main parts of the project were:

- Analysis of the presence of *T.b. brucei* 24-SMT mRNA in both procyclic (Tsetse fly stage) and blood stream (human stage) forms of the parasite by Northern and Western Blot;
- Purification of the *T.b. brucei* 24-SMT protein:
  - ✓ Isolation of the *T.b. brucei* 24-SMT gene;
  - ✓ Cloning the *T.b. brucei* 24-SMT gene in an expression vector;
  - ✓ Over expression of the 24-SMT protein;
  - ✓ Purification of the 24-SMT protein;
  - ✓ Measure the specific activity of the 24-SMT protein;
- Analysis of both procyclic and blood stream forms of the *T.b. brucei* parasite in culture with azasterols (WSP844 and WSP879) by electron microscopy.

VIII.2 Northern and Western blottings

In the 1970s, E.M. Southern[182] developed a method for locating a particular sequence of DNA within a complex mixture. This technique came to be known as Southern blotting. As a particular sense of humour, scientists who used a similar method for locating a sequence of RNA, named this method Northern blotting also known as Northern hybridization or RNA hybridization.

VIII.2.1 Northern blotting

We wanted to investigate if the *T.b. brucei* 24-SMT enzyme was expressed in the blood stream form (bsf) of the parasite. Thus, we first carried out a Northern blotting[183] in order to check whether or not the 24-SMT gene is transcribed in RNA in the bsf of the parasite.

The RNA experiment must be carefully prepared as RNase solutions and glassware can be contaminated by RNA. Ensuring that the RNA preparation remains undegraded after electrophoresis, blotting and hybridization, are required to obtain a good RNA signal. All solutions should be prepared using sterile deionised water. All the glassware should be autoclaved and used with extreme caution. Gloves are indispensable in order to avoid contamination of the glassware by RNA presence on the skin. Northern blot RNA analysis[183] was carried out following a few steps as described next:

- Isolation of RNA for both procyclic and bsf of the parasites;
- Run an agarose gel;
- RNA transfer from the agarose gel to a membrane support;
- Hybridization analysis with labelled DNA probes.

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[182] Brown, T.; Mackey, K. Analysis of DNA Sequences by Blotting and Hybridization. *Curr. Prot. Mol. Biol.* **1997**, *2.9&2.10*, 1-20.

[183] Brown, T.; Mackey, K. Analysis of RNA by Northern and Slot Blot Hybridization. *Curr. Prot. Mol. Biol.* **1997**, *4.9*, 1-16.

VIII.2.1.1 Isolation and agarose gel of the mRNA

Both procyclic and blood stream forms of the parasites were cultured until logarithmic phase when RNA transcription is maximised. The parasites concentrations therefore required are slightly different depending on their respective growth rates. The procyclic form has a complete growth cycle every 8h at 28°C while it is every 12h at 37°C for bsf. Thus, for the procyclic form, $7x10^6$-$1x10^7$ parasites/mL is the best concentration for RNA extraction, whereas for bsf it is $1x10^6$-$1.5x10^6$ parasites/mL. The isolations were achieved when the concentrations were $1x10^7$ parasites/mL for procyclic and $1.6x10^6$ parasites/mL for the blood stream form (Chapter X, Section X.1.2.1). After purification, the RNA concentrations were determined:

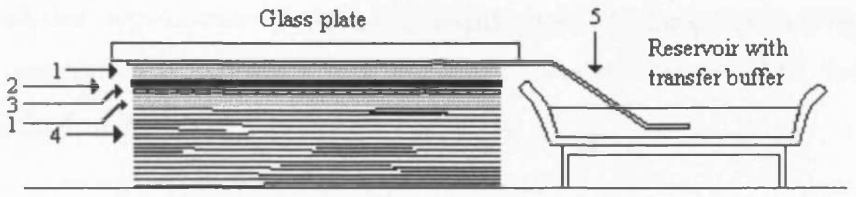
- Procyclic form: 1.0588 µg/µL;
- Bsf: 0.2706 µg/µL.

An agarose gel was run. Agarose gel electrophoresis separates DNA or RNA fragments according to their size. The gel was prepared using formaldehyde that creates denatured conditions. RNA is able to form secondary structures by intramolecular base pairing and therefore must be electrophoresed under denaturing conditions. In the gel, 15 µg of RNA were loaded for both the procyclic and blood stream forms of the parasite. The gel was run for 4 hours at room temperature.

VIII.2.1.2 Transfer of the mRNA

After running the gel, denatured RNA was transferred to a membrane using the "Downward Capillary Transfer" protocol (Southern blot)[182]. Traditionally, a nitrocellulose membrane is used, although nylon or a positively charged nylon membrane may also be used. A nylon membrane has been used because more RNA can bind and it is less fragile than the nitrocellulose membrane. The transfer (Figure VIII.1) was achieved by capillary action with a SSC 20x buffer, overnight at room temperature. Capillary action transfers the

buffer through the gel into the membrane which will bind single stranded RNA.



1: Whatman 3MM
2: Agarose gel
3: Nylon membrane
4: Paper towels (2-3cm)
5: Whatman 3MM wick

Figure VIII.1: mRNA transfer to a nylon membrane

The first transfer attempt resulted in many problems leading to loss of the RNA:

- The paper towels should not touch the Whatman 3MM wick;
- The size of the paper towels, Whatman 3MM, the gel and the membrane must match perfectly;
- The paper towels must be well organised to avoid the formation of grooves;
- The transfer must be carried out without the markers used in the gel.

In the second attempt, the transfer was achieved overnight at room temperature and the RNA fixed on the membrane by irradiation with UV for 5 minutes (this cross links the RNA to the membrane via covalent bonds).

VIII.2.1.3 Hybridization

This process relies on the single stranded DNA (radioactive probe) hybridizing to the RNA on the membrane due to complementary binding. The radio-labelled probe was prepared by PCR of *T.b. brucei* 24-SMT gene using ^{32}P labelled ATP (Section VIII.3.1). An agarose gel was run, 24-SMT DNA

was purified and its radioactive activity determined. It is important that the probe has the correct level of incorporation of ^{32}P label. If the levels of ^{32}P label in the probe are too low, then when hydridisation is carried out, the DNA will give a low signal when hybridised to the RNA.

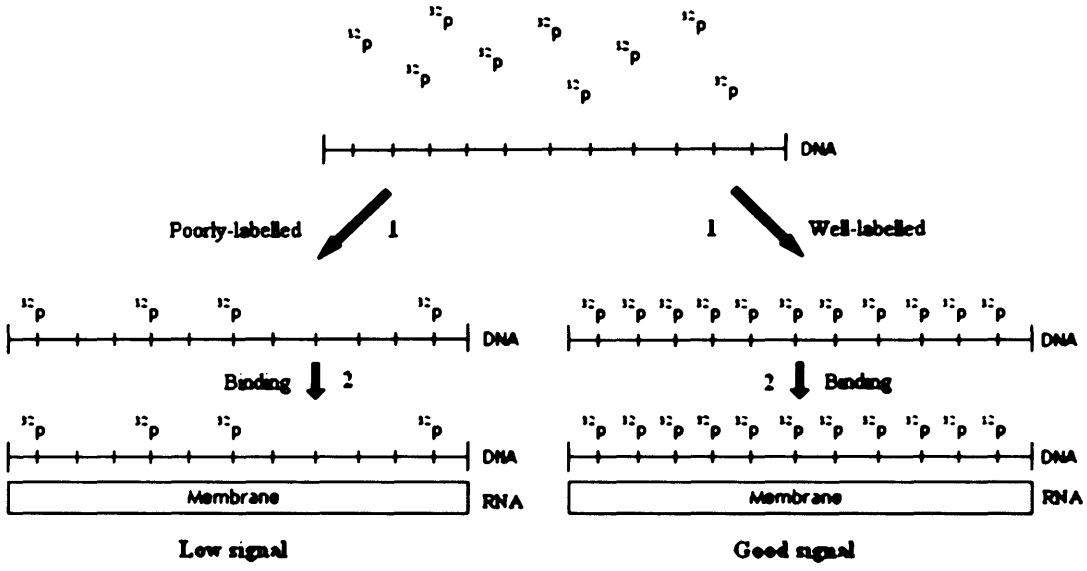


Figure VIII.2: Hybridization of the nylon membrane with a radioactive probe

Before doing the hybridization (Binding), the probe was denatured at 100°C for 10 minutes (Figure VIII.3). The denaturation opens the double-stranded DNA in a single stranded DNA, which will hybridize better with RNA on the membrane.

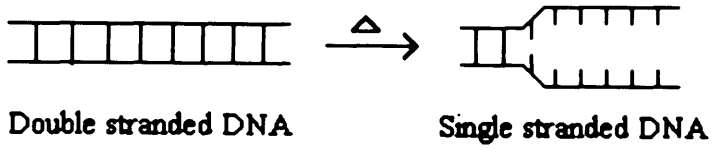


Figure VIII.3: Denaturation of the DNA probe

The activity of the probe was 10^6 counts/min/mL. The labelled probe was then applied in a solution overnight at 42°C that promotes hybridization. The membrane was washed with different SSC/SDS solutions (X.1.2.2) so that any non-specifically bound probe was removed, leaving only probe that was

base-paired to the target DNA. Finally, the radioactively labelled target sequence was visualised by autoradiography (Figure VIII.4). Figure VIII.4 clearly shows a single transcript of 2.6kb in both forms. Densitometric analysis showed that approximately three fold higher levels are present in procyclics. Very interestingly, Northern blot analysis indicated that 24-SMT was also transcribed in the bsf of *T.b. brucei*.

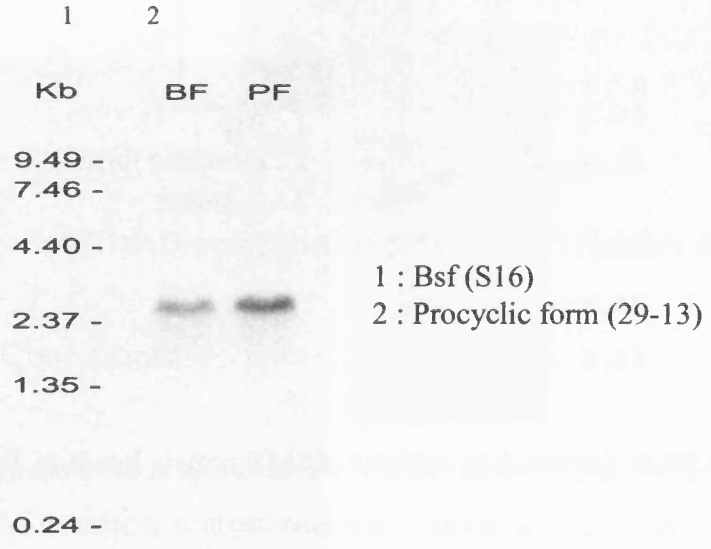


Figure VIII.4: Northern blot analysis of *T.b. brucei* SMT gene expression

VIII.2.2 Western blotting

A Western blotting has recently been carried out by Maria del Carmen Jimenez Jimenez[184] at the Instituto de Parasitologia y Biomedicina "Lopez-Neyra" (Granada, Spain). The Western blot can detect a protein in a mixture of any number of proteins and also give information about its size. This method is dependent on the use of an antibody directed against the desired protein. In this case the antibody raised against recombinant *L. major* 24-SMT was used. The *L. major* 24-SMT shows high sequence similarity to the *T.b. brucei* enzyme.

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[184] Jimenez, M. d. C. J. $\Delta^{24(25)}$-Esterol Metiltransferasa de *Leishmania major*: Funcion Biologica y Estudios Bioquimicos de la Enzima Recombinante. *Thesis. Instituto de Parasitologia y Biomedicina "López-Neyra". Consejo Superior de Investigaciones Científicas (C.S.I.C.) Granada* **2003**.

Fortunately, the antibody to *L. major* 24-SMT showed cross-reactivity to the *T.b. brucei* 24-SMT enzyme. A band of the expected molecular mass was observed in both bsf and procyclic of *T.b. brucei* (Figure VIII.5). The level was approximately three fold higher in the procyclic form as determined by densitometric analysis.

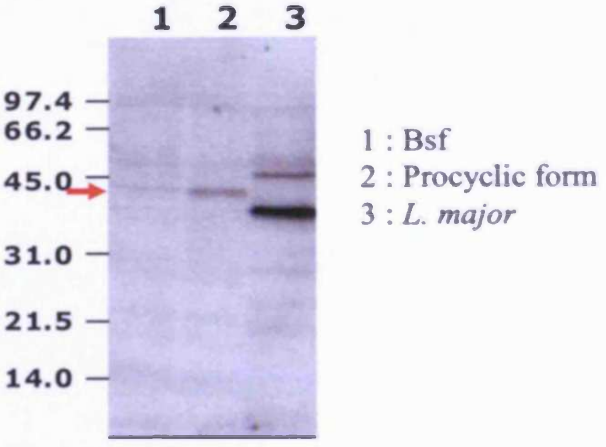

Figure VIII.5: Western blot analysis of SMT protein levels of *T.b. brucei*

The Western blot analysis showed that the protein was expressed in the bsf of *T.b. brucei*, although the level of expression appeared to be less than in procyclic. It was now important to prove that it was also functional. This has been shown by carrying out enzyme assays with radio-labelled desmosterol. Conversion of desmosterol to methylsterols by cellular extracts of both forms of *T.b. brucei* was investigated (Figure VIII.6). The specific activity of 24-SMT found in the procyclic form was 2.2pmol.min^{-1}.mg^{-1} and in the bsf 0.8pmol.min^{-1}.mg^{-1}. As mentioned at the beginning of this chapter, it has been proposed in the literature that ergosterol biosynthesis does not occur in the blood stream form of *T. brucei*[68,70]. Therefore, this result is surprising, as it shows that the 24-SMT is expressed and functional in the bsf *T.b. brucei*.

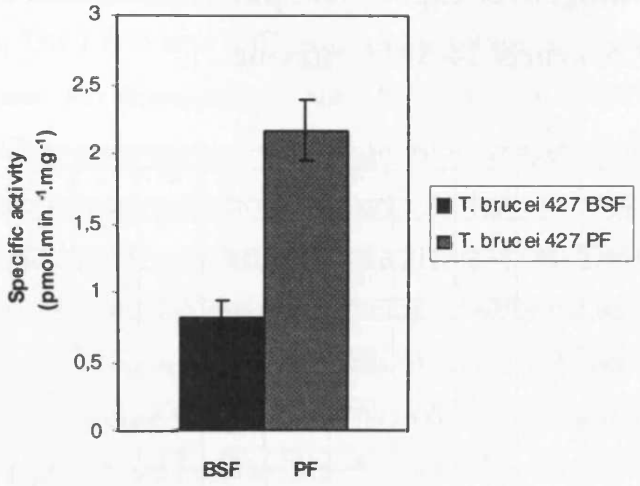

Figure VIII.6: Determination of SMT activity in parasite extracts

VIII.2.4 Conclusion

Trypanosoma brucei brucei RNA was isolated, purified and an agarose gel run. The transfer to a membrane was performed with a SSC 20x buffer, by gravity, using the "Downward Capillary Transfer" protocol. The analysis of the Northern blot after hybridization with a radioactive probe showed the transcription of 24-SMT in bsf *T.b. brucei*. Analysis of a Western blot also showed the presence of the enzyme in bsf parasites.

VIII.3 Cloning, over expression, purification and enzyme assays with the *T.b. brucei* 24-SMT enzyme

1	ATG	TCG	GCC	GGA	TCT	CGT	GGC	CCA	CTT	TCC	CTG	CTC	ATT	GCC	CGT	GAA
1	Met	Ser	Ala	Gly	Ser	Arg	Gly	Pro	Leu	Ser	Leu	Leu	Ile	Ala	Arg	Glu
49	CGT	GAT	GCC	AAT	GGT	GTG	AAT	GGC	GAT	GTG	AAT	GCA	ACG	GCT	GGA	CGC
17	Arg	Asp	Ala	Asn	Gly	Val	Asn	Gly	Asp	Val	Asn	Ala	Thr	Ala	Gly	Arg
97	CTG	CGT	GAC	CGA	TAC	GAT	GGG	AAG	GGT	GCC	TCC	GCC	AGT	GAG	CGA	CGG
33	Leu	Arg	Asp	Arg	Tyr	Asp	Gly	Lys	Gly	Ala	Ser	Ala	Ser	Glu	Arg	Arg
145	CAG	GAT	GCG	ACA	TCG	CTG	ACC	AAC	GAG	TAC	TAC	GAT	ATC	GTT	ACA	GAT
49	Gln	Asp	Ala	Thr	Ser	Leu	Thr	Asn	Glu	Tyr	Tyr	Asp	Ile	Val	Thr	Asp
193	TTC	TAT	GAA	TAC	GGT	TGG	GGG	CAA	AAC	TTC	CAC	TTT	GCT	CCT	CGG	TAC
65	Phe	Tyr	Glu	Tyr	Gly	Trp	Gly	Gln	Asn	Phe	His	Phe	Ala	Pro	Arg	Tyr
241	ATG	AAT	GAG	ACG	TTC	TAC	GAA	TCA	CTC	GCA	CGG	TAT	GAG	TAC	TTT	CTT
81	Met	Asn	Glu	Thr	Phe	Tyr	Glu	Ser	Leu	Ala	Arg	Tyr	Glu	Tyr	Phe	Leu
289	GCG	TAC	CAT	GCT	CAG	TTC	AAA	CCG	ACG	GAC	ACT	GTG	CTA	GAC	GTT	GGC
97	Ala	Tyr	His	Ala	Gln	Phe	Lys	Pro	Thr	Asp	Thr	Val	Leu	Asp	Val	Gly
337	TGC	GGG	ATT	GGT	GGG	CCA	GCA	CGA	AAC	ATG	GTA	CGG	TTC	ACA	TCG	TGC
113	Cys	Gly	Ile	Gly	Gly	Pro	Ala	Arg	Asn	Met	Val	Arg	Phe	Thr	Ser	Cys
385	AAC	GTG	ATG	GGT	GTA	AAT	AAT	AAT	GAG	TAT	CAA	ATA	AAT	CGC	GCG	CGG
129	Asn	Val	Met	Gly	Val	Asn	Asn	Asn	Glu	Tyr	Gln	Ile	Asn	Arg	Ala	Arg
433	CAG	CAC	GAT	TCC	CGC	TAC	GGG	ATG	AGC	GGT	AAA	ATT	AAC	TAC	ACT	AAG
145	Gln	His	Asp	Ser	Arg	Tyr	Gly	Met	Ser	Gly	Lys	Ile	Asn	Tyr	Thr	Lys
481	ACC	GAC	TTC	TGT	AAC	ATG	TGC	TTT	GGC	GAC	AAC	GAG	TTC	GAC	GGA	GCA
161	Thr	Asp	Phe	Cys	Asn	Met	Cys	Phe	Gly	Asp	Asn	Glu	Phe	Asp	Gly	Ala
529	TAC	GCC	ATC	GAG	GCG	ACA	TGC	CAC	TCA	GAG	AGT	AAG	GTA	AAG	TGC	TAT
177	Tyr	Ala	Ile	Glu	Ala	Thr	Cys	His	Ser	Glu	Ser	Lys	Val	Lys	Cys	Tyr
577	AGT	GAA	GTG	TTT	CGC	GCT	ATC	AAA	CCT	GGT	GCC	TAC	TTT	ATG	CTG	TAC
193	Ser	Glu	Val	Phe	Arg	Ala	Ile	Lys	Pro	Gly	Ala	Tyr	Phe	Met	Leu	Tyr
625	GAG	TGG	TGT	TTG	ACG	GAC	CTG	TAT	GAC	CCG	GCA	AAT	GAG	GAA	CAC	CAG
209	Glu	Trp	Cys	Leu	Thr	Asp	Leu	Tyr	Asp	Pro	Ala	Asn	Glu	Glu	His	Gln
673	CGT	GTC	CGG	CAT	GGT	ATC	GAA	CTT	GGC	GAC	GGT	CTT	CCT	GAA	CTC	GAC
225	Arg	Val	Arg	His	Gly	Ile	Glu	Leu	Gly	Asp	Gly	Leu	Pro	Glu	Leu	Asp
721	ACG	ATG	CGG	CAG	GTT	GTC	GCA	GCG	GTA	AAG	GCC	GCC	GGT	TTC	GTT	GTG
241	Thr	Met	Arg	Gln	Val	Val	Ala	Ala	Val	Lys	Ala	Ala	Gly	Phe	Val	Val
769	GAA	GAG	AGC	TTT	GAC	ATG	GCG	GAA	CGA	TTC	GAA	AGT	GGC	GAG	CCG	AAG
257	Glu	Glu	Ser	Phe	Asp	Met	Ala	Glu	Arg	Phe	Glu	Ser	Gly	Glu	Pro	Lys
817	AGC	GTC	CCG	TGG	TAT	GAG	CCA	CTG	CAG	GGG	AGT	TAC	ACG	TCG	CTG	AGT
273	Ser	Val	Pro	Trp	Tyr	Glu	Pro	Leu	Gln	Gly	Ser	Tyr	Thr	Ser	Leu	Ser
865	GGA	CTT	CGG	GCG	ACC	CCT	GCA	GGG	CGC	TGG	TTG	ACG	AGT	GTA	ACA	TGT
289	Gly	Leu	Arg	Ala	Thr	Pro	Ala	Gly	Arg	Trp	Leu	Thr	Ser	Val	Thr	Cys
913	CGT	TTG	CTC	GAG	GCT	GTG	CGC	CTC	GCA	CCG	GCA	GGT	ACA	TGT	AAG	GCG
305	Arg	Leu	Leu	Glu	Ala	Val	Arg	Leu	Ala	Pro	Ala	Gly	Thr	Cys	Lys	Ala
961	ACA	GAG	ATC	CTC	GAA	GAA	GGA	GCG	GTG	AAC	CTT	GTC	AAG	GGG	GGC	GAA
321	Thr	Glu	Ile	Leu	Glu	Glu	Gly	Ala	Val	Asn	Leu	Val	Lys	Gly	Gly	Glu
1009	CTT	GGG	ATA	TTC	ACG	CCA	TCC	TTC	TTT	GTG	AAG	GCG	CGT	AAA	CCG	CGT
337	Leu	Gly	Ile	Phe	Thr	Pro	Ser	Phe	Phe	Val	Lys	Ala	Arg	Lys	Pro	Arg
1057	CTT	GGG	GAA	GAG	CTG	TCG	TGC	TAA								
353	Leu	Gly	Glu	Glu	Leu	Ser	Cys									

Figure VIII.7: *Trypanosoma brucei brucei* 24-SMT sequence

The *T.b. brucei* SMT sequence was identified in Genebank[185] on chromosome 10 of the *T.b. brucei* genome. The sequence has 1080 bases (Figure VIII.7) which corresponds to 360 amino acids for the protein. The program BLAST was used to find the sequence. This program compared the

[185] http://www.ncbi.nlm.nih.gov.

Figure VIII.8 shows complete sequence identity whereas the red colour s similarity in sequence between the organisms. Figure VIII.8 shows that th a well conserved sequence (61% identity between *T.b. brucei* and *L. n* and four main regions have been identified. Region I is the sterol bindin conserved in all SMTs enzyme[186,187] while regions II, III and IV are typi *S*-adenosyl-$_L$-methionine dependent methyltransferase enzymes[188,189]. Re (100-109) contains tryptophan amino-acid which is thought to be essenti the interaction with the hydroxyl group at the position 3 of zymostero Region II (141-151) interacts with the SAM[188]. Region III (199-220) a (227-240) interact with each other and with a portion of region I to for central portion of the β-sheet[189].

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[186] Nes, W. D.; He, L.; Mangla, A. T. 4,4,14-α-Trimethyl-9β,19-cyclo-5α-26-homoch 24,26-dien-3β-ol: A Potent Mechanism-Based Inactivator of $\Delta^{24(25)}$- to $\Delta^{25(27)}$ Methyl Transferase. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3449-3452.

[187] Nes, W. D.; McCourt, B. S.; Zhou, W. X.; Ma, J. Z.; Marshall, J. A.; Peek, L. A.; Br M. Overexpression, Purification, and Stereochemical Studies of the Recombina Adenosyl-$_L$-methionine: $\Delta^{24(25)}$- to $\Delta^{24(28)}$-Sterol Methyl Transferase Enzyme *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **1998**, *353*, 297-311.

[188] Kagan, R. M.; Clarke, S. Widespread Occurence of Three Sequence Motifs in Dive Adenosy-$_L$-Methionine-Dependent Methyltransferases Suggests a Common Structu These Enzymes. *Arch. Biochem. Biophys.* **1994**, *310*, 417-427.

[189] Niewmierzycka, A.; Clarke, S. *S*-Adenosy-$_L$-Methionine-Dependent Methylati *Saccharomyces cerevisiae* - Identification of a Novel Protein Arginine Methyltrans *J. Biol. Chem.* **1999**, *274*, 814-824.

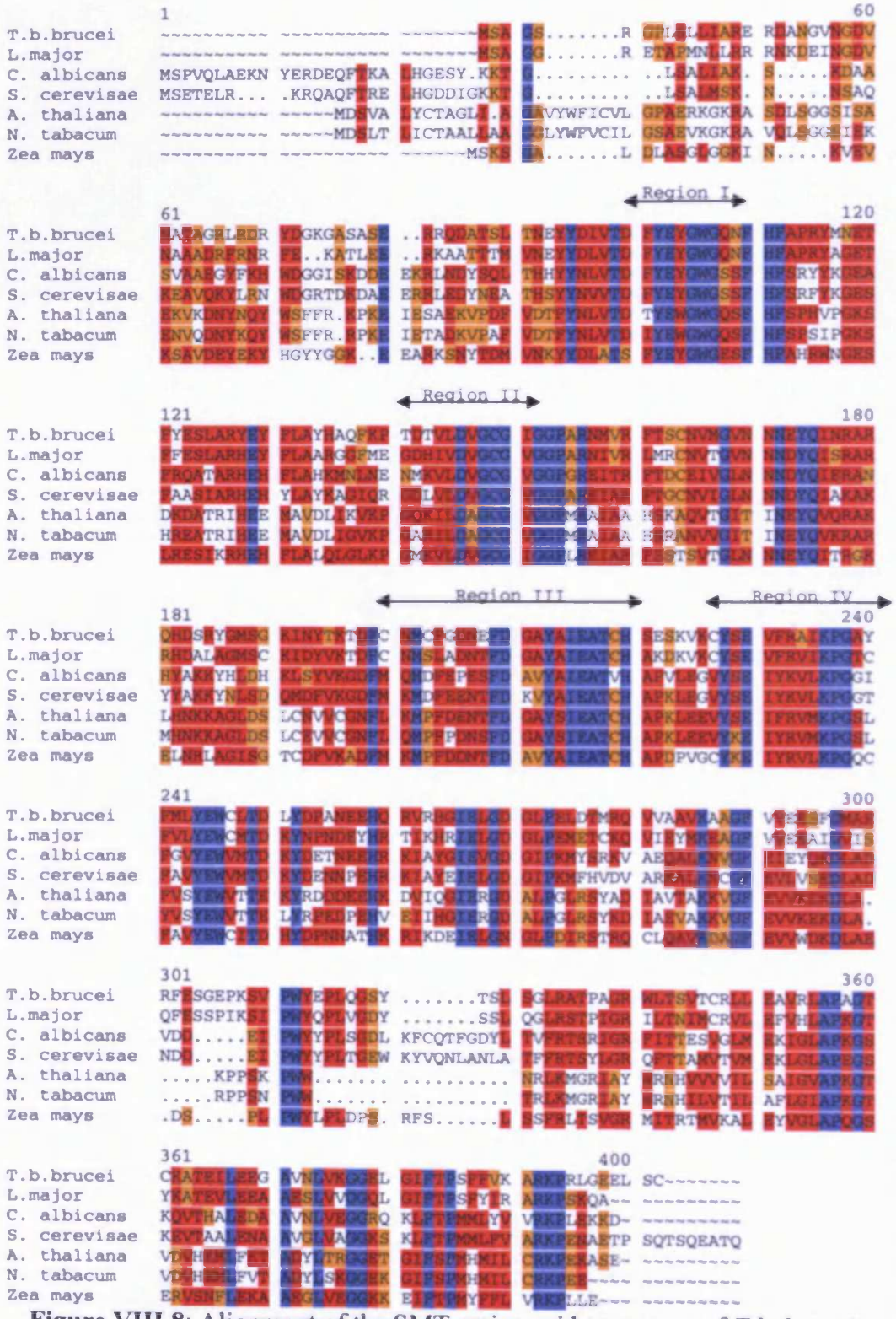


Figure VIII.8: Alignment of the SMT amino acid sequences of *T.b. brucei*, *Leishmania major*, *Candida albicans*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Nicotiana tabacum* and *Zea mays*.

VIII.3.1 Cloning of the *T.b. brucei* 24-SMT gene

The *T.b. brucei* 24-SMT sequence was amplified by PCR (Polymerisation Chain Reaction, Chapter X, Section X.2.2), using *T.b. brucei* DNA and the oligonucleotides TbSMT-1 and TbSMT-2 (Table VIII.2 and VIII.3, Section X.2.1.4). The oligonucleotides TbSMT-1 and TbSMT-2 were designed so they could be used with particular restriction enzymes. The genes resulting from PCR could be cloned into a vector.

Nucleotide TbSMT-1		
Bases	***Nde*I site**	**5' site of the sequence**
GGAATTC	CATATG	TCGGCCGGATCTCGT

Table VIII.2: Design of the oligonucleotide TbSMT-1

Nucleotide TbSMT-2		
Bases	***Bam*HI site**	**Reverse complementary 3' site of the sequence**
CG	GGATCC	TTAGCACGACAGCTCTTCCCC

Table VIII.3: Design of the oligonucleotide TbSMT-2

The oligonucleotides TbSMT-1 and TbSMT-2 were designed following rules:

- TbSMT-1 (Table VIII.2):
  1. A small sequence of bases dependent on the restriction site;
  2. A sequence which can be recognised and cleaved by the restriction enzyme *Nde*I;
  3. The first 15 bases at the 5' end of the *T.b. brucei* 24-SMT gene.

- TbSMT-2 (Table VIII.3):
  1. A small sequence of bases dependent on the restriction site;

2. A sequence which can be recognised and cleaved by the restriction enzyme *Bam*HI;
3. The first 21 bases which are complementary to the 21 bases at the 3' end of the *T.b. brucei* 24-SMT gene.

The choice of the restriction enzymes is based on the *T.b. brucei* 24-SMT sequence. Restriction enzymes, also known as restriction endonucleases, are enzymes that cut a DNA molecule at a particular base sequence. They are essential tools for recombinant DNA technology. The enzyme scans a DNA molecule looking for a particular sequence usually between four and six nucleotides. When the particular sequence is recognised, the enzyme stops and cuts the strands. A wide range of restriction enzymes exist. The programme DNA Strider™ compared the sequences cut by endonucleases to the 24-SMT sequence. The *Bam*HI and *Nde*I sequences did not match with any part of the 24-SMT sequence and therefore were used as restriction sites for the cloning. The technical services of the CSIC prepared the nucleotides TbSMT-1 and TbSMT-2.

PCR was then performed. PCR is the amplification of a small amount of DNA into a larger amount. The sequence to be amplified is shown Figure VIII.9. Red colour corresponds to the starting and ending codons of the *T.b. brucei* 24-SMT. Blue colour represents the 5' end and complementary 3' end present in TbSMT-1 and TbSMT-2 respectively.

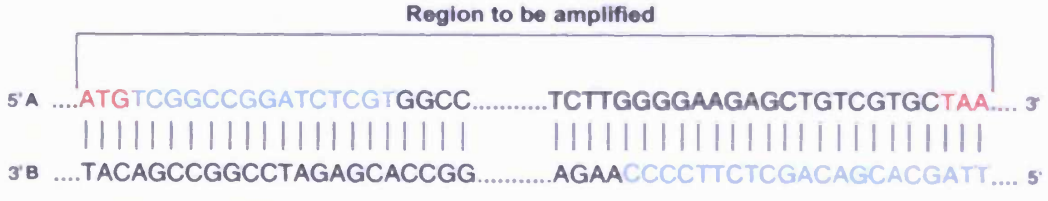


Figure VIII.9: DNA target sequence to be amplified by PCR.

To perform PCR (Section X.2.2), the target sequence is needed (Figure VIII.9); also primers (TbSMT-1 and TbSMT-2) that bind to the target sequence; Taq polymerase (an enzyme involved in replication of DNA) and nucleotides (dNTPs substrates that the DNA Taq polymerase can utilise). The mechanism

of PCR is as follows: DNA is double-stranded. In step **1** (Figure VIII.10), heating to 94°C separates the double-stranded DNA into the two single complementary strands **A** and **B**. Then cooling to 55°C allows the excess primers to hybridize to their complementary sequences in the target DNA. Primer TbSMT-1 and TbSMT-2 bind respectively to strand **B** and **A** at either 3' sites (restriction sites are surrounded by rectangles). This provides a starting point for the replication.

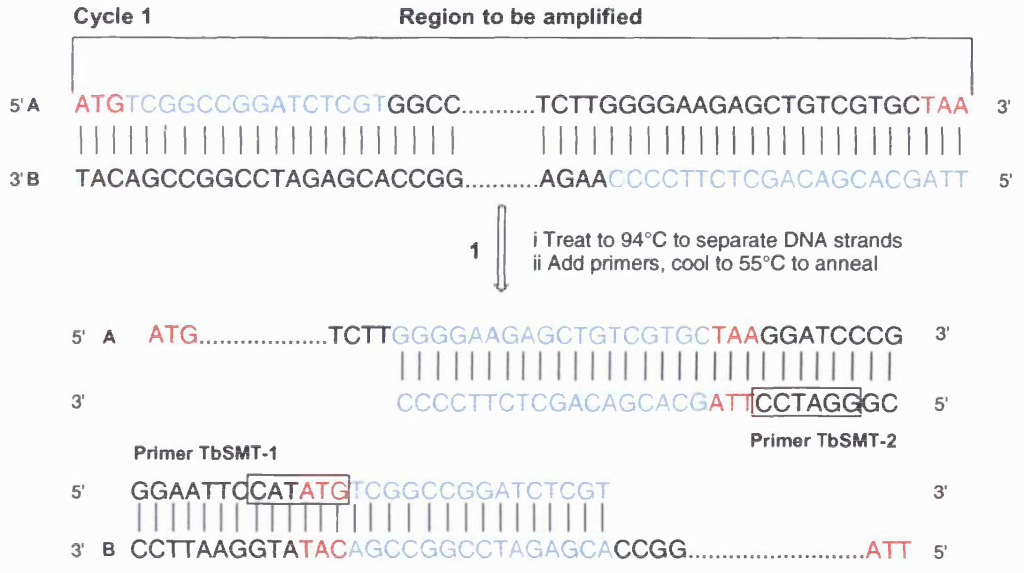


Figure VIII.10: PCR mechanism, Cycle 1

The *Taq* polymerase (Step **2**, Figure VIII.11) extends each primer from its 3' end by polymerization of dNTPs, generating newly synthesized strands **C** and **D**. In the second cycle (Figure VIII.12, step **3**), the original and newly made DNA strands **A**, **B** ,**C** and **D** are separated at 94°C and primers annealed to their complementary sequences at the 3' site, at 55°C. Each annealed primer again is extended by *Taq* polymerase to the end of the other primer sequence at the 5' end and give the strands **E** and **F** (Figure VIII.12, step **4**). Thus the strands synthesized in this cycle exactly equal the length of the region to be amplified including both restriction sites.

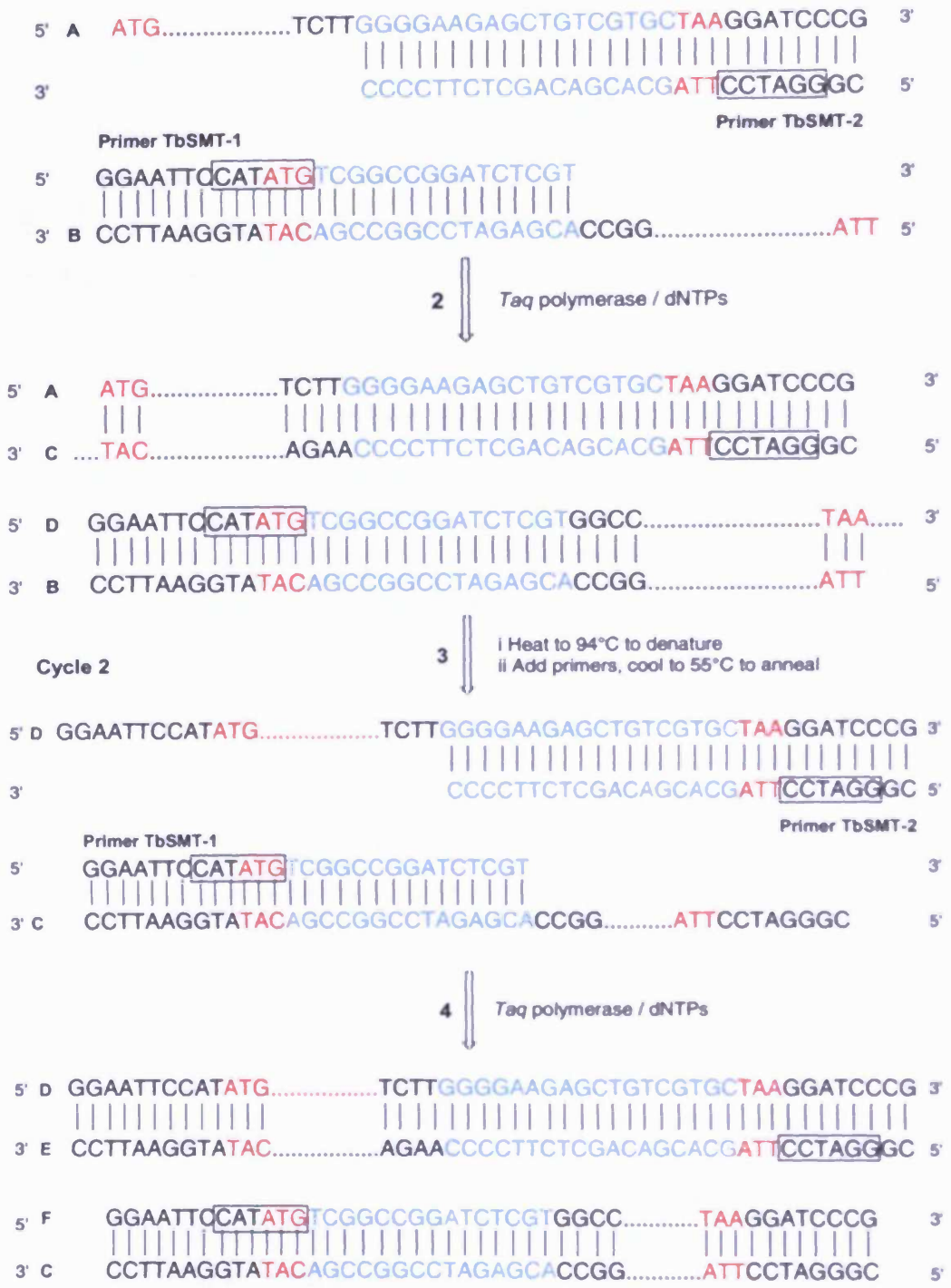


Figure VIII.11: PCR mechanism, cycle 2.

In the third cycle (Figure VIII.12), two double-stranded DNA molecules are generated equal to the sequence of the region to be amplified. Repeated cycles of synthesis, cooling and melting, quickly amplify the

sequence of interest, and the number of copies therefore increases exponentially.

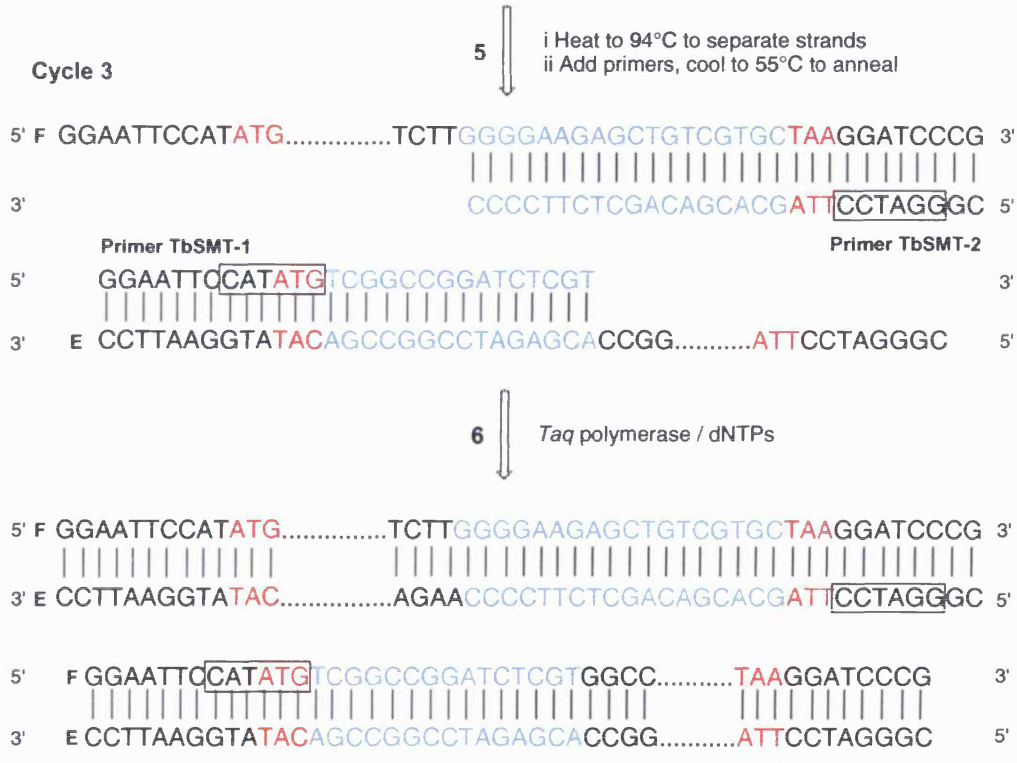
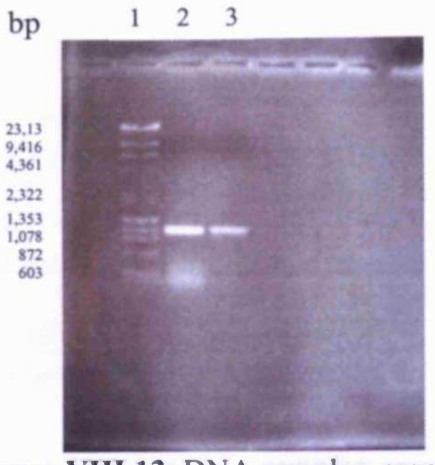


Figure VIII.12: PCR mechanism, Cycle 3.

The PCR product after 30 cycles was checked in an agarose gel (Figure VIII.13). Agarose gel electrophoresis separates DNA fragments according to their size. The gel is finally stained with ethidium bromide which visualises the DNA molecules resolved into bands along the gel. In this case, only one major band was expected around 1080 base pairs. Commercially available standards λ and ϕ were digested with specific enzymes (*Hind*III and *Hae*III, respectively) and used as molecular weight markers[190]. Lanes 2 and 3 (Figure VIII.13) showed a band around 1078 bp which indicates that the specific sequence of *T.b. brucei* 24-SMT DNA was amplified in a good quantity. The band in lane 2 was more intense but the sample was not purified. After using the *kit Wizard PCR Preps DNA Purification System*, pure DNA was obtained (lane 3).

[190] http//www.promega.com.



1: Standards λ and ϕ digested
2: Amplified DNA, non purified
3: Amplified DNA, purified

Figure VIII.13: DNA samples, amplified by PCR, before and after purification

Then, the amplified sequence was cloned into the pGEM-T vector (plasmid)[190] using the T4 DNA ligase (Figure VIII.14). A plasmid is an independent circular self-replicating DNA molecule that carries the insert amplified by PCR. Plasmids are easy to manipulate and isolate using bacteria. There are a wide number of plasmids commercially available, but pGEM-T was chosen because it has the same restriction sites as the insert which will allow the cloning. Also, pGEM-T contains the gene that confers antibiotic resistance to ampicillin; therefore bacteria containing and expressing this plasmid will not be killed by the antibiotic. After transfection, any cells that were not transfected would be killed by the antibiotic. This vector was also chosen for other reasons. First, the vector contains T ends which prevent its recyclisation and therefore, improve the efficiency of the ligation. Secondly, successful transformations can be easily identified following transfection. Clones that contain this plasmid give white colonies whereas the others give blue colonies. Successful cloning interrupts the coding sequence of β-galactosidase (Section VIII.3.2). X-gal (Section X.2.1.3) added to the culture plate would react with β-galactosidase if present, and detected as giving a blue color to the colony. If any bacteria that do not have the plasmid survived the antibiotic, a selection can also be achieved by this means.

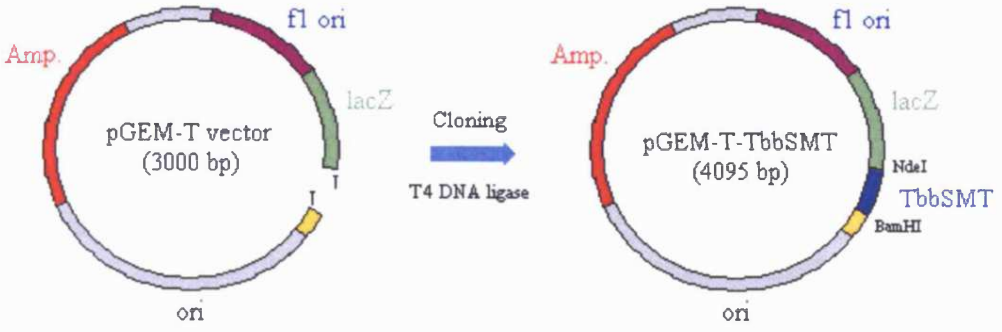


Figure VIII.14: Cloning of *T. brucei brucei* SMT DNA in the pGEM-T vector, pGEM-T-TbbSMT plasmid; ori is the origin of the vector, *lacZ* is the start codon, Amp. is the DNA sequence that gives resistance to Ampicillin when used as an antibiotic.

The ligation was performed with the T4 DNA ligase for 2 hours at room temperature (Chapter X, Section X.2.2). The resulting plasmid pGEM-T-TbbSMT, was transfected into *E. coli* XLI Blue cells by electroporation. The white colonies obtained were checked to ensure they contained the correct plasmid, as follows:

- Extraction of the plasmid from the cells by alkaline lysis (the method of choice for isolating circular plasmid DNA from bacterial cells) using the *kit Wizard® Plus Minipreps DNA Purification System*[190];
- Digestion of the pGEM-T-TbbSMT plasmid by the restriction enzymes *Nde*I and *Bam*HI;
- Analysis of the DNA by an agarose gel (Figure VIII.15): two bands were expected; one for the insert released after the digestion and one for the plasmid.

Seven colonies were checked (lanes 2 to 8, Figure VIII.15). Only the colony 3.1.2 in the third lane showed the release of the insert (1078 bp).

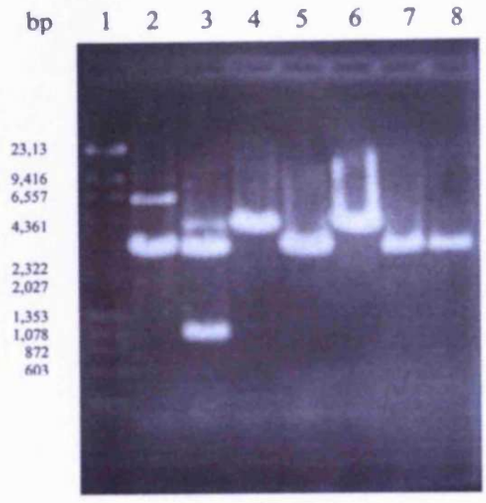


Figure VIII.15: Checking of the cloning in pGEM-T vector

The pGEM-T-TbbSMT plasmid of the colony 3.1.2 was then checked in order to verify that the insert did not contain any mutations that could have happened during the PCR. Two samples were prepared and given to the technical service of the Institute:

- DNA + T7promoter;
- DNA + SP6promoter.

The pGEM-T plasmid contains on one side of the insert the T7 transcription start sequence and on the other side the SP6 transcription start sequence (Figure VIII.16).

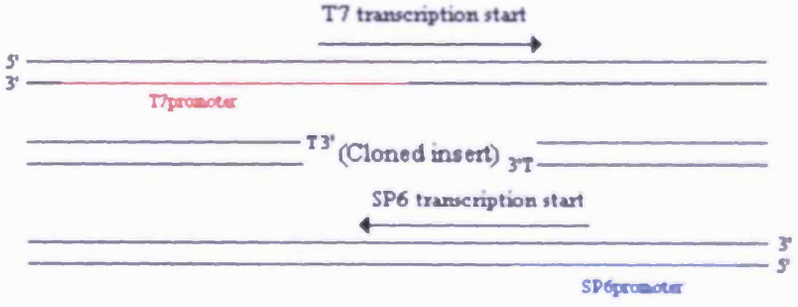


Figure VIII.16: Sequencing of the insert

The T7 promoter and SP6 promoter act such as the oligonucleotides in the PCR experiment. They first bind to their complementary sequence onto the plasmid (blue and red colours, Figure VIII.16) and start building the complementary strand as explained for PCR. Two full complementary sequences were obtained. With the DNA Stride™ program, both sequences were analysed and compared to the original (Figure VIII.8). A 100% identity was observed. Therefore, no mutations occurred during the PCR experiment.

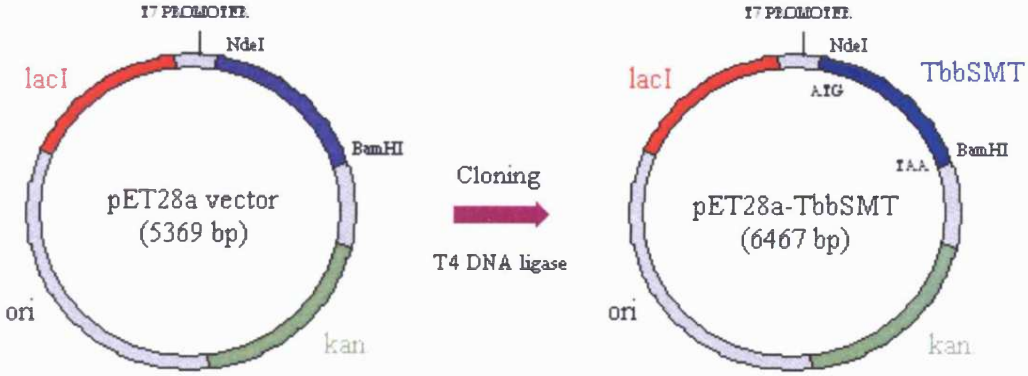


Figure VIII.17: Cloning of *T.brucei brucei* SMT DNA in the pET28a vector, pET28a-TbbSMT plasmid; ori is the origin of the vector, *lacI* represses T7 RNA transcription; Kan. is the DNA sequence that gives resistance to Kanamycin when used as an antibiotic.

The next step was to clone the insert in the expression vector pET28a in order to get the plasmid pET28a-TbbSMT (Figure VIII.17). The plasmid pGEM-T-TbSMT was the source of the insert. No PCR experiment is necessary anymore in order to get the insert. The pET28a vector was chosen because it contains the restriction sites *Nde*I and *Bam*HI, and a specific DNA sequence which confers resistance to kanamycin, an antibiotic used to select the colonies successfully transfected in *E. coli* cells. First, the vector pET28a was prepared by digestion with both enzymes to open the circle, and then dephosphorylated to favor the ligation (Figure VIII.18). Then, the plasmid pGEM-T-TbbSMT was digested with the same two enzymes, *Nde*I and *Bam*HI, to release the insert (Figure VIII.19).

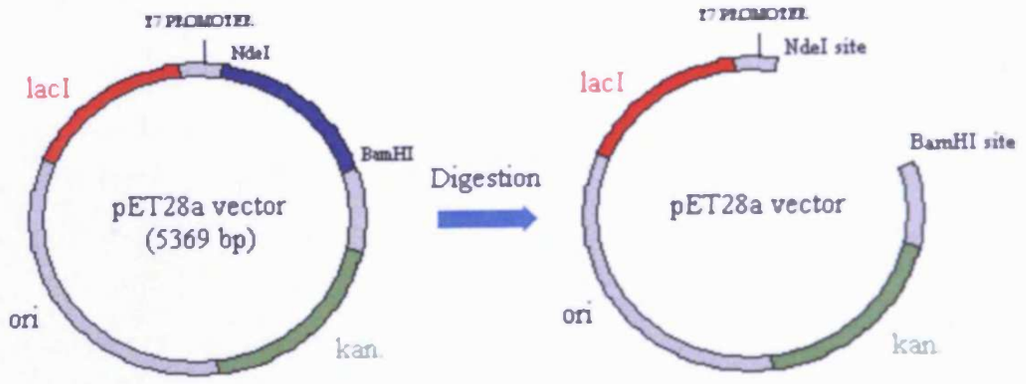


Figure VIII.18: Preparation of the vector Pet28a for the ligation with the insert by digestion with *Nde*I and *Bam*HI and dephosphorylation

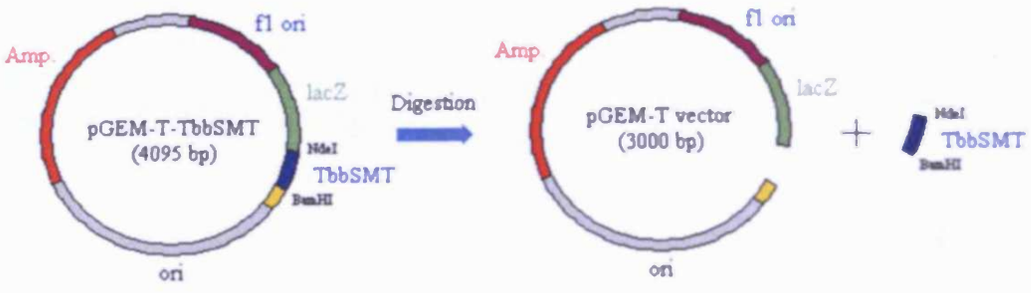


Figure VIII.19: Release of the insert to clone on pET28a by digestion of the plasmid pGEM-T-TbbSMT with *Nde*I and *Bam*HI

The bands with MW corresponding to 1078 bp (DNA from the *T.b. brucei* 24-SMT gene, Figure VIII.20) were removed and the DNA purified using the *Wizard® Plus Minipreps DNA Purification System*[190]. The removal of the bands was carried out under UV light, but the time taken to achieve this procedure was minimised to avoid mutations.

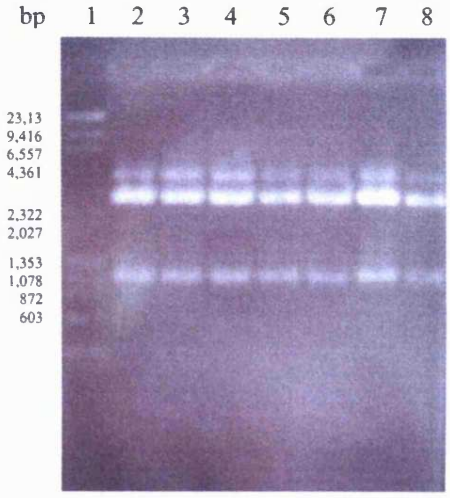


Figure VIII.20: Digestion of the pGEM-T-TbbSMT plasmid by *Nde*I and *Bam*HI enzymes

Finally, the ligation was performed with T4 DNA ligase at 16°C overnight (Figure VIII.21).

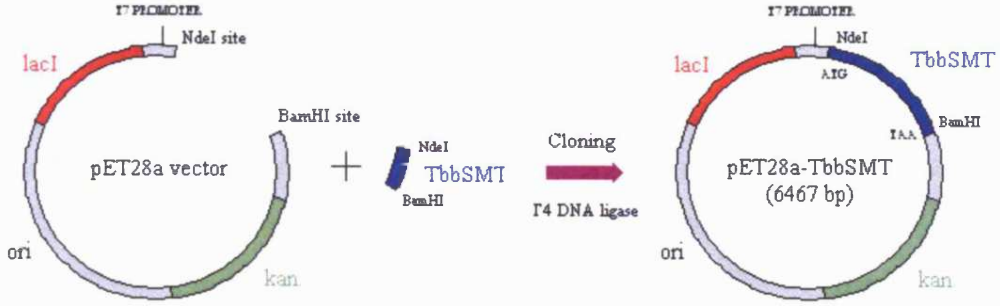
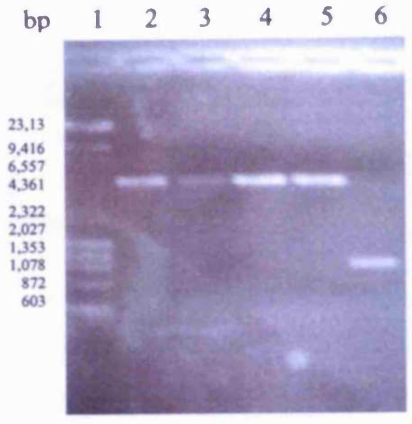


Figure VIII.21: Ligation of the insert in the pET28a vector with T4 DNA ligase

The plasmid was introduced in *E. coli* BL21(DE3) cells by electroporation. Unfortunately, no colonies could be obtained, even after several attempts. No success was achieved using the heat shock method instead of electroporation (Section X.2.2) to transfect the bacteria. Therefore, it was decided to change the conditions. The digestion of pGEM-T-TbbSMT had been carried out by using *Nde*I and *Bam*HI at the same time. Thus, we wanted

to check the efficiency of the two enzymes, *Nde*I and *Bam*HI, by using them sequentially (Figure VIII.22).



1 : Standards λ and ϕ
2 : Vector digested with *Nde*I first
3 : Vector digested with *Nde*I first
4 : Vector digested with *Bam*HI first
5 : Vector digested with *Bam*HI first
6 : TbbSMT insert purified

Figure VIII.22: Checking of the efficiency of *Nde*I and *Bam*HI

In lanes 2 and 3, two different samples of pGEM-T-TbbSMT were digested with *Nde*I, while in lanes 4 and 5 two other samples were digested with *Bam*HI. The agarose gel showed that more insert was released when using *Bam*HI first, as the bands seen in lanes 4 and 5 are more intense. Therefore, in any future experiment that involved the release of the insert, *Bam*HI was used first. Then, it was decided to investigate the ligation conditions. In the previous experiments, the vector pET28a was dephosphorylated following opening the plasmid with the restriction enzymes. Thus, it was decided to investigate ligation with pET28a dephosphorylated or still phosphorylated. Furthermore, a ratio 1/1 of *T.b. brucei* DNA insert and the expression vector pET28a had been used. This time, it was decided to carry out the ligation with different ratios (Table VIII.4).

pET28a	**Phosphorylated**			**Dephosphorylated**		
***T.b. brucei* DNA (eq.)**	1	3	5	1	2	4
pET28a (eq.)	1	1	1	1	1	1
Number of colonies	0	4	1	0	9	0

Table VIII.4: Number of colonies obtained at different ratios of *T.b. brucei* DNA insert and expression vector pET28a

The pET28a still phosphorylated in a ratio 1:1 led to no colonies. A ratio 3:1 and 5:1 led respectively to 4 and 1 colonies. When dephosphorylated pET28a was used, a ratio of 1:1 did not give any colonies as expected. A ratio 2:1 led to 9 colonies whereas a ratio 4:1 did not lead to any colonies. This table showed that the ligation happened in specific conditions, different from those initially investigated. There is no particular explanation, except that ligation is known to be extremely sensitive to the conditions used.

The 14 colonies obtained were checked by digestion of the plasmid pET28a-TbbSMT after extraction from each colony. The numbering is as follows:

- pET28a phosphorylated and a ratio 3/1: colonies 1.1 to 1.4;
- pET28a phosphorylated and a ratio 5/1: colony 2.1
- pET28a dephosphorylated and a ratio 2/1: colonies 3.1 to 3.9

Figures VIII.23 and VIII.24 showed that 11 out of 14 colonies contained the plasmid. The colony 3.8 was selected for sequencing as it showed a good release of the insert. The sequencing of the insert was undertaken using the same strategy as with the pGEM-T vector. This time, the two oligonucleotides used were the T7 promoter and the T7 terminator. The two complementary sequences obtained were compared to the original (Figure VIII.7) with the DNA Strider™ program and 100% identity was found.

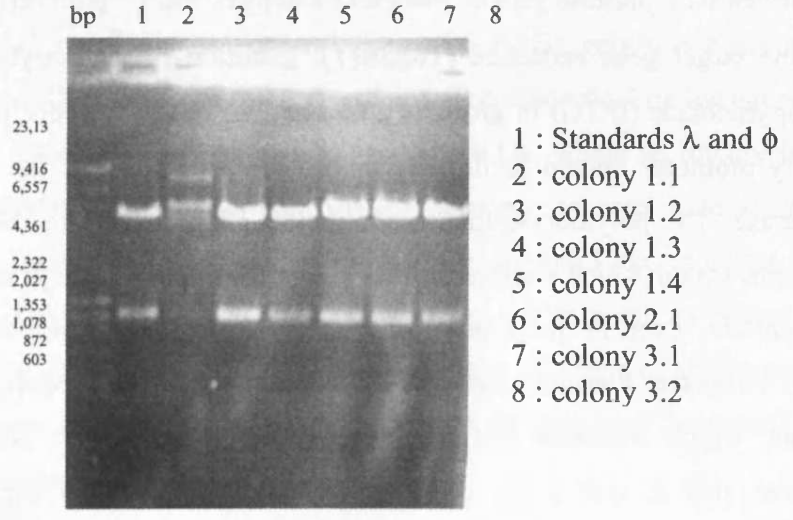


Figure VIII.23: Checking of the cloning in the first 7 colonies, by digestion of the pET28a-TbbSMT plasmid with both *Nde*I and *Bam*HI.

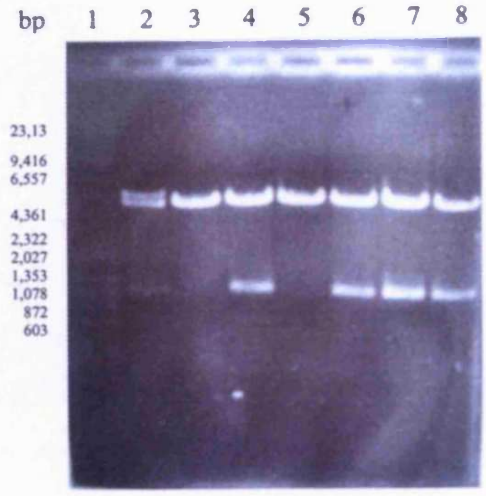


Figure VIII.24: Checking of the cloning in the 7 last colonies, by digestion of the pET28a-TbbSMT plasmid with both *Nde*I and *Bam*HI

VIII.3.2 Over expression of the *T.b. brucei* 24-SMT enzyme

The regulation of protein expression depends on the pET system and the host used. The plasmid pET28a-TbbSMT was introduced by electroporation into *E. coli* BL21(DE3) pLys. This type of system depends on the regulated expression of T7 RNA polymerase in the cell that transcribes the DNA. Recombinant *E. coli* cells are engineered to carry the gene encoding T7 RNA polymerase next to the *lac* promoter (Figure VIII.25). The cell is transformed with plasmid vectors that carry a copy of the T7 promoter and next to it, the target gene sequence (TbbSMT). Addition of Isopropyl-β-D-thio-galactopyranoside (IPTG) in growth culture actives the *lac* promoter which is the only promoter known to directly promote transcription of the T7 RNA polymerase. The polymerase then binds to the T7 promoter on the plasmid expression vector which starts the process of transcription at high rate of the target protein. Even in the absence of IPTG, there is some expression of T7 RNA polymerase. Plasmids like pET28a contain the coding sequence of *lac* repressor which represses the transcription of the T7 RNA polymerase. Moreover, this *E. coli* strain provides additional stability by containing a compatible chloramphenicol resistant plasmid that provides a small amount of T7 lysozyme which is a natural inhibitor of T7 RNA polymerase.

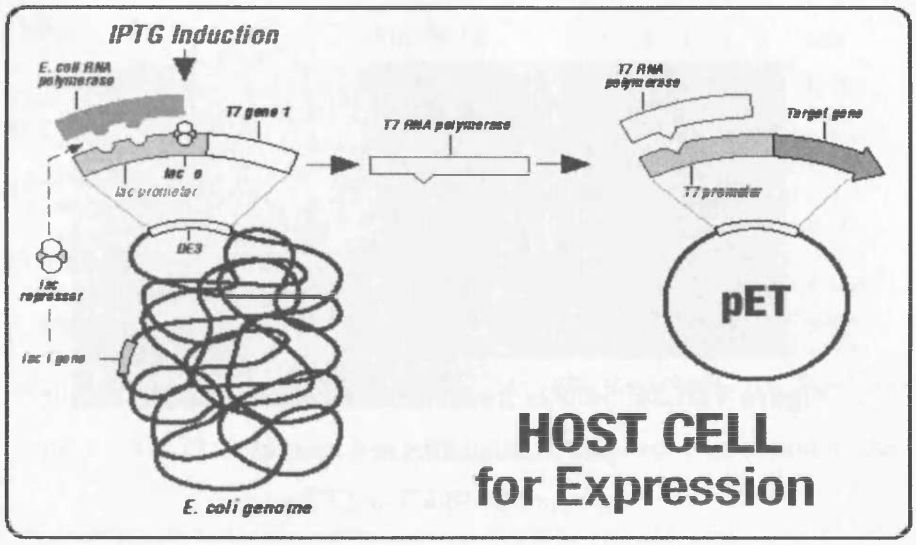


Figure VIII.25: Over expression mechanism of a target protein by IPTG[191]

The over expression was initiated with IPTG at 19°C taking aliquots each hour up to 7 hours and finally overnight. A buffer (Section X.2.2) was added to the aliquots and after sonication and centrifugation, two phases were separated:

- The liquid phase was the soluble fraction;
- The solid phase, after sonication with more buffer, was the inclusion body.

The over expression was also carried out with the pET28a plasmid itself transfected into *E. coli* as a control. Four SDS-PAGE gels have been run on the fractions collected to identify the proteins. The theoretical molecular weight of the *T.b. brucei* 24-SMT protein is around 42kDa, where an intense band was expected. Figures VIII.26 and VIII.27 showed the over expression in the soluble fractions obtained respectively for the pET28a transfect and the pET28a-TbbSMT transfect. In both gels, from t = 0h to o/n, no major changes were observed. Figure VIII.27 did not show any evidence of an intense band at 42kDa compared to figure VIII.26, which would have been the case if the over expression had worked.

[191] http://www.emdbiosciences.com.

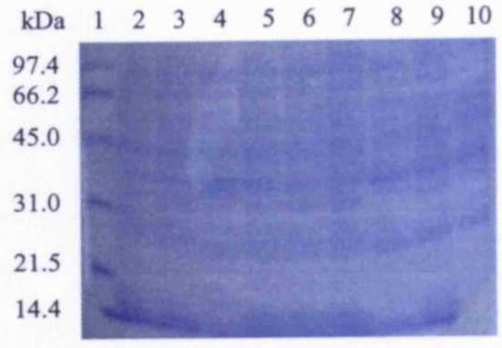


Figure VIII.26: Soluble fractions of the over expression of the pET28a transfect as a control

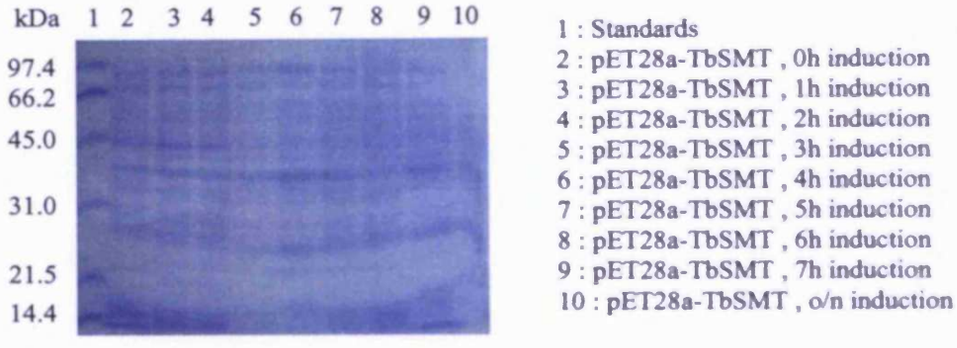


Figure VIII.27: Soluble fractions of the over expression of the pET28a-TbbSMT transfect

Figures VIII.28 and VIII.29 showed the over expression in the inclusion body fractions obtained respectively for the pET28a transfect and the pET28a-TbbSMT transfect. Again, in both gels no major changes were observed and no additional band at 42kDa was observed (Figure VIII.29).

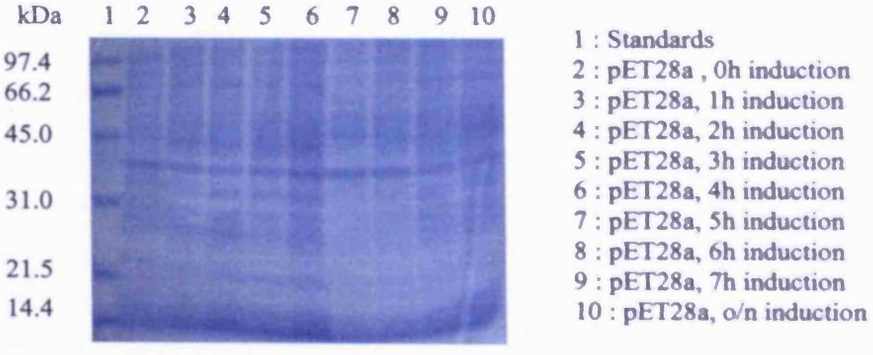


Figure VIII.28: Inclusion body fractions of the over expression of the pET28a transfect as a control

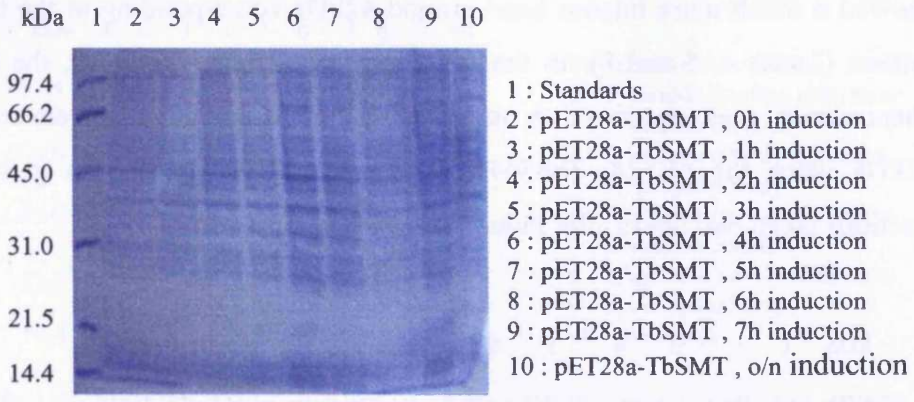


Figure VIII.29: Inclusion body fractions of the over expression of the pET28a-TbbSMT transfect

Any recombinant protein expressed in *E. coli* may interfere in normal functioning of the cell and therefore may be toxic to the bacteria. The degree of toxicity varies from protein to protein and might be enough to prevent vigorous growth, the establishment of the plasmid and in our case the over expression of the target protein. Thus, it was decided to carry out the over expression with the host cells of the plasmid, *E. coli* BL21(DE3) instead of *E. coli* BL21(DE3)pLys. This was performed at 37°C by IPTG (Figure VIII.30 and VIII.31).

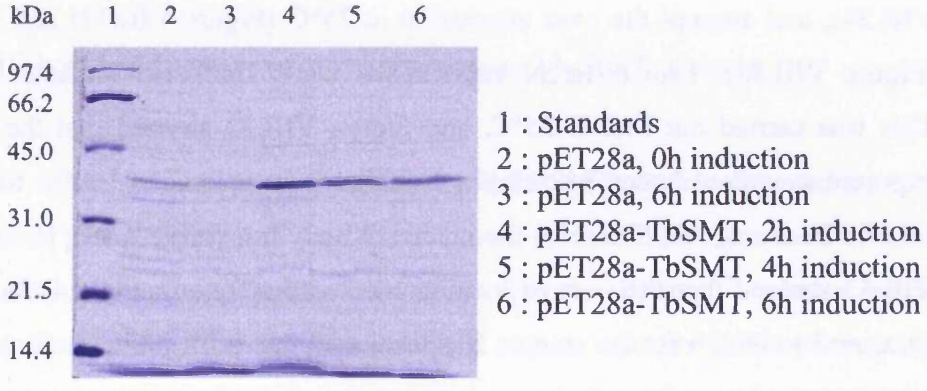


Figure VIII.30: Soluble fractions of the over expression of the pET28a transfect as a control and the pET28-TbbSMT transfect

In the soluble fractions (Figure VIII.30), the over expression of the pET28a transfect showed the appearance of different light bands (Lanes 2 and 3). On the other hand, the over expression of the plasmid pET28a-TbbSMT

showed a much more intense band around 42kDa corresponding to the target protein (Lanes 4, 5 and 6). In the inclusion body (Figure VIII.31), the same phenomenon was observed. A much more intense band appeared around 42kDa when the pET28a-TbbSMT was over expressed. In both gels, the fractions taken two hours after induction gave the best data.

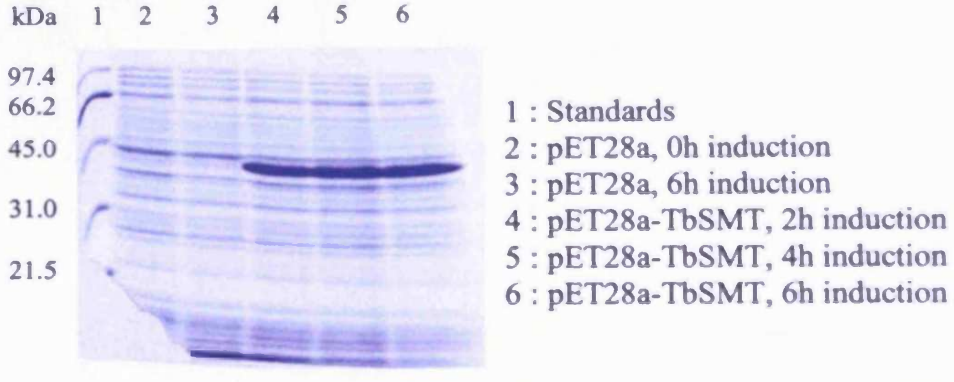


Figure VIII.31: Inclusion body fractions of the over expression of the pET28a transfect as a control and the pET28-TbbSMT transfect

Unfortunately, after a few transformations, the over expression of the protein was no longer observed. It was then decided to carry out the transformation in *E. coli* BL21(DE3) from the original colony 3.8 (Figure VIII.24), and attempt the over expression at 25°C (Figure VIII.32) and 37°C (Figure VIII.33). Two different experiments were conducted for each case. This was carried out first at 25°C, and Figure VIII.32 showed that the over expression worked better for sample 1 (Lanes 3 compared to lane 2 for the soluble fractions, and 5 to 4 for the inclusion body fractions). At 37°C, Figure VIII.33 showed that the over expression worked better for sample 4 (lanes 7 compared to lane 6 for the soluble fractions, and 9 to 8 for the inclusion body fractions).

From those good colonies, samples in 15% glycerol were frozen at -80°C as a stock for further uses.

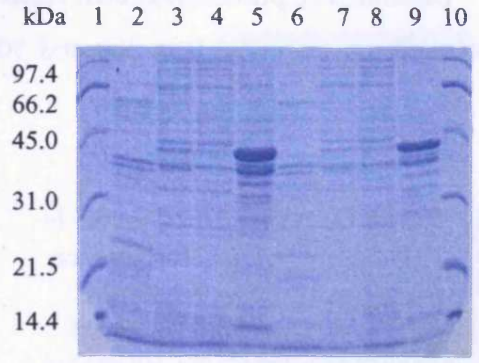


Figure VIII.32: Over expression of the pET28a vector itself and pET28-TbbSMT vector at 25°C, soluble fractions and inclusion body fractions

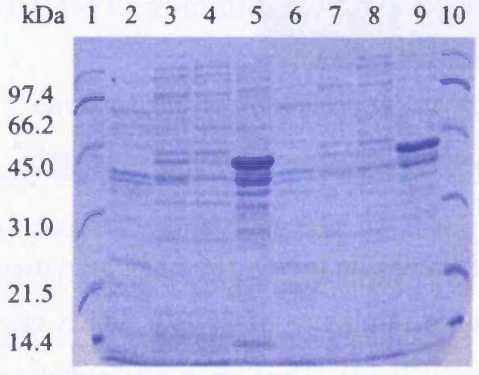


Figure VIII.33: Over expression of the pET28a vector itself and pET28-TbbSMT vector at 37°C, soluble fractions and inclusion body fractions

VIII.3.3 Purification of the *T.b. brucei* 24-SMT protein

The purification of the *T.b. brucei* 24-SMT protein obtained from the plasmid pET28a-TbbSMT transformed in *E. coli* BL21(DE3) was achieved by column chromatography. The column used was a HisTrap™ column, usually used for histidine-Tagged proteins. pET vectors contain different sequences adjacent to the cloning sites that encode a number of peptide "tags" which perform localization, detection or purification functions when fused with the target protein. Several of these peptides are small in size and the detection reagents for them are extremely specific and sensitive. pET28a contains a His•Tag gene sequence. When the over expression is carried out, this sequence is also transcribed and will allow the purification of the target protein by

binding specifically in the HisTrap™ column. The protein was then eluted with different concentrations of imidazole solutions: 10, 20, 100, 300 and 500mM (Figure VIII.34).

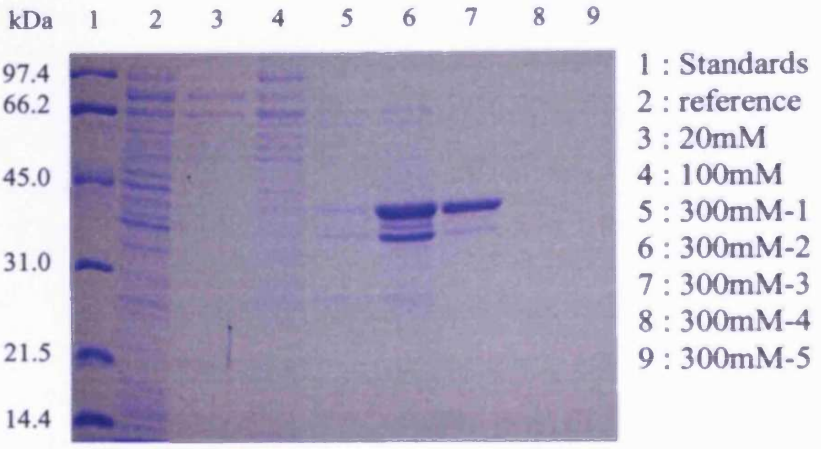


Figure VIII.34: Purification of the target protein by affinity column chromatography using increased concentration solutions of imidazole

The first low concentrations of imidazole (up to 100mM) were used to solubilise a maximum of impurity and a minimum of protein which can be observed in lanes two to four (Figure VIII.34). Then, at a concentration of 300mM of imidazole, the target protein was released from the column. Lanes 5 and 8 showed a small amount of release while lanes 6 and 7 showed a large amount. Lane 6 also showed the presence of another protein around 38kDa. Nevertheless, samples 300mM-1, 2, 3 and 4 were all mixed together and washed. After centrifugation, the protein was obtained, 90-95% pure (Figure VIII.35).

It has been observed that without washing, the protein was not stable more than a day in solution with imidazole. However, doing several washings led to further loss of the protein. Therefore, only one washing filtration was then carried out, and the protein could then be stored at 0°C in solution.

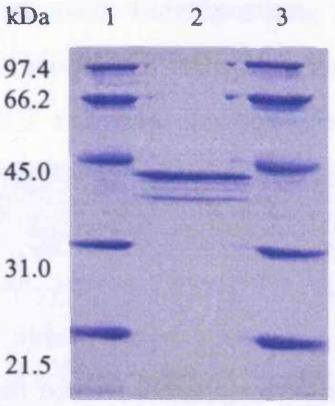
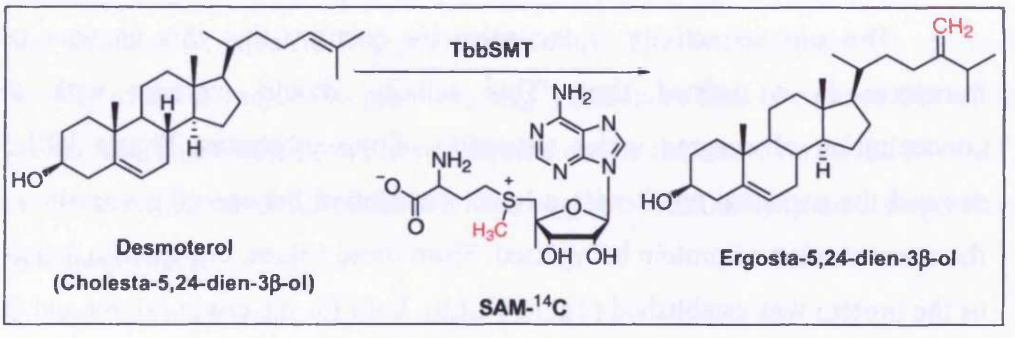


Figure VIII.35 *T.b. brucei* 24-SMT protein 90-95% pure

VIII.3.4 Enzymatic activity of the *T.b. brucei* 24-SMT protein

The enzymatic activity of the protein was studied using the method described by Nes et al.[192], which consists of methylating the 24 position of desmosterol, using *S*-adenosyl-$_L$-methionine labelled with ^{14}C (SAM-^{14}C) as a methyl donor. Using this method, it is possible to quantify the radio-labelled methyl incorporated in desmosterol by the *T.b. brucei* 24-SMT. The mechanism of incorporation is showed in Scheme VIII.1.



SchemeVIII.1: Reaction of methylation of desmosterol catalysed by the His-*T.b. brucei* SMT protein; in red is the methyl radio-labelled incorporated.

[192] Nes, W. D.; Janssen, G. G.; Bergenstrahle, A. Structural Requirements for Transformation of Substrates by the (*S*)-Adenosyl-$_L$-Methionine - $\Delta^{24(25)}$-Sterol Methyl Transferase. *J. Biol. Chem.* **1991**, *266*, 15202-15212.

The activity of the protein was evaluated from the *E. coli* extracts BL21(DE3) transformed with the plasmid pET28a-TbbSMT, and induced by IPTG at 37°C during 2 hours, which over expressed the *T.b. brucei* 24-SMT protein. This experiment was carried out with the extract and not the purified protein in order to compare the result with previous SMT activity studies achieved with extracts. The activity assay was carried out with increasing concentrations of over expressed *T.b. brucei* enzyme (Table VIII.5), saturated desmosterol and SAM-^{14}C solutions. Therefore, the limited factor is the protein concentration.

Protein (mg / mL)	Activity (pmol / min)
0.033	19.7
0.067	44.7
0.133	72.3

Table VIII.5: *T.b. brucei* 24-SMT activities using different protein concentrations

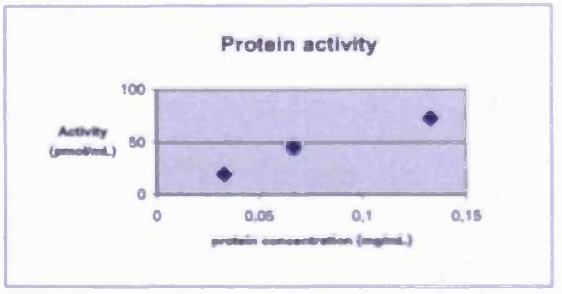


Figure VIII.36: *T.b. brucei* 24-SMT activities graph

The protein activity is the substrate quantity that this enzyme can transform in a defined time. This activity should increase with the concentration of enzyme under saturation of the substrates. Figure VIII.36 showed the expected result with a linear correlation between the activity and the concentration of protein being used. From these values, the specific activity of the protein was established (Table VIII.6), both for the crude extract and the purified enzyme. This allows estimation of the recovery of protein after purification by HisTrap™ column chromatography, which in this particular case is quite good with a recovery of 40%. The Table VIII.6 also shows that the specific activity of *T.b. brucei* 24-SMT protein is 318 pmol.min^{-1}.mg^{-1}. Other specific activities have also been determined[184]. The specific activities of SMTs from *Leishmania major* and *Saccharomyces cerevisiae* were respectively evaluated at 66 and 134 pmol.min^{-1}.mg^{-1}. Therefore, the turn over

of the *T.b. brucei* protein is respectively five and two times higher than in *L. major* and *S. cerevisiae*.

Step	mL	Concentration (mg/mL)	Protein (mg)	Specific Activity (pmol/min/mg)	Units (pmol/min)	Purification (fold)	Recovery (%)
Crude extract	7,4	10,5725	78,3	0,553	43,271	1	100
HisTrap column	2,4	0,022729	0,05455	318,075	17,351	575,2	40,1

Table VIII.6: Specific activity of the *T.b. brucei* 24-SMT protein

VIII.3.5 Conclusion

The *T.b. brucei* 24-SMT gene sequence was cloned in an expression vector, the pET28a. The cloning worked better using a 2/1 ratio *T.b. brucei* DNA/pET28a with a dephosphorylated vector. The plasmid pET28a-TbbSMT was transformed in *E. coli* BL21(DE3) and the over expression performed at 25°C or 37°C by IPTG. The protein was obtained almost pure after purification by HisTrap™ affinity column chromatography.

The specific activity of the *T.b. brucei* 24-SMT protein was determined and found to be equal to 318 $pmol.min^{-}1.mg^{-1}$. This is five times the specific activity of the *L. major* (66 $pmolmin^{-1}mg^{-1}$) and more than two times that of *S. cerevisiae*. This result shows how different can be the turn over of the 24-SMT enzymes which nevertheless have a high degree of similarity in their respective gene sequences.

VIII.4 Electron microscopy

In Chapter VI, the biological evaluation of azasterol derivatives showed some promising data. Amide or amine derivatives showed good *in vitro* activities, especially against the *Trypanosoma brucei rhodesiense* strain. However the mode of action of these compounds is unknown. It is possible that they will have an effect on the ultra-structure of the parasites due to effects on

the membrane. Therefore, it was decided to study the effect(s) of the compounds **4c** (WSP 844, IC_{50} = 120nM) and **20c** (WSP 879, IC_{50} = 12 nM) on the ultra-structure of both procyclic and blood stream forms of the *T.b. brucei* parasites. We wanted to investigate and compare the mode of action of these derivatives in both procyclic and bsf strains of *T.b. brucei*, although the change from *rhodesiense* to *brucei* might have an effect. To achieve it, electron microscopy could be used. This is a powerful tool which in theory allows a resolution of 0.005nm but in practice gives a resolution of 0.1nm (100000 times better than human eye). Electron microscopy allows the observation of the parasite's organelles, which might give an indication of the mode of action of these derivatives.

VIII.4.1 Results

A study on the growth of the parasites at different concentrations of inhibitors was first carried out in order to establish which concentration would be the most appropriate for the final experiment. A control with chloroform (the solvent used to dissolve the inhibitors) was also carried out. The parasites were cultured in the appropriate solutions until reaching the logarithmic phase as previously described for the Northern blotting (Section VIII.2.1.1). Then, the experiment was started with the following parasite concentrations:

- Procyclic form: $1x10^6$ parasites/mL;
- Bsf: 25000 parasites/mL.

The different concentrations that we worked with to study the inhibition growth of the parasite were as shown in Table VIII.7:

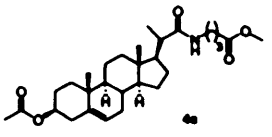
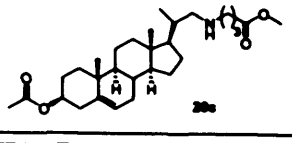
Compound	Concentration (µM)	
	Procyclic Form	Bsf
	5, 10, 40, 80	1, 3, 5, 10
	3, 5, 8, 10	1, 3, 5, 10

Table VIII.7: Concentrations in µM of inhibitors used for the parasite growth

Figure VIII.37 shows the growth inhibition of the bsf of the *T.b. brucei* parasite with compound **20c**. It has been observed that at a concentration of 10µM, there was a total growth inhibition while the other concentrations (1, 3 and 5µM) led to a partial growth inhibition.

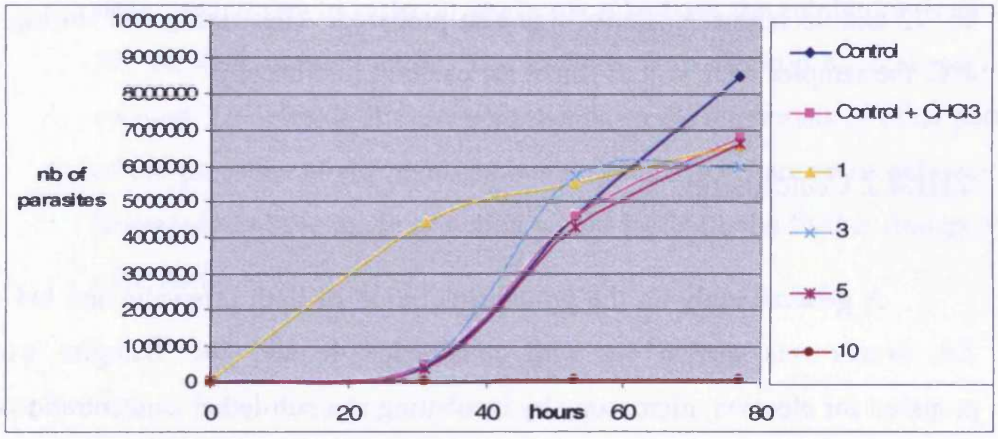


Figure VIII.37: Growth inhibition effect on the bsf of the parasite with compound **20c**

This experiment was also carried out with the procyclic form (Figure VIII.38) and with compound **4c**. Results presented Figure VIII.38 were not as good as in the previous experiment, with 10µM concentration giving a low growth inhibition compared to control.

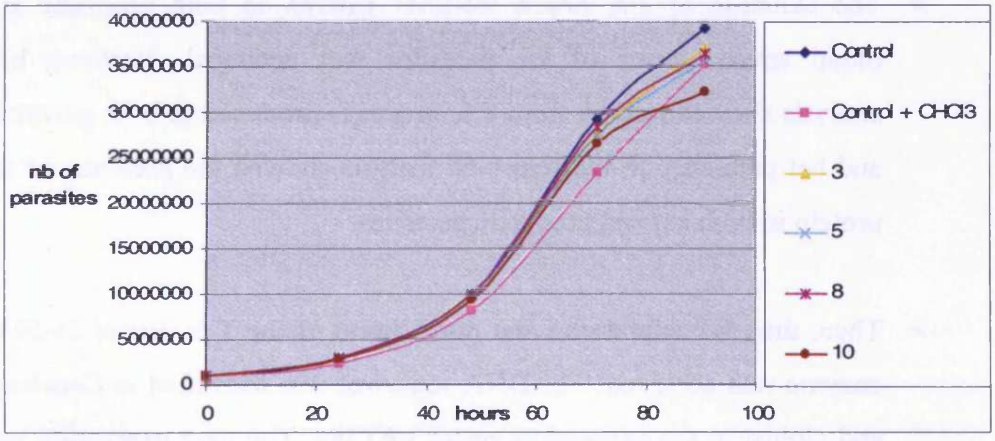


Figure V.38: Growth inhibition effect on the procyclic form of the parasite with compound **20c**

Thus, in order to prepare samples for electron microscopy, it was decided to incubate both procyclic and bsf parasites at concentrations of 20μM for compound **4c** and 5 μM for compound **20c**. The samples were prepared with parasites in the logarithmic phase. They had a final volume of 30 mL, and were prepared 3 times for each form of parasites, in order to do the fixing after 48, 72 and 96 hours. A control was also prepared. After fixing and storing at 4°C, the samples were sent to Brazil for electron microscopy.

VIII.4.2 Conclusion

A general study on the growth inhibition of both procyclic and bsf of *T.b. brucei* was carried out with compounds **4c** and **20c**. Samples were prepared for electron microscopy by incubating at a sub-lethal concentration of compounds at varying time-points. The cells were then fixed and sent to Brazil. Results are still being expected and should allow understanding the effects of the azasterols derivatives on the ultra-structure shape of both forms of parasites. The observation of membrane disruption for example is expected to occur.

VIII.5 General conclusion

- The isolation of *T.b. brucei* 24-SMT mRNA in both procyclic and blood stream forms of the parasites was achieved. Northern blot analysis showed mRNA from *T.b. brucei* is produced in both procyclic and bsf parasites. A Western blot analysis showed the presence of the protein in both bsf and procyclic parasites.

- Then, the over expression and purification of the *T.b. brucei* 24-SMT enzyme was achieved. The DNA sequence was identified in Genebank and cloned in the expression vector pET28a. The over expression was performed with IPTG in *E. coli* BL21(DE3) cells. The purification was carried out by column chromatography and gave protein with a high

level of purity. The specific activity of the protein was determined to be 318.075 pmol.min^{-1}.mg^{-1}, which is 5 times the specific activity of the *L. major* enzyme and more than two times that of *S. cerevisiae*.

- Finally, cultures of both procyclic and blood stream forms of parasites were carried out in order to study the effects of the inhibitors **4c** and **20c** on their ultra-structure. The analysis from electron microscopy is awaited. The effects of these compounds on the membrane or other part of the parasites might highlight some specific mechanism or pathway. Knowledge of the mode of action would facilitate the further design of inhibitors.

CHAPTER IX

CHEMICAL EXPERIMENTAL

IX.1 General Experimental Details

When applicable, all glassware was oven-dried overnight and all reactions were carried out under nitrogen atmosphere. Sensitive liquids and solutions were transferred via syringe or cannula and were introduced into reaction vessels through rubber septa.

All dry solvents, ethanol, methanol, dichloromethane, tetrahydrofuran, dimethylformamide were purchased from Aldrich or Fluka in Sure Seal bottles.

Analytical TLC was performed on silica-gel 60 F254 plates, purchased from Merck. Visualisation of spots was effected by one of the following techniques: (a) UV illumination, (b) immersion of the plate in a 3% solution of ninhydrin in ethanol followed by heating and (c) immersion of the plate in a 48 $g.L^{-1}$ solution of phosphomolibdic acid in methanol followed by heating.

Column chromatography was carried out on Sorbsil C60A (40-60 micron) silica gel purchased from Merck.

NMR spectra were recorded on a Bruker Avance DPX 300 MHz spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C or a Bruker DPX 500 MHz spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts are reported downfield in parts per million using $CDCl_3$ as an internal reference (δ = 7.26 for ^{1}H and δ = 77.16 for ^{13}C) unless otherwise stated, and coupling constants (*J* values) are in hertz.

I.R. spectra were recorded on a Perkin Elmer 1600 series FTIR spectrometer. Compounds were ground with KBr and spectra recorded in reflectance mode.

Melting points were determined with a Gallenkamp melting point apparatus and are uncorrected.

Mass spectra and exact mass measurements were performed respectively on a Waters ZQ4000 and a Finnigan MAT 95XP at EPSRC National Mass Spectrometry Service Centre in the Chemistry Department, University of Wales Swansea, Swansea, Wales, UK.

The steroid nomenclature as previously described in "Guidelines to nomenclature" is as followed Figure IX.1:

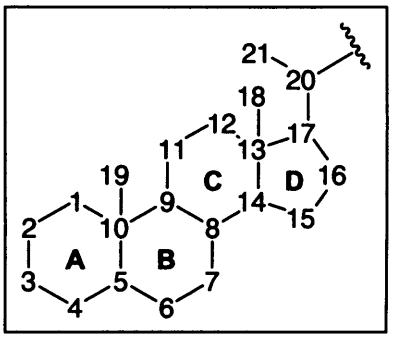
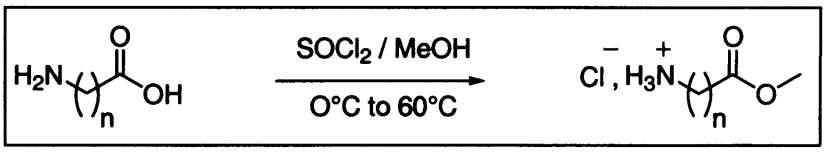


Figure IX.1: Steroid nucleus numbering

IX.2 General procedures

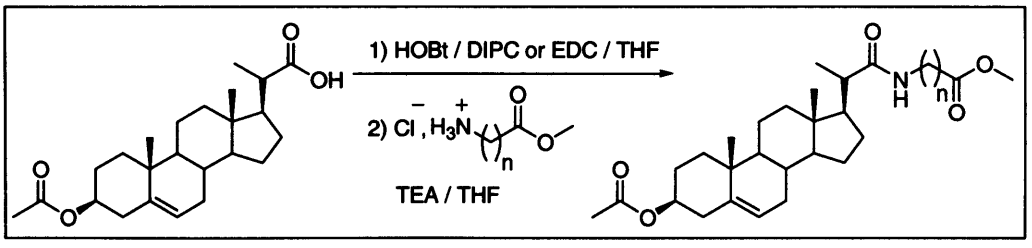
General procedure A:



Scheme IX.1: Esterification procedure

Thionyl chloride (3 eq.) was added dropwise to a stirred solution of amino acid **2** (1 eq.) and methanol (10 eq.), at 0°C. The resulting solution was then heated to reflux in methanol (0.4 mL/mmol) overnight. The solvent and thionyl chloride in excess were then removed under reduced pressure.

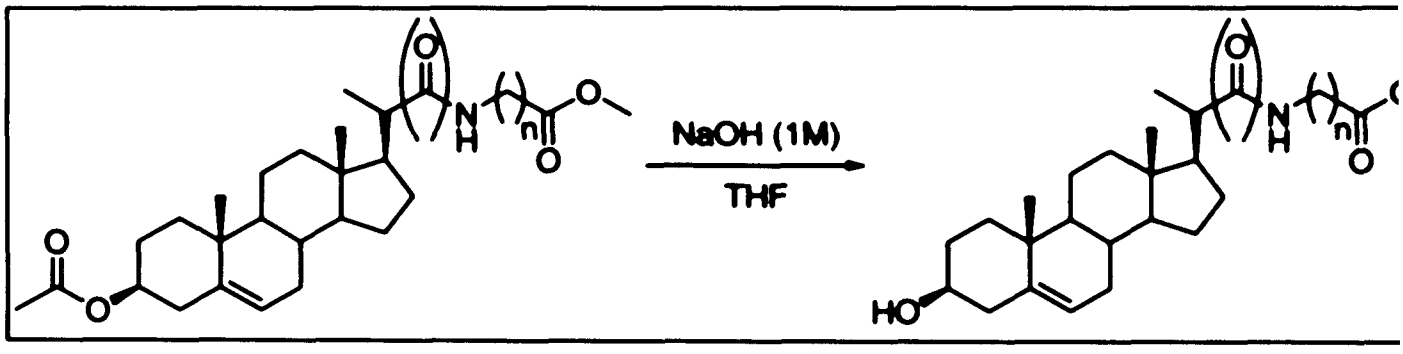
General procedure B:



Scheme IX.2: Coupling procedure

organic layer was washed with water (10 mL), a saturated solution of K_2C (2•10 mL), HCl 1M (2•10 mL), and finally water again (2•10 mL). solution was dried with magnesium sulfate and the remaining solvents remo under reduced pressure. The crude was purified by column chromatography silica gel using hexane/AcOEt (80/20) as eluant.

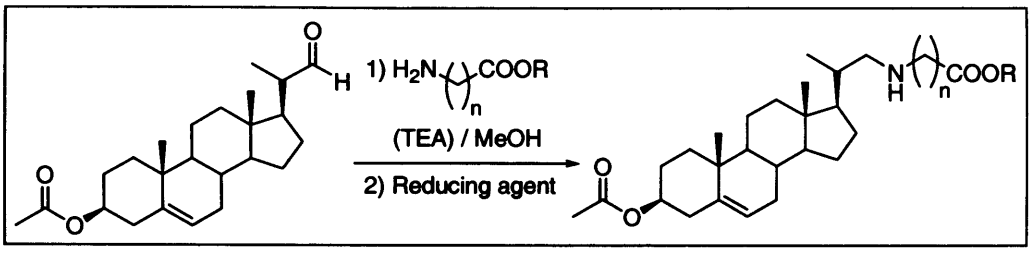
General procedure C:



Scheme IX.3: Hydrolysis procedure

Sodium hydroxide solution (1M, 10 eq.) was added to the start material (1.0 eq.) dissolved in THF (25 mL/mmol) at room temperature. T mixture was stirred for various length of time. Then, THF was removed un reduced pressure. The resulting mixture was diluted with water (50 mL/mm and a hydrochloric acid solution (1M) was added until the pH was neutral. T product was filtered, washed with AcOEt, and dried under vacuum.

General procedure D:

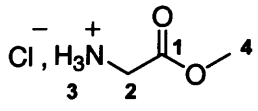


Scheme IX.4: Reductive amination procedure

n-Amino acid **2** (1.2 eq.) or triethylamine (1.4 eq.) with methyl n-amino ester **3** (1.2 eq.) in a methanol solution (12 mL/mmol) were stirred 30 minutes. Then, 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (1 eq.) was added as a solid and the solution stirred 30 more minutes. Finally, triacetoxy sodium borohydride or sodium cyanoborohydride as a solid or in a THF solution (1.2 eq.) was added and the mixture stirred at room temperature overnight. The solution was diluted with water (10 mL) and extracted with chloroform (20 mL). The organic layer was washed with a saturated solution of sodium chloride (10 mL), dried, and the solvent removed under reduced pressure. The crude was purified by column chromatography ($CHCl_3$/MeOH: 90/10).

IX.3 Synthesis of the amide derivatives (Chapter IV)

Methyl 2-aminoethanoate, monohydrochloric acid (3a)



$C_3H_8NO_2Cl$
MW = 125.55 g.mol^{-1}

Following the **general procedure A** starting from thionyl chloride (6.0 mL, 82.21 mmol, 3.0 eq.) and glycine (2.043 g, 27.21 mmol, 1.0 eq.) in methanol (10 mL, 246.88 mmol, 9.1 eq.), compound **3a** was isolated as a white solid (3.351 g, 98%).

^{1}H NMR (300MHz, MeOH): δ 3.85 (m, 5H, H^2 and H^4);

^{13}C NMR (75MHz, MeOH): δ 41.4 (C^2), 53.9 (C^4), 169.4 (C^1);

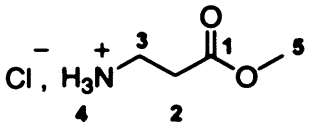
Mp = 174-175°C; **R_f** = 0.06 ($CHCl_3$/MeOH: 50/50);

Anal.Calcd. for $C_3H_8NO_2Cl \cdot 0.1H_2O$: % Theory: C, 28.29; H, 6.49; N, 11.00; **% Found:** C, 28.59; H, 6.48; N, 10.90 ;

LRMS, m/z (EI^+ mode): 89 ($[M-HCl]^+$, 100%);

HRMS (EI^+ mode), **Calculated for $C_3H_7NO_2$ ($[M-HCl]^+$):** 89.0477; **Found:** 89.0474 .

Methyl 3-aminopropanoate, monohydrochloric acid (3b)



$C_4H_{10}NO_2Cl$
MW = 139.58 g.mol^{-1}

Following the **general procedure A** starting from thionyl chloride (5.0 mL, 68.50 mmol, 3.0 eq.) and β-alanine (2.042 g, 22.92 mmol, 1.0 eq.) in methanol (10 mL, 246.88 mmol, 10.8 eq.), compound **3b** was isolated as a white solid (3.061 g, 96%).

1H NMR (300MHz, MeOH): δ 2.80 (t, 2H, H^2, J = 6.5), 3.24 (t, 2H, H^3, J = 6.5), 3.76 (s, 3H, H^5);

^{13}C NMR (75MHz, MeOH): δ 32.5 (C^2), 36.9 (C^3), 53.1 (C^5), 173.0 (C^1);

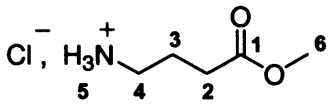
Mp = 88-90°C (Litt.[132]: **Mp** = 106°C); **R_f** = 0.08 ($CHCl_3$/MeOH: 50/50);

Anal.Calcd. for $C_4H_{10}NO_2Cl \cdot 0.1H_2O$: % Theory: C, 33.98; H, 7.27; N, 9.91; **% Found:** C, 34.06; H, 7.22; N, 9.82 ;

LRMS, m/z (EI^+ mode): 104 ($[M-Cl]^+$, 100%);

HRMS (EI^+ mode), **Calculated for $C_4H_{10}NO_2$ ($[M-Cl]^+$):** 104.0712; **Found:** 104.0716 .

Methyl 4-aminobutanoate, monohydrochloric acid (3c)

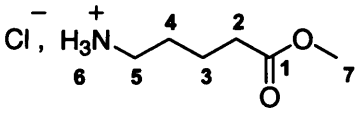


$C_5H_{12}NO_2Cl$
MW = 153.61 g.mol^{-1}

Following the **general procedure A** starting from thionyl chloride (3.4 mL, 46.58 mmol, 2.3 eq.) and 4-aminobutanoic acid (2.076 g, 20.13 mmol, 1.0 eq.) in methanol (8 mL, 197.50 mmol, 9.8 eq.), compound **3c** was isolated as a white solid (3.067 g, 99%).

1H NMR (300MHz, MeOH): δ 1.96 (qt, 2H, H^3, J= 7.4), 2.51 (t, 2H, H^2, J= 7.2), 3.00 (t, 2H, H^4, J= 7.6), 3.71 (s, 3H, H^6);
^{13}C NMR (75MHz, MeOH): δ 24.1 (C^3), 31.7 (C^2), 40.5 (C^4), 52.7 (C^6), 174.9 (C^1);
IR (KBr, cm^{-1}): 1585.8 ($\nu_{NH_3^+ bending}$), 1729.2 (ν_{CO} ester);
Mp = 105-107°C; **R_f** = 0.07 ($CHCl_3$/MeOH: 50/50);
Anal.Calcd. for $C_5H_{12}NO_2Cl \cdot 0.2H_2O$: % Theory: C, 38.20; H, 7.95; N, 8.91; **% Found:** C, 38.30; H, 7.75; N, 8.94 ;
LRMS, m/z (FAB^+ mode): 118 ($[M\text{-}Cl]^+$, 100%);
HRMS (FAB^+ mode), **Calculated for $C_5H_{12}NO_2$ ($[M\text{-}Cl]^+$):** 118.0868; **Found:** 118.0872 .

Methyl 5-aminopentanoate, monohydrochloric acid (3d)



$C_6H_{14}NO_2Cl$
MW = 167.63 g.mol^{-1}

Following the **general procedure A** starting from thionyl chloride (4 mL, 54.80 mmol, 2.5 eq.) and 5-aminopentanoic acid (2.555 g, 21.81 mmol, 1.0 eq.) in methanol (8 mL, 197.50 mmol, 9.1 eq.), compound **3d** was isolated as a white solid (3.557 g, 97%).

^{1}H NMR (300MHz, MeOH): δ 1.71 (m, 4H, H^3 and H^4), 2.43 (t, 2H, H^2, J = 6.7), 2.95 (t, 2H, H^5, J= 6.0), 3.69 (s, 3H, H^7);

^{13}C NMR (75MHz, MeOH): δ 23.1 and 28.3 (C^3 and C^4), 34.3 (C^2), 40.8 (C^5), 52.5 (C^7), 175.6 (C^1);

IR (KBr, cm^{-1}): 1571.9 ($\nu_{NH_3^+ bending}$), 1733.9 (ν_{CO} ester);

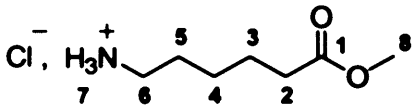
Mp = 122-123°C; **R_f** = 0.07 (CH_3Cl/MeOH: 50/50);

Anal.Calcd. for $C_6H_{14}NO_2Cl \cdot 0.3H_2O$: % Theory: C, 41.65; H, 8.50; N, 8.09; **% Found:** C, 41.61; H, 8.21; N, 8.12 ;

LRMS, m/z (FAB^+ mode): 132 ($[M-Cl]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_6H_{14}NO_2$ ($[M-Cl]^+$):** 132.1025; **Found:** 132.1027 .

Methyl 6-aminohexanoate, monohydrochloric acid (3e)



$C_7H_{16}NO_2Cl$
MW = 181.66 g.mol^{-1}

Following the **general procedure A** starting from thionyl chloride (4 mL, 54.80 mmol, 2.6 eq.) and 6-aminohexanoic acid (2.764 g, 21.07 mmol, 1.0 eq.) in methanol (8 mL, 197.50 mmol, 9.4 eq.), compound **3e** was isolated as a white solid (3.784 g, 99%).

^{1}H NMR (300MHz, MeOH): δ 1.44 (m, 2H, H^4), 1.68 (quint, 4H, H^3 and H^5, J= 7.6), 2.39 (t, 2H, H^2, J = 7.3), 2.94 (t, 2H, H^6, J= 7.6), 3.68 (s, 3H, H^8);

^{13}C NMR (75MHz, MeOH): δ 25.8 and 28.6 (C^3 and C^5), 27.3 (C^4), 34.8 (C^2), 41.0 (C^6), 52.4 (C^8), 176.0 (C^1);

IR (KBr, cm^{-1}): 1580.0 ($\nu_{NH_3^+ bending}$), 1730.9 (ν_{CO} ester);

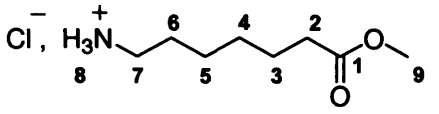
Mp = 81-82°C (Litt.[132]: **Mp** = 125°C); **R_f** = 0.09 (CH_3Cl/MeOH: 50/50);

Anal.Calcd. for $C_7H_{16}NO_2Cl \cdot 0.2H_2O$: % Theory: C, 45.38; H, 8.92; N, 7.56; **% Found:** C, 45.43; H, 8.85; N, 7.60 ;

LRMS, m/z (ES^+ mode): 146 ($[M-Cl]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_7H_{15}NO_2$ ($[M-Cl]^+$):** 145.1103; **Found:** 145.1104 .

Methyl 7-aminoheptanoate, monohydrochloric acid (3f)

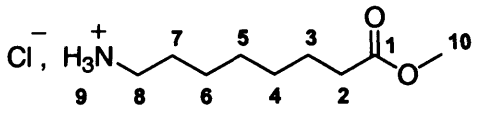


$C_8H_{18}NO_2Cl$
MW = 195.69 g.mol^{-1}

Following the **general procedure A** starting from thionyl chloride (1 mL, 13.70 mmol, 5.2 eq.) and 7-aminoheptanoic acid (0.386 g, 2.66 mmol, 1.0 eq.) in methanol (2.0 mL, 49.38 mmol, 18.6 eq.), compound **3f** was isolated as a white solid (0.516 g, 99%).

^{1}H NMR (300MHz, MeOH): δ 1.44 (m, 4H, H^4 and H^5), 1.69 (m, 4H, H^3 and H^6), 2.38 (t, 2H, H^2, J= 7.3), 2.96 (t, 2H, H^7, J= 7.6), 3.69 (s, 3H, H^9);
^{13}C NMR (75MHz, MeOH): δ 26.1 and 28.8 (C^3 and C^6), 27.5 and 30.0 (C^4 and C^5), 34.9 (C^2), 41.1 (C^7), 52.4 (C^9), 176.2 (C^1);
IR (KBr, cm^{-1}): 1574.1 ($\nu_{NH_3^+ bending}$), 1737.4 (ν_{CO} ester);
Mp = 109-111°C; **R_f** = 0.09 (CH_3Cl/MeOH: 50/50);
Anal.Calcd. for $C_8H_{18}NO_2Cl$·0.1H_2O: % Theory: C, 48.65; H, 9.29; N, 7.09; **% Found:** C, 48.73; H, 9.23; N, 7.01 ;
LRMS, m/z (ES^+ mode): 160 ($[M+H]^+$, 100%);
HRMS (ES^+ mode), **Calculated for $C_8H_{18}NO_2$ ($[M-Cl]^+$):** 160.1338; **Found:** 160.1333 .

Methyl 8-aminoctanoate, monohydrochloric acid (3g)



$C_9H_{20}NO_2Cl$
MW = 209.71 g.mol^{-1}

^{13}C NMR (75MHz, MeOH): δ 26.3 and 28.9 (C^3 and C^7), 27.7, 30.2 and 30 (C^4, C^5 and C^6), 35.1 (C^2), 41.2 (C^8), 52.4 (C^{10}), 176.3 (C^1);

IR (KBr, cm^{-1}): 1583.1 ($\nu_{NH_3^+ bending}$), 1736.6 (ν_{CO} ester);

Mp = 102-104°C; **R_f** = 0.09 (CH_3Cl/MeOH: 50/50);

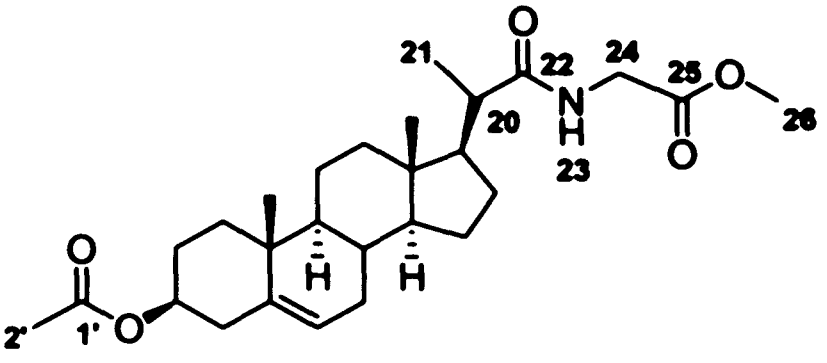
Anal.Calcd. for $C_9H_{20}NO_2Cl \cdot 0.2H_2O$: % Theory: C, 50.67; H, 9.64; N, 6.5

% Found: C, 50.91; H, 9.58; N, 6.52 ;

LRMS, m/z (ES^+ mode): 174 ($[M-Cl]^+$, 100%);

HRMS (ES^+mode), **Calculated for $C_9H_{20}NO_2$ ($[M-Cl]^+$):** 174.1494; **Foun** 174.1488 .

3β-acetoxy-23,24-bisnor-5-en-22-(methyl ethanoate) amide (4a)



$C_{27}H_{41}NO_5$
MW = 459.62 g.mol^{-1}

1- Following the **general procedure B** starting from HOBt (0.65 g, 4.81 mmo 1.1 eq.), 3β-acetoxy-5-cholenic acid **1** (1.75 g, 4.50 mmol, 1.0 eq.) and DIP (1.1 mL, 7.08 mmol, 1.6 eq.) in THF (10 mL), and methyl 2-aminoethanoa **3a** (0.66 g, 5.26 mmol, 1.2 eq.) and triethylamine (1.6 mL, 11.50 mmol, 2.6 e in THF (10 mL), compound **4a** was isolated as a white solid (1.459 g, 83%).

2- Following the **general procedure B** starting from HOBt (286 mg, 2.12 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (804 mg, 1.76 mmol, 1.0 eq.) and EDC (384 mg, 2.00 mmol, 1.1 eq.) in THF (9 mL), and, methyl 2-aminoethanoate **3a** (313 mg, 2.49 mmol, 1.4 eq.) and triethylamine (0.38 mL, 2.73 mmol, 1.6 eq) in THF (9 mL), compound **4a** was isolated as a white solid (164 mg, 20%).

^{1}H NMR (500MHz, $CDCl_3$): δ 0.72 (s, 3H, H^{18}), 1.06 (s, 3H, H^{19}), 1.25 (d, 3H, H^{21}, J= 6.8), 2.07 (s, 3H, $H^{2'}$), 0.90 to 2.00 (m, 18H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}), 2.22 (m, 1H, H^{20}), 2.35 (d, 2H, H^4, J= 4.9), 3.82 (s, 3H, H^{26}), 4.07 (d, 2H, H^{24}, J= 5.1), 4.63 (m, 1H, H^3), 5.39 (m, 1H, H^6), 5.88 (t, 1H, H^{23}, J= 4.9);

^{13}C NMR (125MHz, $CDCl_3$): δ 12.1 (C^{18}), 17.6 (C^{21}), 19.3 (C^{19}), 21.5 ($C^{2'}$), 36.6 (C^{10}), 38.1 (C^4), 41.0 (C^{24}), 42.4 (C^{13}), 44.6 (C^{20}), 52.4 (C^{26}), 21.0, 24.4, 27.5, 27.8, 31.8, 31.9, 37.0, 39.5, 49.9, 52.8, 56.2 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 73.9 (C^3), 122.5 (C^6), 139.6 (C^5), 170.5 ($C^{1'}$), 170.7 (C^{25}), 176.7 (C^{22});

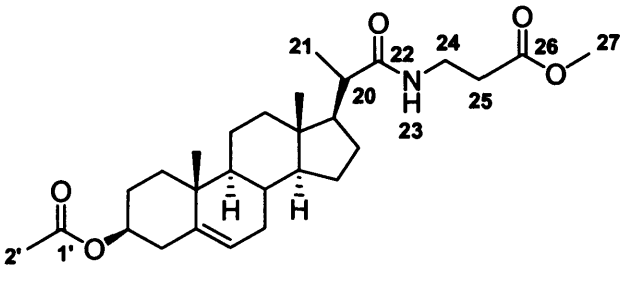
Mp = 189-190°C; **R_f** = 0.16 (Hexane/AcOEt: 60/40);

Anal.Calcd. for $C_{27}H_{41}NO_5$*0.7H_2O: % Theory: C, 68.67; H, 9.05; N, 2.97 **% Found:** C, 68.52; H, 8.86; N, 2.79 ;

LRMS, m/z (ES^+ mode): 482.5 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{27}H_{41}NO_5Na$ ($[M+Na]^+$):** 482.2882; **Found:** 482.2891 .

3β-acetoxy-23,24-bisnor-5-en-22-(methyl propanoate) amide (4b)



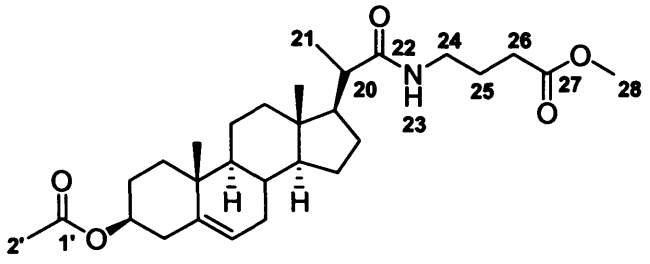
$C_{28}H_{43}NO_5$
MW = 473.64 g.mol^{-1}

1- Following the **general procedure B** starting from HOBt (0.65 g, 4.81 mmol, 1.1 eq.), 3β-acetoxy-5-cholenic acid **1** (1.75 g, 4.50 mmol, 1.0 eq.) and DIPC (1.1 mL, 7.08 mmol, 1.6 eq.) in THF (10 mL), and, methyl 3-aminopropanoate **3b** (734 mg, 5.26 mmol, 1.2 eq.) and triethylamine (1.6 mL, 11.50 mmol, 2.6 eq) in THF (10 mL), compound **4b** was isolated as a white solid (1.936 g, 91%).

2- Following the **general procedure B** starting from HOBt (286 mg, 2.12 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (803 mg, 1.76 mmol, 1.0 eq.) and EDC (384 mg, 2.00 mmol, 1.1 eq.) in THF (9 mL), and, methyl 3-aminopropanoate **3b** (336 mg, 2.41 mmol, 1.4 eq.) and triethylamine (0.38 mL, 2.73 mmol, 1.6 eq) in THF (9 mL), compound **4b** was isolated as a white solid (181 mg, 22%).

^{1}H NMR (500MHz, $CDCl_3$): δ 0.71 (s, 3H, H^{18}), 1.05 (s, 3H, H^{19}), 1.18 (d, 3H, H^{21}, J= 6.7), 2.03 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 19H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}), 2.32 (d, 2H, H^4, J= 4.5), 2.56 (t, 2H, H^{25}, J= 7.0), 3.52 (m, 2H, H^{24}), 3.73 (s, 3H, H^{27}), 4.62 (m, 1H, H^3), 5.38 (m, 1H, H^6), 5.98 (t, 1H, H^{23}, J= 5.8);
^{13}C NMR (125MHz, $CDCl_3$): δ 12.1 (C^{18}), 17.6 (C^{21}), 19.3 (C^{19}), 21.4 ($C^{2'}$), 33.8 (C^{25}), 34.6 (C^{24}), 36.6 (C^{10}), 38.1 (C^4), 42.3 (C^{13}), 44.8 (C^{20}), 51.8 (C^{27}), 21.0, 24.3, 27.4, 27.8, 31.8, 31.9, 37.0, 39.5, 49.9, 52.8, 56.2 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 73.9 (C^3), 122.5 (C^6), 139.7 (C^5), 170.5 ($C^{1'}$), 173.3 (C^{26}), 176.6 (C^{22});
Mp = 156-158°C; **R_f** = 0.12 (Hexane/AcOEt: 60/40);
Anal.Calcd. for $C_{28}H_{43}NO_5 \cdot 0.3H_2O$: % Theory: C, 70.20; H, 9.17; N, 2.92 **% Found:** C, 70.31; H, 9.22; N, 2.84;
LRMS, m/z (ES^+ mode): 496.6 ($[M+Na]^+$, 100%), 474.6 ($[M+H]^+$, 13%);
HRMS (ES^+ mode), **Calculated for $C_{28}H_{43}NO_5Na$ ($[M+Na]^+$):** 496.3039; **Found:** 496.3019 .

3β-acetoxy-23,24-bisnor-5-en-22-(methyl butanoate) amide (4c)



$C_{29}H_{45}NO_5$
MW = 487.67 g.mol^{-1}

1- Following the **general procedure B** starting from HOBt (1.104 g, 8.18 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (3.001 g, 6.57 mmol, 1.0 eq.) and DIPC (1.4 mL, 9.02 mmol, 1.4 eq.) in THF (15 mL), and, methyl 4-aminobutanoate **3c** (1.212 g, 7.90 mmol, 1.2 eq.) and triethylamine (1.5 mL, 10.78 mmol, 1.6 eq) in THF (15 mL), compound **4c** was isolated as a white solid (2.226 g, 70%).

2- Following the **general procedure B** starting from HOBt (546 mg, 4.04 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (1.497 g, 3.28 mmol, 1.0 eq.) and EDC (747 mg, 3.90 mmol, 1.2 eq.) in THF (10 mL), and, methyl 4-aminobutanoate **3c** (605 mg, 3.94 mmol, 1.2 eq.) and triethylamine (0.6 mL, 4.31 mmol, 1.3 eq) in THF (10 mL), compound **4c** was isolated as a white solid (541 mg, 34%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.74 (s, 3H, H^{18}), 1.07 (s, 3H, H^{19}), 1.22 (d, 3H, H^{21}, J= 6.7), 2.06 (s, 3H, $H^{2'}$), 0.90 to 2.20 (m, 21H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}), 2.40 (m, 4H, H^4 and H^{26}), 3.32 (m, 2H, H^{24}), 3.71 (s, 3H, H^{28}), 4.69 (m, 1H, H^3), 5.42 (m, 1H, H^6), 5.68 (t, 1H, H^{23}, J= 5.4);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.5 (C^{18}), 18.1 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 25.1 (C^{25}), 31.9 (C^{26}), 37.0 (C^{10}), 38.5 (C^4), 39.1 (C^{24}), 42.7 (C^{13}), 45.4 (C^{20}), 52.1 (C^{28}), 21.4, 24.8, 28.0, 28.2, 32.2, 32.3, 37.4, 39.9, 50.3, 53.2, 56.6 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.3 (C^3), 122.9 (C^6), 140.1 (C^5), 170.9 ($C^{1'}$), 177.1 (C^{22} and C^{27});

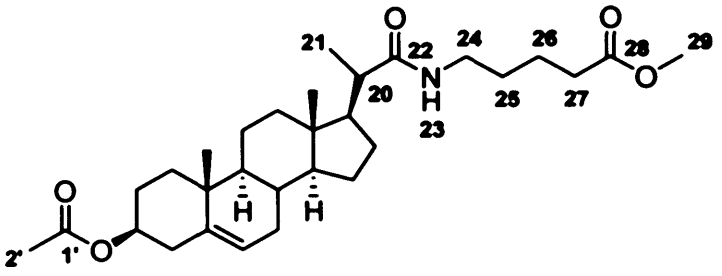
Mp = 159-160°C; R_f = 0.10 (Hexane/AcOEt: 60/40);

Anal.Calcd. for $C_{29}H_{45}NO_5$: % Theory: C, 71.42; H, 9.30; N, 2.87 **% Found:** C, 71.17; H, 9.33; N, 2.83 ;

LRMS, m/z (ES^+ mode): 510.0 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{29}H_{45}NO_5Na$ ($[M+Na]^+$):** 510.3195; **Found:** 510.3187 .

3β-acetoxy-23,24-bisnor-5-en-22-(methyl pentanoate) amide (4d)



$C_{30}H_{47}NO_5$
MW = 501.70 g.mol^{-1}

1- Following the **general procedure B** starting from HOBt (1.119 g, 8.29 mmol, 1.3 eq.), 3β-acetoxy-5-cholenic acid **1** (3.021 g, 6.61 mmol, 1.0 eq.) and DIPC (1.4 mL, 9.02 mmol, 1.4 eq.) in THF (15 mL), and, methyl 5-aminopentanoate **3d** (1.320 g, 7.88 mmol, 1.2 eq.) and triethylamine (2 mL, 14.38 mmol, 2.2 eq.) in THF (15 mL), compound **4d** was isolated as a white solid (2.644 g, 80%).

2- Following the **general procedure B** starting from HOBt (275 mg, 2.04 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (800 mg, 1.75 mmol, 1.0 eq.) and EDC (380 mg, 1.98 mmol, 1.1 eq.) in THF (9 mL), and, methyl 5-aminopentanoate **3d** (398 mg, 2.38 mmol, 1.4 eq.) and triethylamine (0.35 mL, 2.52 mmol, 1.4 eq) in THF (9 mL), compound **4d** was isolated as a white solid (194 mg, 22%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.79 (s, 3H, H^{18}), 1.12 (s, 3H, H^{19}), 1.27 (d, 3H, H^{21}, J = 6.7), 2.16 (s, 3H, $H^{2'}$), 1.00 to 2.20 (m, 23H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}), 2.45 (m, 4H, H^4 and H^{27}), 3.32 (m,

2H, H^{24}), 3.79 (s, 3H, H^{29}), 4.70 (m, 1H, H^{3}), 5.47 (m, 1H, H^{6}), 5.50 (t, 1H, H^{23}, J= 5.5);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.5 (C^{18}), 18.1 (C^{21}), 19.7 (C^{19}), 21.8 ($C^{2'}$), 33.9 (C^{27}), 37.0 (C^{10}), 38.5 (C^{4}), 39.1 (C^{24}), 42.7 (C^{13}), 45.4 (C^{20}), 52.0 (C^{29}), 21.4, 22.5, 24.8, 28.0, 28.2, 29.5, 32.2, 32.3, 37.4, 39.9, 50.3, 53.2, 56.6 (C^{1}, C^{2}, C^{7}, C^{8}, C^{9}, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}), 74.4 (C^{3}), 122.9 (C^{6}), 140.1 (C^{5}), 171.0 ($C^{1'}$ and C^{28}), 177.0 (C^{22});

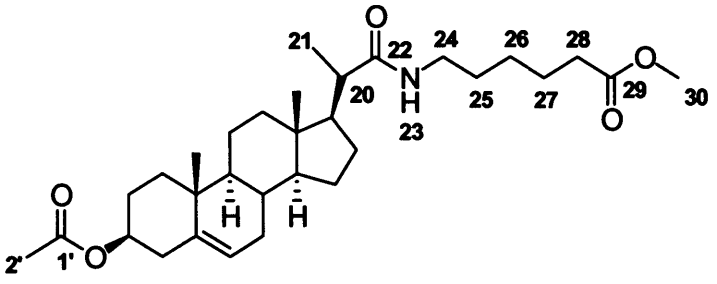
Mp = 165-166°C; **R_f** = 0.10 (Hexane/AcOEt: 60/40);

Anal.Calcd. for $C_{30}H_{47}NO_5$*0.3H_2O: % Theory: C, 71.05; H, 9.46; N, 2.76 **% Found:** C, 71.02; H, 9.41; N, 2.71 ;

LRMS, m/z (ES^+ mode): 524.0 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{30}H_{47}NO_5Na$ ($[M+Na]^+$):** 524.3352; **Found:** 524.3359 .

3β-acetoxy-23,24-bisnor-5-en-22-(methyl hexanoate) amide (4e)



$C_{31}H_{49}NO_5$
MW = 515.72 g.mol^{-1}

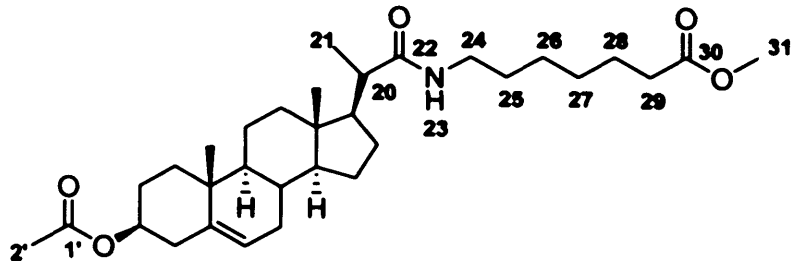
1- Following the **general procedure B** starting from HOBt (723 mg, 5.36 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (2.001 g, 4.38 mmol, 1.0 eq.) and DIPC (1.0 mL, 6.44 mmol, 1.5 eq.) in THF (10 mL), and, methyl 6-aminohexanoate **3e** (980 mg, 5.39 mmol, 1.2 eq.) and triethylamine (1.4 mL, 10.06 mmol, 2.3 eq.) in THF (10 mL), compound **4e** was isolated as a white solid (1.465 g, 65%).

2- Following the **general procedure B** starting from HOBt (276 mg, 2.04 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (801 mg, 1.75 mmol, 1.0 eq.) and EDC (380 mg, 1.98 mmol, 1.1 eq.) in THF (9 mL), and, methyl 6-

aminohexanoate **3e** (427 mg, 2.35 mmol, 1.3 eq.) and triethylamine (0.35 mL, 2.52 mmol, 1.4 eq) in THF (9 mL), compound **4e** was isolated as a white solid (276 mg, 31%).

^{1}H NMR (500MHz, $CDCl_3$): δ 0.71 (s, 3H, H^{18}), 1.04 (s, 3H, H^{19}), 1.20 (d, 3H, H^{21}, J= 6.7), 2.08 (s, 3H, $H^{2'}$), 1.00 to 2.10 (m, 25H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.33 (m, 4H, H^4 and H^{28}), 3.26 (m, 2H, H^{24}), 3.70 (s, 3H, H^{30}), 4.62 (m, 1H, H^3), 5.40 (m, 2H, H^6 and H^{23});
^{13}C NMR (125MHz, $CDCl_3$): δ 12.1 (C^{18}), 17.7 (C^{21}), 19.3 (C^{19}), 21.5 ($C^{2'}$), 33.9 (C^{28}), 36.6 (C^{10}), 38.1 (C^4), 38.9 (C^{24}), 42.3 (C^{13}), 45.0 (C^{20}), 51.5 (C^{30}), 21.0, 24.4, 24.4, 26.3, 27.6, 27.8, 29.3, 31.8, 31.9, 37.0, 39.5, 49.9, 52.8, 56.2 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}), 73.9 (C^3), 122.5 (C^6), 139.6 (C^5), 170.5 ($C^{1'}$ and C^{29}), 176.5 (C^{22});
Mp = 151-152°C; **R_f** = 0.12 (Hexane/AcOEt: 60/40);
Anal.Calcd. for $C_{31}H_{49}NO_5 \cdot 0.3H_2O$: % Theory: C, 71.45; H, 9.59; N, 2.69 **% Found:** C, 71.33; H, 9.54; N, 2.63 ;
LRMS, m/z (ES^+ mode): 538.0 ($[M+Na]^+$, 100%);
HRMS (ES^+ mode), **Calculated for $C_{31}H_{49}NO_5Na$ ($[M+Na]^+$):** 538.3508; **Found:** 538.3496 .

3β-acetoxy-23,24-bisnor-5-en-22-(methyl heptanoate) amide (4f)



$C_{32}H_{51}NO_5$
MW = 529.75 g.mol^{-1}

1- Following the **general procedure B** starting from HOBt (230 mg, 1.70 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (628 mg, 1.37 mmol, 1.0 eq.) and DIPC (0.4 mL, 2.58 mmol, 1.9 eq.) in THF (5 mL), and, methyl 7-aminoheptanoate **3f** (301 mg, 1.54 mmol, 1.1 eq.) and triethylamine (0.3 mL,

2.16 mmol, 1.6 eq.) in THF (5 mL), compound **4f** was isolated as a white solid (452 mg, 62%).

2- Following the **general procedure B** starting from HOBt (195 mg, 1.44 mmol, 1.1 eq.), 3β-acetoxy-5-cholenic acid **1** (606 mg, 1.33 mmol, 1.0 eq.) and EDC (269 mg, 1.40 mmol, 1.1 eq.) in THF (9 mL), and, methyl 7-aminoheptanoate **3f** (295 mg, 1.51 mmol, 1.1 eq.) and triethylamine (0.25 mL, 1.80 mmol, 1.4 eq) in THF (9 mL), compound **4f** was isolated as a white solid (141 mg, 20%).

^{1}H NMR (500MHz, $CDCl_3$): δ 0.72 (s, 3H, H^{18}), 1.07 (s, 3H, H^{19}), 1.19 (d, 3H, H^{21}, J= 6.7), 2.08 (s, 3H, $H^{2'}$), 1.00 to 2.10 (m, 27H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}), 2.31 (m, 4H, H^4 and H^{29}), 3.24 (m, 2H, H^{24}), 3.70 (s, 3H, H^{31}), 4.63 (m, 1H, H^3), 5.38 (m, 2H, H^6 and H^{23});

^{13}C NMR (125MHz, $CDCl_3$): δ 12.1 (C^{18}), 17.7 (C^{21}), 19.3 (C^{19}), 21.4 ($C^{2'}$), 33.9 (C^{29}), 36.6 (C^{10}), 38.1 (C^4), 39.1 (C^{24}), 42.3 (C^{13}), 45.0 (C^{20}), 51.5 (C^{31}), 21.0, 24.3, 24.8, 26.5, 27.6, 27.8, 28.7, 29.5, 31.8, 31.9, 37.0, 39.5, 49.9, 52.9, 56.2 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}), 73.9 (C^3), 122.5 (C^6), 139.7 (C^5), 170.5 ($C^{1'}$ and C^{30}), 176.5 (C^{22});

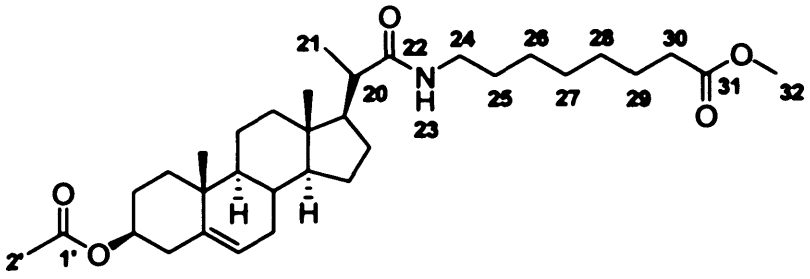
Mp = 146-147°C; **R_f** = 0.17 (Hexane/AcOEt: 60/40);

Anal.Calcd. for $C_{32}H_{51}NO_5$*0.2H_2O: % Theory: C, 72.06; H, 9.71; N, 2.71 **% Found:** C, 72.17; H, 9.74; N, 2.58 ;

LRMS, m/z (ES^+ mode): 552.0 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{32}H_{51}NO_5Na$ ($[M+Na]^+$):** 552.3665; **Found:** 552.3662 .

3β-acetoxy-23,24-bisnor-5-en-22-(methyl octanoate) amide (4g)



$C_{33}H_{53}NO_5$
MW = 543.78 g.mol^{-1}

1- Following the **general procedure B** starting from HOBt (1.104 g, 8.18 mmol, 1.2 eq.), 3β-acetoxy-5-cholenic acid **1** (3.059 g, 6.69 mmol, 1.0 eq.) and DIPC (1.4 mL, 9.02 mmol, 1.4 eq.) in THF (15 mL), and, methyl 8-aminooctanoate **3g** (1.618 g, 7.72 mmol, 1.2 eq.) and triethylamine (1.4 mL, 10.06 mmol, 1.5 eq.) in THF (15 mL), compound **4g** was isolated as a white solid (2.719 g, 75%).

2- Following the **general procedure B** starting from HOBt (221 mg, 1.64 mmol, 1.1 eq.), 3β-acetoxy-5-cholenic acid **1** (687 mg, 1.50 mmol, 1.0 eq.) and EDC (305 mg, 1.59 mmol, 1.1 eq.) in THF (8 ml), and, methyl 8-aminooctanoate **3g** (408 mg, 1.95 mmol, 1.3 eq.) and triethylamine (0.30 ml, 2.16 mmol, 1.4 eq) in THF (8 ml), compound **4g** was isolated as a white solid (172 mg, 21%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.71 (s, 3H, H^{18}), 1.06 (s, 3H, H^{19}), 1.20 (d, 3H, H^{21}, J = 6.7), 2.08 (s, 3H, $H^{2'}$), 1.00 to 2.10 (m, 29H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}, H^{29}), 2.34 (m, 4H, H^4 and H^{30}), 3.25 (m, 2H, H^{24}), 3.68 (s, 3H, H^{32}), 4.61 (m, 1H, H^3), 5.38 (t, 1H, H^{23}, J = 5.6), 5.40 (m, 1H, H^6);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.1 (C^{18}), 17.7 (C^{21}), 19.3 (C^{19}), 21.4 ($C^{2'}$), 34.0 (C^{30}), 36.6 (C^{10}), 38.1 (C^4), 39.2 (C^{24}), 42.3 (C^{13}), 45.0 (C^{20}), 51.5 (C^{32}), 21.0, 24.3, 24.8, 26.7, 27.6, 27.8, 28.9, 29.0, 29.6, 31.8, 31.9, 37.0, 39.5, 49.9, 52.9, 56.2 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}, C^{29}), 73.9 (C^3), 122.5 (C^6), 139.7 (C^5), 170.5 ($C^{1'}$ and C^{31}), 176.4 (C^{22});

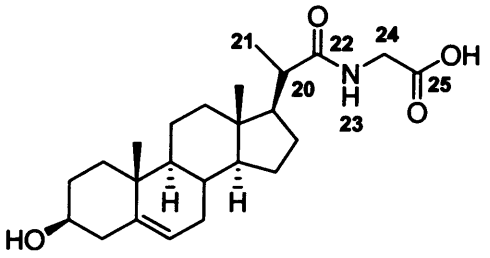
Mp = 139-140°C; $\mathbf{R_f}$ = 0.20 (Hexane/AcOEt: 60/40);

Anal.Calcd. for $C_{33}H_{53}NO_5 \cdot 0.2H_2O$: % Theory: C, 72.41; H, 9.83; N, 2.56 **% Found:** C, 72.37; H, 9.85; N, 2.55 ;

LRMS, m/z (ES^+ mode): 566.0 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{33}H_{53}NO_5Na$ ($[M+Na]^+$):** 566.3821; **Found:** 566.3823 .

3β-ol-23,24-bisnor-5-en-22-(ethanoic acid) amide (8a)



$C_{24}H_{37}NO_4$
MW = 403.55 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl ethanoate) amide **4a** (0.200 g, 0.44 mmol, 1.0 eq.) and sodium hydroxide (4.3 mL, C = 1M, 4.3 mmol, 9.8 eq.) in THF (10 mL), compound **8a** was isolated as a white solid (135 mg, 77%).

^{1}H NMR (300MHz, DMSO): δ 0.67 (s, 3H, H^{18}), 0.92 (s, 3H, H^{19}), 1.03 (d, 3H, H^{21}, J= 6.8), 0.80 to 2.30 (m, 20H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}), 3.40 (m, 2H, H^3 and H^{20}), 3.68 (d, 2H, H^{24}, J= 5.0), 4.62 (s, 1H, OH), 5.28 (m, 1H, H^6), 8.00 (t, 1H, H^{23}, J= 5.0);

^{13}C NMR (75MHz, DMSO): δ 11.3 (C^{18}), 16.8 (C^{21}), 18.6 (C^{19}), 35.5 (C^4), 36.3 (C^{10}), 41.1 (C^{13}), 41.6 (C^{24}), 41.8 (C^{20}), 20.0, 23.3, 26.2, 30.8, 30.8, 30.9, 35.5, 38.6, 49.0, 51.8, 55.4 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 69.4 (C^3), 119.8 (C^6), 140.7 (C^5), 170.8 (C^{25}), 175.4 (C^{22});

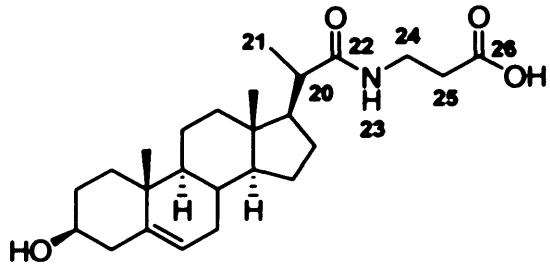
Mp = 220-222°C; $\mathbf{R_f}$ = 0.06 (Chloroform/Methanol: 90/10);

Anal.Calcd. for $C_{24}H_{37}NO_4 \cdot 1.3H_2O$: % Theory: C, 67.51; H, 9.35; N, 3.28 **% Found:** C, 67.19; H, 9.01; N, 3.12 ;

LRMS, m/z (Cl^+ mode): 404.3 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{24}H_{38}NO_4$ ($[M+H]^+$):** 404.2795; **Found:** 404.2791 .

3β-ol-23,24-bisnor-5-en-22-(propanoic acid) amide (8b)

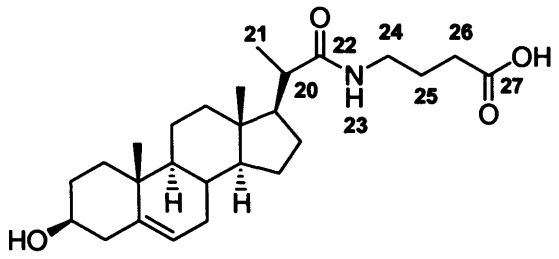


$C_{25}H_{39}NO_4$
MW = 417.58 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl propanoate) amide **4b** (0.208 g, 0.44 mmol, 1.0 eq.) and sodium hydroxide (4.3 mL, C = 1M, 4.3 mmol, 9.8 eq.) in THF (10 mL), compound **8b** was isolated as a white solid (150 mg, 82%).

^{1}H NMR (300MHz, DMSO): δ 0.62 (s, 3H, H^{18}), 0.86 (s, 3H, H^{19}), 0.94 (d, 3H, H^{21}, J = 6.7), 0.80 to 2.10 (m, 21H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}), 2.20 (t, 2H, H^{25}, J = 7.0), 3.17 (m, 3H, H^3 and H^{24}), 5.22 (m, 1H, H^6), 5.55 (s, 1H, OH), 7.78 (t, 1H, H^{23}, J = 5.5);
^{13}C NMR (75MHz, DMSO): δ 12.8 (C^{18}), 18.2 (C^{21}), 20.0 (C^{19}), 32.3 (C^{25}), 36.2 (C^{10}), 36.9 (C^4), 42.6 (C^{13}), 43.1 (C^{20}), 43.4 (C^{24}), 21.5, 24.2, 27.5, 32.2, 32.3, 32.3, 36.1, 39.5, 50.5, 53.4, 56.8, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 70.9 (C^3), 121.3 (C^6), 142.1 (C^5), 174.6 (C^{26}), 176.3 (C^{22});
Mp = 226-227°C; **R_f** = 0.24 (Chloroform/Methanol: 90/10);
Anal.Calcd. for $C_{25}H_{39}NO_4 \cdot 2.1H_2O \cdot 0.5HCl$: % Theory: C, 63.40; H, 9.30; N, 2.96 **% Found:** C, 63.45; H, 9.20; N, 3.04 ;
LRMS, m/z (Cl^+ mode): 417.9 ($[M+H]^+$, 100%);
HRMS (ES^+ mode), **Calculated for $C_{25}H_{40}NO_4$ ($[M+H]^+$):** 418.2952; **Found:** 418.2952 .

3β-ol-23,24-bisnor-5-en-22-(butanoic acid) amide (8c)

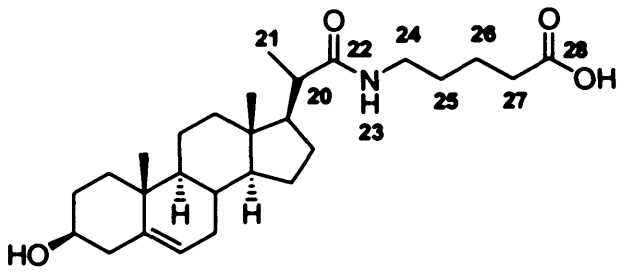


$C_{26}H_{41}NO_4$
MW = 431.61 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl butanoate) amide **4c** (0.112 g, 0.23 mmol, 1.0 eq.) and sodium hydroxide (2.1 mL, C = 1M, 2.1 mmol, 9.1 eq.) in THF (6.2 mL), compound **8c** was isolated as a white solid (84 mg, 85%).

^{1}H NMR (300MHz, DMSO): δ 0.65 (s, 3H, H^{18}), 0.94 (s, 3H, H^{19}), 0.99 (d, 3H, H^{21}, J= 6.5), 0.80 to 2.20 (m, 25H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}), 2.98 (m, 2H, H^{24}), 3.24 (m, 1H, H^3), 4.64 (s, 1H, OH), 5.24 (m, 1H, H^6), 7.75 (t, 1H, H^{23}, J= 5.8), 12.07 (s, 1H, COOH);
^{13}C NMR (75MHz, DMSO): δ 12.3 (C^{18}), 17.8 (C^{21}), 19.5 (C^{19}), 24.9 (C^{25}), 31.4 (C^{26}), 36.4 (C^{10}), 37.9 (C^4), 42.0 (C^{13}), 42.6 (C^{24}), 43.0 (C^{20}), 21.0, 24.3, 27.1, 31.7, 31.8, 31.8, 37.3, 39.5, 49.9, 52.7, 56.3, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 70.4 (C^3), 120.8 (C^6), 141.6 (C^5), 174.6 (C^{27}), 176.0 (C^{22});
Mp = 231-232 °C; **R_f** = 0.12 ($CHCl_3$/MeOH: 90/10);
Anal.Calcd. for $C_{26}H_{41}NO_4$•1.1HCl: % Theory: C, 66.20; H, 9.00; N, 2.97 **% Found:** C, 66.14; H, 8.78; N, 2.98 ;
LRMS, m/z (ES^+ mode): 454.3 ($[M+Na]^+$, 100%);
HRMS (ES^+ mode), **Calculated for $C_{26}H_{41}NO_4Na$ ($[M+Na]^+$):** 454.2933; **Found:** 454.2922 .

3β-ol-23,24-bisnor-5-en-22-(pentanoic acid) amide (8d)



$C_{27}H_{43}NO_4$
MW = 445.63 g.mol^{-1}

1- Following the **general procedure C** overnight, starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl pentanoate) amide **4d** (0.200 g, 0.40 mmol, 1.0 eq.) and sodium hydroxide (4.0 mL, C = 1M, 4.0 mmol, 10.0 eq.) in THF (20 mL), compound **8d** was isolated as a white solid (15 mg, 8%).

2- Following the **general procedure C** for 24h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl pentanoate) amide **4d** (98 mg, 0.20 mmol, 1.0 eq.) and sodium hydroxide (2.0 mL, C = 1M, 2.0 mmol, 10.0 eq.) in THF (6 mL), compound **8d** was isolated as a white solid (46 mg, 53%).

3- Following the **general procedure C** for 36h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl pentanoate) amide **4d** (102 mg, 0.20 mmol, 1.0 eq.) and sodium hydroxide (2.0 mL, C = 1M, 2.0 mmol, 10.0 eq.) in THF (6 mL), compound **8d** was isolated as a white solid (48 mg, 53%).

4- Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl pentanoate) amide **4d** (123 mg, 0.25 mmol, 1.0 eq.) and sodium hydroxide (2.0 mL, C = 1M, 2.0 mmol, 8.0 eq.) in THF (6 mL), compound **8d** was isolated as a white solid (103 mg, 95%).

^{1}H NMR (300MHz, DMSO): δ 0.66 (s, 3H, H^{18}), 0.94 (s, 3H, H^{19}), 1.00 (d, 3H, H^{21}, J= 6.9), 0.80 to 2.20 (m, 27H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}), H^{27}), 2.98 (m, 2H, H^{24}), 3.24 (m, 1H, H^3), 4.62 (s, 1H, OH), 5.27 (m, 1H, H^6), 7.72 (t, 1H, H^{23}, J= 5.3);

^{13}C NMR (75MHz, DMSO): δ 12.3 (C^{18}), 17.8 (C^{21}), 19.5 (C^{19}), 33.8 (C^{27}), 36.4 (C^{10}), 38.1 (C^{4}), 42.0 (C^{13}), 42.6 (C^{24}), 43.0 (C^{20}), 21.0, 22.3, 24.3, 27.1, 29.0, 31.7, 31.8, 31.8, 37.3, 39.5, 49.9, 52.7, 56.3, (C^{1}, C^{2}, C^{7}, C^{8}, C^{9}, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}), 70.4 (C^{3}), 120.8 (C^{6}), 141.6 (C^{5}), 174.9 (C^{28}), 175.8 (C^{22});

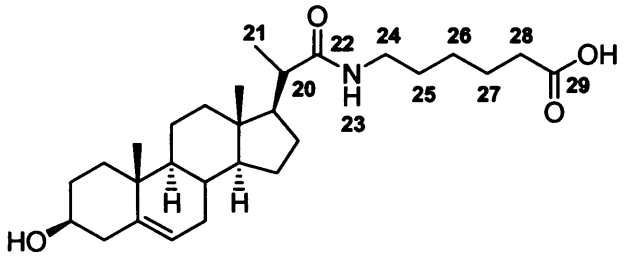
Mp = 215-216 °C; **R_f** = 0.15 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{27}H_{43}NO_4$•1.6HCl•0.1DIPU: % Theory: C, 64.18; H, 8.98; N, 3.24 **% Found:** C, 64.25; H, 8.93; N, 3.35 ;

LRMS, m/z (ES^+ mode): 468.3 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{27}H_{43}NO_4Na$ ($[M+Na]^+$):** 468.3090; **Found:** 468.3105 .

3β-ol-23,24-bisnor-5-en-22-(hexanoic acid) amide (8e)



$C_{28}H_{45}NO_4$
MW = 459.66 g.mol^{-1}

1- Following the **general procedure C** for 5h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl hexanoate) amide **4e** (388 mg, 0.75 mmol, 1.0 eq.) and sodium hydroxide (7.0 mL, C = 1M, 7.0 mmol, 9.3 eq.) in THF (20 mL), compound **8e** was isolated as a white solid (64 mg, 19%).

2- Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl hexanoate) amide **4e** (226 mg, 0.44 mmol, 1.0 eq.) and sodium hydroxide (221 mg, 5.53 mmol, 12.6 eq.) in THF (6 mL), compound **8e** was isolated as a white solid (10 mg, 05%).

3- Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl hexanoate) amide **4e** (110 mg, 0.21 mmol,

1.0eq.) and sodium hydroxide (2.0 mL, C = 1M, 2.0 mmol, 9.5 eq.) in THF (5.9 mL), compound **8e** was isolated as a white solid (79 mg, 81%).

^{1}H NMR (300MHz, DMSO): δ 0.65 (s, 3H, H^{18}), 0.94 (s, 3H, H^{19}), 0.99 (d, 3H, H^{21}, J= 6.7), 0.80 to 2.20 (m, 29H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}), 2.96 (m, 2H, H^{24}), 3.26 (m, 1H, H^3), 4.61 (s, 1H, OH), 5.25 (m, 1H, H^6), 7.69 (t, 1H, H^{23}, J = 5.6), 12.00 (s, 1H, COOH);

^{13}C NMR (75MHz, DMSO): δ 12.3 (C^{18}), 17.8 (C^{21}), 19.5 (C^{19}), 34.0 (C^{28}), 36.4 (C^{10}), 38.3 (C^4), 42.0 (C^{13}), 42.6 (C^{24}), 43.0 (C^{20}), 21.0, 24.3, 24.5, 26.3, 27.1, 29.2, 31.7, 31.8, 31.8, 37.3, 39.5, 50.0, 52.7, 56.3, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}), 70.4 (C^3), 120.8 (C^6), 141.6 (C^5), 174.8 (C^{29}), 175.8 (C^{22});

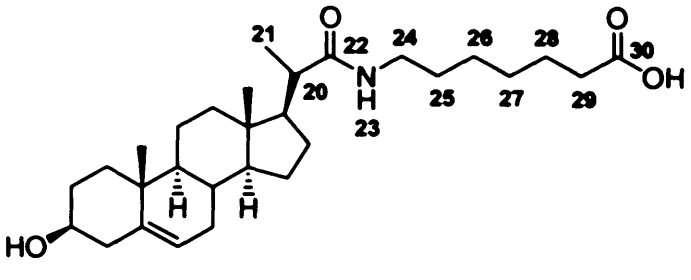
Mp = 178-179 °C; **R_f** = 0.18 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{28}H_{45}NO_4$•1.5HCl: % Theory: C, 65.38; H, 9.11; N, 2.72 **% Found:** C, 65.42; H, 9.01; N, 2.78 ;

LRMS, m/z (ES^+ mode): 482.3 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{28}H_{45}NO_4Na$ ($[M+Na]^+$):** 482.3246; **Found:** 482.3250 .

3β-ol-23,24-bisnor-5-en-22-(heptanoic acid) amide (8f)



$C_{29}H_{47}NO_4$
MW = 473.69 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl heptanoate) amide **4f** (120 mg, 0.23 mmol, 1.0 eq.) and sodium hydroxide (1.9 mL, C = 1M, 1.9 mmol, 8.3 eq.) in THF (5.8 mL), compound **8f** was isolated as a white solid (92 mg, 86%).

^{1}H NMR (300MHz, DMSO): δ 0.66 (s, 3H, H^{18}), 0.96 (s, 3H, H^{19}), 1.00 (d, 3H, H^{21}, J= 6.5), 0.80 to 2.20 (m, 31H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}, H^{29}), 2.97 (m, 2H, H^{24}), 3.26 (m, 1H, H^3), 5.26 (m, 1H, H^6), 7.68 (t, 1H, H^{23}, J= 5.4);

^{13}C NMR (75MHz, DMSO): δ 12.3 (C^{18}), 17.8 (C^{21}), 19.5 (C^{19}), 34.3 (C^{29}), 36.4 (C^{10}), 38.4 (C^4), 42.0 (C^{13}), 42.6 (C^{24}), 43.0 (C^{20}), 20.9, 24.3, 25.0, 26.5, 27.1, 28.7, 29.3, 31.7, 31.8, 31.8, 37.3, 39.5, 50.0, 52.7, 56.3, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}), 70.4 (C^3), 120.8 (C^6), 141.6 (C^5), 175.1 (C^{30}), 175.7 (C^{22});

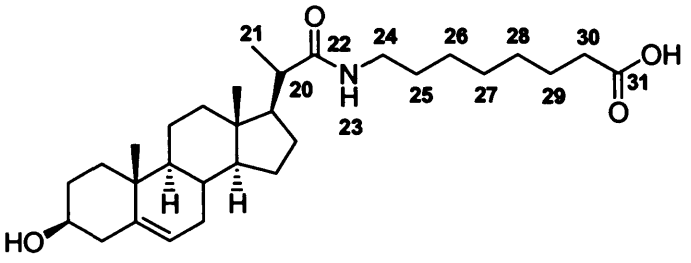
Mp = 136-138 °C; **R_f** = 0.19 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{27}H_{43}NO_4$•0.7HCl•0.1DIPU: % Theory: C, 69.45; H, 9.67; N, 3.27 **% Found:** C, 69.40; H, 9.83; N, 3.38;

LRMS, m/z (ES^+ mode): 496.3 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{29}H_{47}NO_4Na$ ($[M+Na]^+$):** 496.3403; **Found:** 496.3407 .

3β-ol-23,24-bisnor-5-en-22-(octanoic acid) amide (8g)



$C_{30}H_{49}NO_4$
MW = 487.71 g.mol^{-1}

1- Following the **general procedure C** for 120h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl octanoate) amide **4g** (128 mg, 0.24 mmol, 1.0 eq.) and sodium hydroxide (2.0 mL, C = 1M, 2.0 mmol, 8.3 eq.) in THF (5.5 mL), compound **8g** was isolated as a white solid (79 mg, 69%).

2- Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(methyl octanoate) amide **4g** (114 mg, 0.21 mmol, 1.0

eq.) and sodium hydroxide (1.9 mL, C = 1M, 1.9 mmol, 9.1 eq.) in THF (5.6 mL), compound **8g** was isolated as a white solid (83 mg, 81%).

^{1}H NMR (300MHz, DMSO): δ 0.65 (s, 3H, H^{18}), 0.93 (s, 3H, H^{19}), 0.99 (d, 3H, H^{21}, J= 6.6), 0.80 to 2.20 (m, 33H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}, H^{29}, H^{30}), 2.96 (m, 2H, H^{24}), 3.25 (m, 1H, H^3), 4.64 (s, 1H, OH), 5.25 (m, 1H, H^6), 7.68 (t, 1H, H^{23}, J= 5.5), 12.01 (s, 1H, COOH);

^{13}C NMR (75MHz, DMSO): δ 12.3 (C^{18}), 17.8 (C^{21}), 19.5 (C^{19}), 34.0 (C^{30}), 36.4 (C^{10}), 38.4 (C^4), 42.0 (C^{13}), 42.6 (C^{24}), 43.0 (C^{20}), 21.0, 24.2, 24.8, 26.6, 27.1, 28.8, 28.9, 29.4, 31.7, 31.8, 31.8, 37.3, 39.5, 50.0, 52.7, 56.3, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}, C^{29}), 70.4 (C^3), 120.8 (C^6), 141.6 (C^5), 174.9 (C^{31}), 175.7 (C^{22});

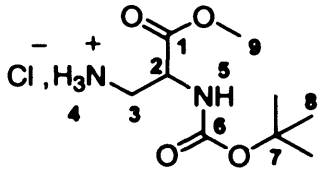
Mp = 163-164 °C; **R_f** = 0.20 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{30}H_{49}NO_4$•0.7HCl: % Theory: C, 70.21; H, 9.76; N, 2.73 **% Found:** C, 70.42; H, 9.69; N, 2.72 ;

LRMS, m/z (ES^+ mode): 510.3 ($[M+Na]^+$, 100%);

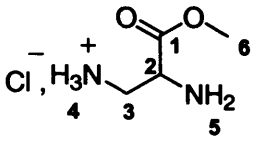
HRMS (ES^+ mode), **Calculated for $C_{30}H_{49}NO_4Na$ ($[M+Na]^+$):** 510.3559; **Found:** 510.3551 .

Attempted synthesis of Boc-Dap-OMe (10)



$C_9H_{19}N_2O_4Cl$
MW =254.71

1- Following the **general procedure A** starting from thionyl chloride (0.5 mL, 6.85 mmol, 4.6 eq.) and Boc-Dap-OH (0.305 g, 1.50 mmol, 1.0 eq.) in methanol (2 mL, 49.44 mmol, 33.0 eq.), analyses by NMR and MS showed the byproduct **11**.



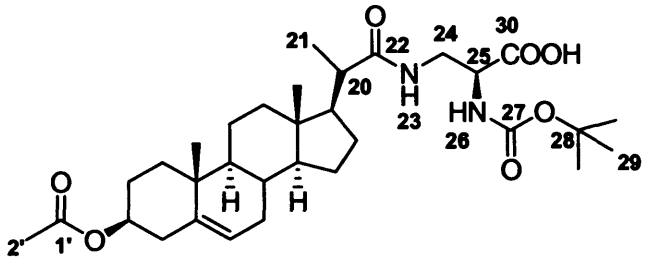
Byproduct 11

$C_4H_{11}N_2O_2Cl$
MW = 154.60

^{1}H NMR (300MHz, DMSO): δ 3.46 (dd, 1H, H^3, $J_{3\text{-}3'}$ = 13.5 Hz, $J_{3\text{-}2}$ = 5.5), 3.54 (dd, 1H, $H^{3'}$, $J_{3'\text{-}3}$ = 13.5 Hz, $J_{3'\text{-}2}$ = 8.1), 3.96 (s, 3H, H^6), 4.47 (dd, 1H, H^2, $J_{2\text{-}3}$ = 5.5, $J_{2\text{-}3'}$ = 8.1);
LRMS, m/z (ES^+ mode): 119 ($[M\text{-}Cl]^+$, 100%).

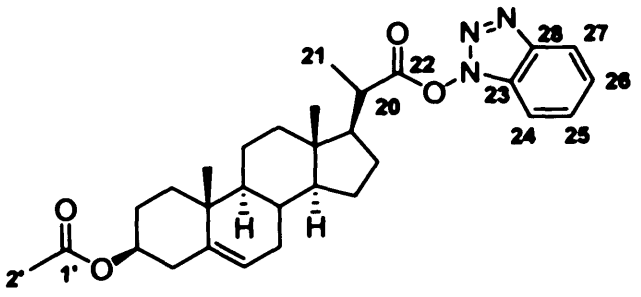
2- Following the **general procedure A** at room temperature starting from thionyl chloride (0.5 mL, 6.85 mmol, 4.57 eq.) and Boc-Dap-OH (0.305 g, 1.50 mmol, 1.0 eq.) in methanol (2 mL, 49.44 mmol, 33.0 eq.), analyses by NMR and MS showed the by-product **11**.

3β-acetoxy-23,24-bisnor-5-en-22-(3-(2-Boc-amino)propanoic acid) amide (14a)



$C_{32}H_{50}N_2O_7$
MW = 574.75

1- Following the **general procedure B** for 6h starting from HOBt (87 mg, 0.64 mmol, 1.1 eq.), 3β-acetoxy-5-cholenic acid **1** (228 mg, 0.59 mmol, 1.0 eq.), DIPC (0.21 mL, 1.35 mmol, 2.3 eq.) and Boc-Dap-OH (145 mg, 0.72 mmol, 1.2 eq.) in THF (3 mL), analyses by MS of the crude showed only the intermediate **6** (98mg, 33%).



$C_{30}H_{39}N_3O_4$
MW = 505.65

^{1}H NMR (300MHz, $CHCl_3$): δ 0.87 (s, 3H, H^{18}), 1.09 (s, 3H, H^{19}), 1.57 (d, 3H, H^{21}, J= 6.9), 2.08 (s, 3H, $H^{2'}$), 1.00 to 2.20 (m, 18H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}), 2.37 (d, 2H, H^4, J= 6.9), 2.97 (m, 2H, H^{20}), 4.65 (m, 1H, H^3), 5.43 (m, 1H, H^6), 7.45 (m, 2H, H^{25} and H^{26}), 7.59 (t, 1H, H^{27}, J= 7.6), 8.11 (d, 1H, H^{24}, J= 8.4);
LRMS, m/z (ES^+ mode): 559.6 ($[M+2Na]^+$, 15%).

2- HOBt (102 mg, 0.76 mmol, 1.2 eq.), TBTU (249 mg, 0.78 mmol, 1.2 eq.), and 3β-acetoxy-5-cholenic acid **1** (298 mg, 0.65 mmol, 1.0 eq.), were dissolved in dry DMF (8 mL). Then, DIPEA (0.4 mL, 2.30 mmol, 3.5 eq.) was added and the solution was stirred 30 minutes. Boc-Dap-OH (153 mg, 0.75 mmol, 1.2 eq.) in suspension in DMF (8 mL) was then added, and the resulting solution stirred overnight. The reaction was diluted with water, extracted with Chloroform (2•15 mL), dried with magnesium sulfate and the remaining solvents removed under reduced pressure. The crude was purified by column chromatography on silica gel using an elution of hexane/AcOEt (80/20) first, and $CHCl_3$/MeOH (90/10) then. The compound **14a** was isolated as a white solid (39 mg, 10%).

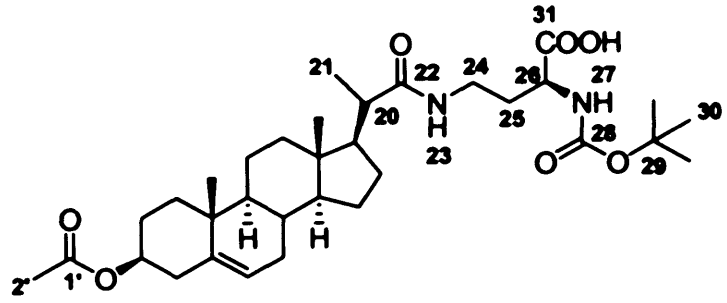
3- HOBt (128 mg, 0.95 mmol, 1.5 eq.), TBTU (296 mg, 0.92 mmol, 1.4 eq.), and 3β-acetoxy-5-cholenic acid **1** (295 mg, 0.65 mmol, 1.0 eq.), were dissolved in dry DMF (8 mL). Then, DIPEA (0.4 mL, 2.30 mmol, 3.5 eq.) was added and the solution was stirred 1h. Boc-Dap-OH (227 mg, 1.11 mmol, 1.7 eq.) in suspension in DMF (10 mL) was then added, and the resulting solution stirred for 24h. The reaction was diluted with water, extracted with chloroform (2•15 mL), dried with magnesium sulfate and the remaining solvents removed under

reduced pressure. The crude was purified by column chromatography on silica gel using an elution of hexane/AcOEt (80/20) first, and $CHCl_3$/MeOH (90/10) then. The compound **14a** was isolated as a white solid (177 mg, 48%).

4- HOBt (138 mg, 1.02 mmol, 1.4 eq.), TBTU (323 mg, 1.01 mmol, 1.4 eq.), and 3β-acetoxy-5-cholenic acid **1** (344 mg, 0.75 mmol, 1.0 eq.), were dissolved in dry DMF (8 mL). Then, DIPEA (0.44 mL, 2.53 mmol, 3.4 eq.) was added and the solution was stirred 1h. Boc-Dap-OH (203 mg, 1.00 mmol, 1.3 eq.) in suspension in DMF (10 mL) was then added, and the resulting solution stirred 48h. The reaction was diluted with water, extracted with chloroform (2*15 mL), dried with magnesium sulfate and the remaining solvents removed under reduced pressure. The crude was purified by column chromatography on silica gel using an elution of hexane/AcOEt (80/20) first, and $CHCl_3$/MeOH (90/10) then. The compound **14a** was isolated as a white solid (239 mg, 55%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.65 (s, 3H, H^{18}), 0.98 (s, 3H, H^{19}), 1.15 (d, 3H, H^{21}, J= 6.6), 1.42 (s, 9H, H^{29}), 2.00 (s, 3H, $H^{2'}$), 0.80 to 2.0 (m, 18H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}), 2.13 (m, 1H, H^{20}), 2.28 (d, 2H, H^4, J= 6.7), 3.40 (m, 1H, H^{24}), 3.76 (m, 1H, $H^{24'}$), 4.14 (m, 1H, H^{25}), 4.56 (m, 1H, H^3), 5.32 (m, 1H, H^6), 6.14 and 6.49 (2m, 2H, H^{23} and H^{26});
^{13}C NMR (75MHz, $CDCl_3$): δ 12.6 (C^{18}), 18.0 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 28.8 (C^{29}), 37.0 (C^{10}), 38.5 (C^4), 42.4 (C^{20}), 42.7 (C^{13}), 44.8 (C^{24}), 55.8 (C^{25}), 21.4, 24.8, 27.8, 28.2, 32.3, 32.3, 37.4, 39.9, 50.3, 53.1, 56.6, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.3 (C^3), 81.0 (C^{28}), 122.9 (C^6), 140.0 (C^5), 157.1 (C^{27}), 171.0 ($C^{1'}$ and C^{30}), 179.4 (C^{22});
Mp = 158-160 °C; **R_f** = 0.17 ($CHCl_3$/MeOH: 90/10);
LRMS, m/z (ES^+ mode): 619.3 ($[M+2Na-H]^+$, 100%);
HRMS (ES^+ mode), **Calculated for $C_{32}H_{49}N_2O_7Na_2$ ($[M+2Na-H]^+$):** 619.3335; **Found:** 619.3340 .

3β-acetoxy-23,24-bisnor-5-en-22-(4-(2-Boc-amino)butanoic acid) amide (14b)



$C_{33}H_{52}N_2O_7$
MW = 588.78

1- HOBt (309 mg, 2.29 mmol, 1.7 eq.), TBTU (5875 mg, 1.83 mmol, 1.4 eq.), and 3β-acetoxy-5-cholenic acid **1** (611 mg, 1.34 mmol, 1.0 eq.), were dissolved in dry DMF (15 mL). Then, DIPEA (0.5 mL, 2.88 mmol, 2.2 eq.) was added and the solution was stirred 1h. Boc-Dab-OH (392 mg, 1.80 mmol, 1.3 eq.) in suspension in DMF (15 mL) was then added, and the resulting solution stirred overnight. The reaction was diluted with water, extracted with Chloroform (2•25 mL), dried with magnesium sulfate and the remaining solvents removed under reduced pressure. The crude was purified by column chromatography on silica gel using an elution of hexane/AcOEt (80/20) first, and $CHCl_3$/MeOH (90/10) then. The compound **14b** was isolated as a white solid (256 mg, 33%).

2- HOBt (138 mg, 1.02 mmol, 1.2 eq.), TBTU (325 mg, 1.01 mmol, 1.2 eq.), and 3β-acetoxy-5-cholenic acid **1** (330 mg, 0.85 mmol, 1.0 eq.), were dissolved in dry DMF (7 mL). Then, DIPEA (0.2 mL, 1.15 mmol, 1.4 eq.) was added and the solution was stirred 1h. Boc-Dab-OH (215 mg, 0.99 mmol, 1.2 eq.) in suspension in DMF (8 mL) was then added, and the resulting solution stirred 48h. The reaction was diluted with water, extracted with chloroform (2•15 mL), dried with magnesium sulfate and the remaining solvents removed under reduced pressure. The crude was purified by column chromatography on silica gel using an elution of hexane/AcOEt (80/20) first, and $CHCl_3$/MeOH (90/10) then. The compound **14b** was isolated as a white solid (191 mg, 45%).

^{1}H NMR (500MHz, $CDCl_3$): δ 0.62 (s, 3H, H^{18}), 0.95 (s, 3H, H^{19}), 1.11 (d, 3H, H^{21}, J= 6.6), 1.36 (s, 9H, H^{30}), 1.96 (s, 3H, $H^{2'}$), 0.80 to 2.0 (m, 20H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{25}), 2.10 (m, 1H, H^{20}), 2.25 (d, 2H, H^4, J= 6.7), 2.94 (m, 1H, H^{24}), 3.53 (m, 1H, $H^{24'}$), 4.09 (m, 1H, H^{26}), 4.51 (m, 1H, H^3), 5.29 (m, 1H, H^6), 5.50 and 6.53 (2m, 2H, H^{23} and H^{27});

^{13}C NMR (125MHz, $CDCl_3$): δ 12.1 (C^{18}), 17.6 (C^{21}), 19.3 (C^{19}), 21.4 ($C^{2'}$), 28.4 (C^{30}), 29.7 (C^{25}), 36.2 (C^{24}), 36.6 (C^{10}), 38.1 (C^4), 42.4 (C^{13}), 44.8 (C^{20}), 56.2 (C^{26}), 21.0, 24.4, 27.6, 27.8, 31.9, 31.9, 37.0, 39.6, 50.0, 52.7, 56.2, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 73.9 (C^3), 80.0 (C^{29}), 122.5 (C^6), 139.7 (C^5), 155.8 (C^{28}), 170.5 ($C^{1'}$ and C^{31}), 178.4 (C^{22});

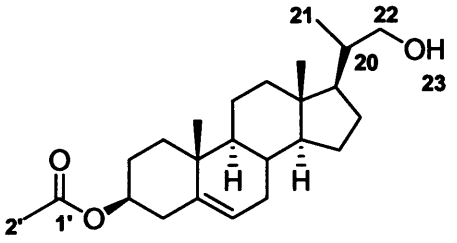
Mp = 156-158 °C; **R_f** = 0.27 ($CHCl_3$/MeOH: 90/10);

LRMS, m/z (ES^+ mode): 589.2 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{33}H_{53}N_2O_7$ ($[M+H]^+$):** 589.3847; **Found:** 589.3849 .

IX.4 Synthesis of the amine derivatives (Chapter V)

3β-acetoxy-23,24-bisnor-chol-5-en-22-ol (17)



$C_{24}H_{38}O_3$
MW = 374.56 g.mol^{-1}

A solution of borane-dimethyl sulfide complex (5.6 mL, C = 2M, 11.20 mmol, 1.3 eq.) was added dropwise to a solution of 3β-acetoxy-5-cholenic acid **1** (4.032 g, 8.82 mmol, 1.0 eq.) in THF (70 mL) kept below 0°C. The reaction mixture was stirred overnight at room temperature. Then it was diluted with water (2*10 mL), and extracted with Et_2O (30 mL). The organic layer was washed with a saturated solution of K_2CO_3 (2*10 mL), a saturated solution of NaCl (10 mL), dried over sodium sulfate, and the solvent removed to afford the

product **17** (3.245 g, 98%), after purification by column chromatography (Hexane/AcOEt: 80/20) as a white precipitate.

^{1}H NMR (300MHz, $CDCl_3$): δ 0.84 (s, 3H, H^{18}), 1.16 (s, 3H, H^{19}), 1.19 (d, 3H, H^{21}, J = 6.6), 2.17 (s, 3H, $H^{2'}$), 1.0 to 2.20 (m, 19H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}), 2.46 (d, 2H, H^4, J = 7.6 Hz), 3.50 (dd, 1H, H^{22}, $J_{22\text{-}22'}$ = 10.2, $J_{22\text{-}20}$ = 7.1), 3.79 (dd, 1H, $H^{22'}$, $J_{22'\text{-}22}$ = 10.2, $J_{22'\text{-}20}$ = 2.8), 4.74 (m, 1H, H^3), 5.51 (m, 1H, H^6);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 17.2 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 37.0 (C^{10}), 38.5 (C^4), 42.8 (C^{13}), 21.4, 24.8, 28.2, 28.2, 32.3, 32.3, 37.4, 39.2, 40.0, 50.4, 52.8, 56.8 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}), 68.4 (C^{22}), 74.4 (C^3), 123.0 (C^6), 140.1 (C^5), 171.0 ($C^{1'}$);

IR (KBr, cm^{-1}): 1263.9 (ν_{CO} acetyl), 1729.8 (ν_{CO} ester), 3266.3 ($\nu_{O\text{-}H}$);

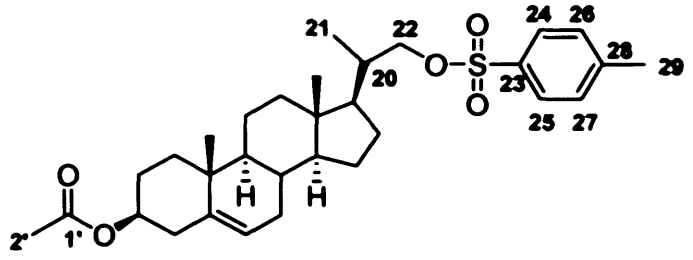
Mp = 136-137°C; **R_f** = 0.42 (hexane/AcOEt: 80/20);

Anal.Calcd. for $C_{24}H_{38}O_3$: % Theory: C, 76.96; H, 10.23; **% Found:** C, 76.61; H, 10.28;

LRMS, m/z (ES^+ mode): 397 ($[M+Na]^+$, 64%);

HRMS (ES^+ mode), **Calculated for $C_{24}H_{38}O_3Na$ ($[M+Na]^+$):** 397.2719; **Found:** 397.2720 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(p-toluensulfonyloxy) (18)



$C_{31}H_{44}O_5S$
MW = 528.74 g.mol^{-1}

3β-acetoxy-23,24-bisnor-chol-5-en-22-ol **17** (1.488 g, 3.97 mmol, 1.0 eq.) was dissolved in a solution of 1:1 pyridine/DCM (40 mL), and cooled to 0°C. Then p-toluensulfonylchloride (1.003 g, 5.27 mmol, 1.3 eq.) was added as a solid at 0°C. The solution was stirred at 4°C for 24h. The reaction mixture was poured

into water (20 mL), extracted with chloroform (30 mL), the organic layer washed with a saturated solution of copper (II) sulfate hydrate, dried over magnesium sulfate and the solvent removed to afford the product **18** (1.751 g, 83%), after purification by column chromatography (Hexane/AcOEt: 80/20), as a white precipitate.

^{1}H NMR (300MHz, $CDCl_3$): δ 0.70 (s, 3H, H^{18}), 1.04 (d, 3H, H^{21}, J = 6.8), 1.05 (s, 3H, H^{19}), 2.09 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 19H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}), 2.37 (d, 2H, H^4, J = 6.9), 2.51 (s, 3H, H^{29}), 3.82 (dd, 1H, H^{22}, $J_{22\text{-}22'}$ = 9.2, $J_{22\text{-}20}$ = 6.6), 4.05 (dd, 1H, $H^{22'}$, $J_{22'\text{-}22}$ = 9.2, $J_{22'\text{-}20}$ = 3.0), 4.65 (m, 1H, H^3), 5.42 (m, 1H, H^6), 7.40 (d, 2H, H^{26} and H^{27}, J = 7.9), 7.84 (d, 2H, H^{24} and H^{25}, J = 8.3).

^{13}C NMR (75MHz, $CDCl_3$): δ 12.2 (C^{18}), 17.3 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 22.1 (C^{29}), 37.0 (C^{10}), 38.5 (C^4), 42.8 (C^{13}), 21.4, 24.7, 27.9, 28.2, 32.2, 32.2, 36.6, 37.4, 39.8, 50.3, 52.2, 56.7 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}), 74.3 (C^3), 76.1 (C^{22}), 122.9 (C^6), 128.3 (C^{26} and C^{27}), 130.2 (C^{24} and C^{25}), 133.5 (C^{28}), 140.1 (C^5), 145.0 (C^{23}), 171.0 ($C^{1'}$);

IR (KBr, cm^{-1}): 1174.4 ($\nu_{S=O}$), 1243.3 ($\nu_{C\text{-}O}$), 1726.5 (ν_{CO} ester);

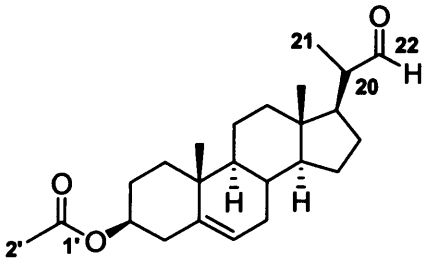
Mp = 109-110°C; **R_f** = 0.63 (Hexane/AcOEt: 80/20);

Anal.Calcd. for $C_{31}H_{44}O_5S$: % Theory: C, 70.42; H, 8.39; S, 6.06 **% Found:** C, 70.83; H, 8.56; S, 5.91 ;

LRMS, m/z (ES^+ mode): 551 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{31}H_{44}O_5SNa$ ($[M+Na]^+$):** 551.2807; **Found:** 551.2811 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-al (23)



$C_{24}H_{36}O_3$
MW = 372.54 g.mol^{-1}

3β-acetoxy-23,24-bisnor-chol-5-en-22-ol **17** (1.010 g, 2.70 mmol, 1.0 eq.) and pyridinium chlorochromate (1.113 g, 5.16 mmol, 1.9 eq.) were dissolved in DCM (20 mL), with molecular sieves (3Å). The solution was stirred at room temperature overnight, poured onto a silica pad, and washed with DCM (1.5 L). The filtrate was collected, concentrated and purified by column chromatography (Hexane/AcOEt: 80/20) to afford the product **23** (0,749 g, 75%), as a white solid.

^{1}H NMR (300MHz, $CDCl_3$): δ 0.79 (s, 3H, H^{18}), 1.08 (s, 3H, H^{19}), 1.19 (d, 3H, H^{21}, J= 6.8), 2.09 (s, 3H, $H^{2'}$), 1.00 to 2.10 (m, 18H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}), 2.40 (m, 3H, H^4 and H^{20}), 4.67 (m, 1H, H^3), 5.43 (m, 1H, H^6), 9.63 (d, 1H, H^{22}, J= 3.3);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.7 (C^{18}), 13.9 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 37.0 (C^{10}), 38.5 (C^4), 43.4 (C^{13}), 49.9 (C^{20}), 21.4, 25.1, 27.5, 28.2, 32.2, 32.3, 37.4, 39.8, 50.4, 51.4, 56.3 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.3 (C^3), 122.9 (C^6), 140.1 (C^5), 171.0 ($C^{1'}$), 205.6 (C^{22});

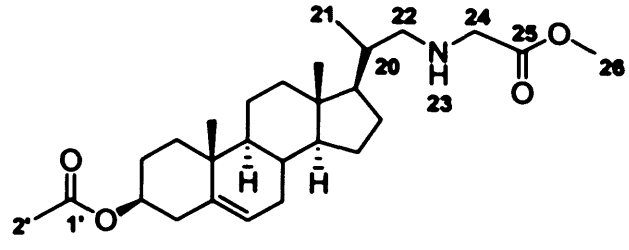
Mp = 105-106°C; **R_f** = 0.56 (Hexane/AcOEt: 80/20);

Anal.Calcd. for $C_{24}H_{36}O_3$: % Theory: C, 77.38; H, 9.74; **% Found:** C, 77.07; H, 9.94 ;

LRMS, m/z (ES^+ mode): 395 ($[M+Na]^+$, 46%);

HRMS (ES^+ mode), **Calculated for $C_{24}H_{36}O_3Na$ ($[M+Na]^+$):** 395. 2562; **Found:** 395.2570 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ethanoate) amine (20a)



$C_{27}H_{43}NO_4$
MW = 445.63 g.mol^{-1}

Following the **general procedure D** starting from triethylamine (120 µL, 0.86 mmol, 1.6 eq), methyl 2-aminoethanoate **3a** (84 mg, 0.67 mmol, 1.3 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (199 mg, 0.53 mmol, 1.0 eq.) and sodium cyanoborohydride (0.65 mL, 0.65 mmol, 1.2 eq.) in methanol (6 mL), compound **20a** was isolated as a white solid (118 mg, 50%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.75 (s, 3H, H^{18}), 1.08 (s, 3H, H^{19}), 1.09 (d, 3H, H^{21}, J = 6.8), 2.09 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 19H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}), 2.37 (d, 2H, H^4, J= 7.8), 2.41 (m, 1H, H^{22}), 2.64 (dd, 1H, $H^{22'}$, J= 3.0; J= 11.2), 3.43 (d, 2H, H^{24}, J= 2.9), 3.79 (s, 3H, H^{26}), 4.65 (m, 1H, H^3), 5.42 (m, 1H, H^6);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.3 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 37.0 (C^{10}), 37.1 (C^{20}), 38.5 (C^4), 42.8 (C^{13}), 51.7 (C^{24}), 52.2 (C^{26}), 55.7 (C^{22}), 21.4, 24.7, 28.2, 28.4, 32.3, 32.3, 37.4, 40.0, 50.4, 54.3, 56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.4 (C^3), 123.0 (C^6), 140.1 (C^5), 171.0 ($C^{1'}$), 173.6 (C^{25});

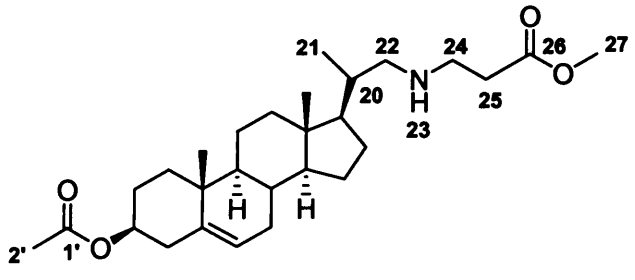
Mp = 74-75°C; **R_f** = 0.80 (Chloroform/Methanol: 90/10);

Anal.Calcd. for $C_{27}H_{43}NO_4$: % Theory: C, 72.77; H, 9.73; N, 3.14 **% Found:** C, 72.53; H, 9.86; N, 3.16 ;

LRMS, m/z (ES^+ mode): 446.2 ($[M+H]^+$, 100%); 468.2 ($[M+Na]^+$, 19%);

HRMS (ES^+ mode), **Calculated for $C_{27}H_{44}NO_4$ ($[M+H]^+$):** 446.3270; **Found:** 446.3277 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl propanoate) amine (20b)

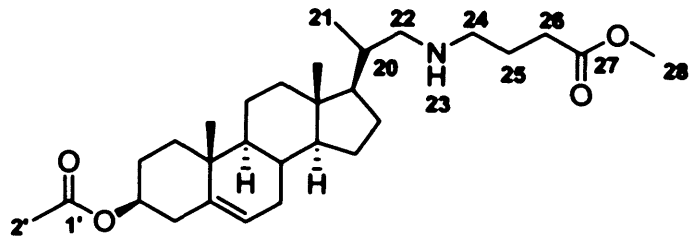


$C_{28}H_{45}NO_4$
MW = 459.66 g.mol^{-1}

Following the **general procedure D** starting from triethylamine (120 µL, 0.86 mmol, 1.6 eq), methyl 3-aminopropanoate **3b** (92 mg, 0.66 mmol, 1.2 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (200 mg, 0.54 mmol, 1.0 eq.) and sodium cyanoborohydride (0.65 mL, 0.65 mmol, 1.2 eq.) in methanol (6 mL), compound **20b** was isolated as a white solid (133 mg, 54%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.74 (s, 3H, H^{18}), 1.04 (d, 3H, H^{21}, J = 6.5), 1.07 (s, 3H, H^{19}), 2.09 (s, 3H, $H^{2'}$), 0.80 to 2.10 (m, 19H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}), 2.32 (m, 1H, H^{22}), 2.36 (d, 2H, H^4, J= 8.5), 2.58 (t, 2H, H^{25}, J= 6.5), 2.68 (dd, 1H, $H^{22'}$, J= 3.1; J= 11.5), 2.94 (m, 2H, H^{24}), 3.74 (s, 3H, H^{27}), 4.64 (m, 1H, H^3), 5.42 (m, 1H, H^6);
^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 18.1 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 34.9 (C^{25}), 36.8 (C^{20}), 37.0 (C^{10}), 38.5 (C^4), 42.9 (C^{13}), 45.4 (C^{24}), 52.0 (C^{27}), 55.7 (C^{22}), 21.4, 24.8, 28.2, 28.4, 32.3, 32.3, 37.4, 40.1, 50.4, 54.6, 56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.4 (C^3), 123.0 (C^6), 140.1 (C^5), 171.0 ($C^{1'}$), 173.8 (C^{26});
Mp = 83-84°C; **R_f** = 0.50 (Chloroform/Methanol: 90/10);
Anal.Calcd. for $C_{28}H_{45}NO_4 \cdot 0.2H_2O$: % Theory: C, 72.59; H, 9.88; N, 3.02 **% Found:** C, 72.59; H, 9.93; N, 2.98 ;
LRMS, m/z (ES^+ mode): 460.3 ($[M+H]^+$, 100%); 482.3 ($[M+Na]^+$, 64%);
HRMS (ES^+ mode), **Calculated for $C_{28}H_{46}NO_4$ ($[M+H]^+$):** 460.3427; **Found:** 460.3433 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl butanoate) amine (20c)



$C_{29}H_{47}NO_4$
MW = 473.69 g.mol^{-1}

1- Following the **general procedure D** starting from triethylamine (160 µL, 1.15 mmol, 2.1 eq.), methyl 4-aminobutanoate **3c** (169 mg, 1.10 mmol, 2.0 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (206 mg, 0.55 mmol, 1.0 eq.) and sodium cyanoborohydride (70 mg, 1.11 mmol, 2.0 eq.) in methanol (4 mL) with Molecular Sieves (3Å), compound **20c** was isolated as a white solid (100 mg, 38%).

2- Following the **general procedure D** starting from triethylamine (44 µL, 0.32 mmol, 1.1 eq.), methyl 4-aminobutanoate **3c** (49 mg, 0.32 mmol, 1.1 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (103 mg, 0.28 mmol, 1.0 eq.) and sodium cyanoborohydride (24 mg, 0.38 mmol, 1.4 eq.) in methanol (2 mL), compound **20c** was isolated as a white solid (60 mg, 46%).

3- Following the **general procedure D** starting from triethylamine (73 µL, 0.52 mmol, 2.2 eq.), methyl 4-aminobutanoate **3c** (88 mg, 0.57 mmol, 2.4 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (90 mg, 0.24 mmol, 1.0 eq.) and sodium cyanoborohydride (0.55 mL, 0.55 mmol, 2.3 eq.) in methanol (3.5 mL), compound **20c** was isolated as a white solid (64 mg, 56%).

4- Following the **general procedure D** starting from triethylamine (46 µL, 0.33 mmol, 1.4 eq.), methyl 4-aminobutanoate **3c** (49 mg, 0.32 mmol, 1.3 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (89 mg, 0.24 mmol, 1.0 eq.) and triacetoxy sodium borohydride (68 mg, 0.32 mmol, 1.3 eq.) in methanol (3 mL), compound **20c** was isolated as a white solid (49 mg, 43%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.74 (s, 3H, H^{18}), 1.05 (d, 3H, H^{21}, J= 6.8), 1.07 (s, 3H, H^{19}), 2.09 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 21H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}), 2.40 (m, 5H, H^4, H^{26} and H^{22}), 2.71 (m, 3H, H^{24} and $H^{22'}$), 3.73 (s, 3H, H^{28}), 4.65 (m, 1H, H^3), 5.42 (m, 1H, H^6);
^{13}C NMR (75MHz, $CDCl_3$): δ 12.3 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 25.4 (C^{25}), 34.3 (C^{26}), 36.7 (C^{20}), 37.0 (C^{10}), 38.5 (C^4), 42.9 (C^{13}), 49.7 (C^{24}), 52.0 (C^{28}), 55.6 (C^{22}), 21.4, 24.7, 28.2, 28.4, 32.3, 32.3, 37.4, 40.0, 50.4, 54.6,

56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.4 (C^3), 123.0 (C^6), 140.1 (C^5), 171.0 ($C^{1'}$), 174.5 (C^{27});

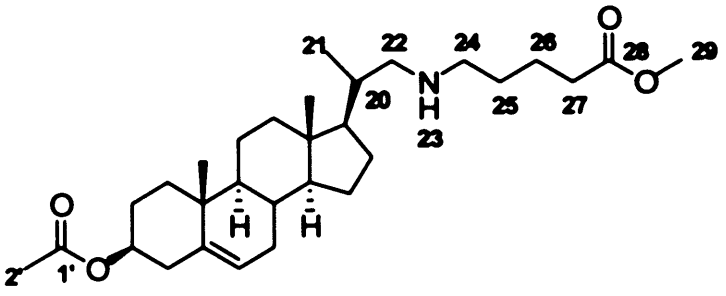
Mp = 94-95 °C; $\mathbf{R_f}$ = 0.30 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{29}H_{47}NO_4 \cdot 0.4H_2O$: % Theory: C, 72.43; H, 10.02; N, 2.91; **% Found:** C, 72.44; H, 10.11; N, 2.78 ;

LRMS, m/z (ES^+ mode): 474.2 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{29}H_{48}NO_4$ ($[M+H]^+$)**: 474.3583; **Found:** 474.3579 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl pentanoate) amine (20d)



$C_{30}H_{49}NO_4$
MW = 487.71 g.mol^{-1}

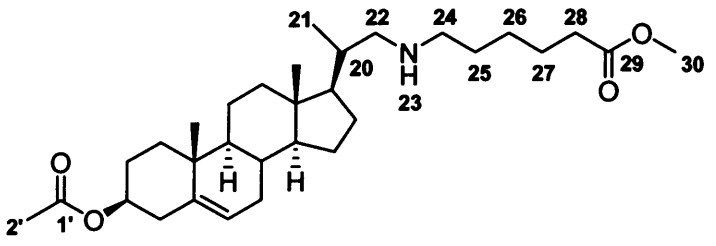
1- Following the **general procedure D** starting from triethylamine (104 µL, 0.75 mmol, 2.3 eq), methyl 5-aminopentanoate **3d** (126 mg, 0.75 mmol, 2.3 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (122 mg, 0.33 mmol, 1.0 eq.) and sodium cyanoborohydride (50 mg, 0.80 mmol, 2.4 eq.) in methanol (4 mL) for 48h, compound **20d** was isolated as a white solid (67 mg, 42%).

2- Following the **general procedure D** starting from triethylamine (61 µL, 0.44 mmol, 1.6 eq.), methyl 5-aminopentanoate **3d** (74 mg, 0.44 mmol, 1.6 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (104 mg, 0.28 mmol, 1.0 eq.) and sodium cyanoborohydride (0.32 mL, 0.32 mmol, 1.1 eq.) in methanol (2 mL), compound **20d** was isolated as a white solid (53 mg, 39%).

3- Following the **general procedure D** starting from triethylamine (37 µL, 0.27 mmol, 2.1 eq.), methyl 5-aminopentanoate **3d** (37 mg, 0.22 mmol, 1.7 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (48 mg, 0.13 mmol, 1.0 eq.) and triacetoxy sodium borohydride (30 mg, 0.14 mmol, 1.1 eq.) in methanol (2 mL) for 36h, compound **20d** was isolated as a white solid (44 mg, 70%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.82 (s, 3H, H^{18}), 1.12 (d, 3H, H^{21}, J = 6.6), 1.14 (s, 3H, H^{19}), 2.16 (s, 3H, $H^{2'}$), 0.90 to 2.20 (m, 23H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}), 2.45 (m, 5H, H^4, H^{27} and H^{22}), 2.74 (m, 3H, H^{24} and $H^{22'}$), 3.80 (s, 3H, H^{29}), 4.73 (m, 1H, H^3), 5.50 (m, 1H, H^6);
^{13}C NMR (75MHz, $CDCl_3$): δ 12.3 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 34.3 (C^{27}), 36.6 (C^{20}), 37.0 (C^{10}), 38.5 (C^4), 42.9 (C^{13}), 49.9 (C^{24}), 52.0 (C^{29}), 55.6 (C^{22}), 21.4, 23.1, 24.7, 28.2, 28.4, 29.4, 32.2, 32.2, 37.4, 40.0, 50.4, 54.6, 56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}), 74.4 (C^3), 123.0 (C^6), 140.1 (C^5), 171.0 ($C^{1'}$), 174.0 (C^{28});
Mp = 85-86 °C; **R_f** = 0.28 ($CHCl_3$/MeOH: 90/10);
Anal.Calcd. for $C_{30}H_{49}NO_4$·0.2H_2O: % Theory: C, 73.34; H, 10.13; N, 2.85; **% Found:** C, 73.19; H, 10.28; N, 2.77 ;
LRMS, m/z (ES^+ mode): 488.4 ($[M+H]^+$, 100%);
HRMS (ES^+ mode), **Calculated for $C_{30}H_{50}NO_4$ ($[M+H]^+$):** 488.3740; **Found:** 488.3756 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl hexanoate) amine (20e)



$C_{31}H_{51}NO_4$
MW = 501.74 g.mol^{-1}

2- Following the **general procedure D** starting from triethylamine (90
0.65 mmol, 1.7 eq), methyl 6-aminohexanoate **3e** (135 mg, 0.74 mmol,
eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (140 mg, 0.38 mmol, 1.0
and sodium cyanoborohydride (50 mg, 0.80 mmol, 2.1 eq.) in methano
mL), compound **20e** was isolated as a white solid (126 mg, 67%).

3- Following the **general procedure D** starting from triethylamine (56
0.40 mmol, 1.5 eq.), methyl 6-aminohexanoate **3e** (68 mg, 0.37 mmol, 1.4 e
3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (101 mg, 0.27 mmol, 1.0 eq.)
sodium cyanoborohydride (0.35 mL, 0.35 mmol, 1.3 eq.) in methanol (2 m
compound **20e** was isolated as a white solid (36 mg, 26%).

4- Following the **general procedure D** starting from triethylamine (24
0.17 mmol, 1.4 eq.), methyl 6-aminohexanoate **3e** (34 mg, 0.19 mmol, 1.6 e
3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (46 mg, 0.12 mmol, 1.0 eq.)
triacetoxy sodium borohydride (65 mg, 0.31 mmol, 2.6 eq.) in methanol (
mL) for 24h, compound **20e** was isolated as a white solid (32 mg, 52%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.72 (s, 3H, H^{18}), 1.01 (d, 3H, H^{21}, J = 6
1.05 (s, 3H, H^{19}), 2.07 (s, 3H, $H^{2'}$), 0.80 to 2.10 (m, 25H, H^1, H^2, H^7, H^8,
H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.35 (m, 5H, H^4, H^{28} and H
2.60 (m, 3H, H^{24} and $H^{22'}$), 3.69 (s, 3H, H^{30}), 4.63 (m, 1H, H^3), 5.40 (m,
H^6);
^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 (C
34.4 (C^{28}), 36.8 (C^{20}), 37.0 (C^{10}), 38.5 (C^4), 42.9 (C^{13}), 50.0 (C^{24}), 51.9 (C

55.9 (C^{22}), 21.4, 24.8, 25.3, 27.3, 28.2, 28.4, 30.0, 32.3, 32.3, 37.4, 40.1, 50.4, 54.7, 56.9 (C^{1}, C^{2}, C^{7}, C^{8}, C^{9}, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}), 74.4 (C^{3}), 123.0 (C^{6}), 140.1 (C^{5}), 171.0 ($C^{1'}$), 174.6 (C^{29});

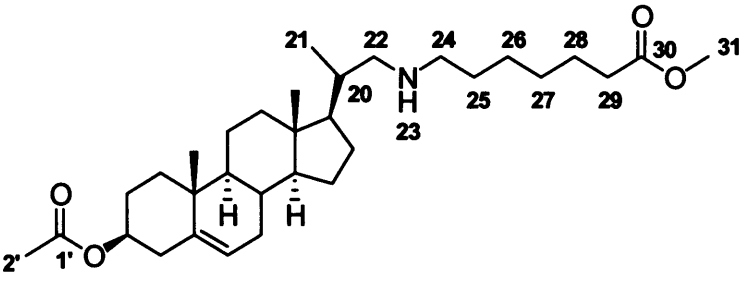
Mp = 75-76 °C; **R_f**= 0.26 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{31}H_{51}NO_4$•0.2H_2O: % Theory: C, 73.68; H, 10.25; N, 2.77; **% Found:** C, 73.61; H, 10.35; N, 2.77 ;

LRMS, m/z (ES^+ mode): 502.5 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{31}H_{52}NO_4$ ($[M+H]^+$):** 502.3896; **Found:** 502.3888 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl heptanoate) amine (20f)



$C_{32}H_{53}NO_4$
MW = 515.77 g.mol^{-1}

1- Following the **general procedure D** starting from triethylamine (170 µL, 1.22 mmol, 3.5 eq.), methyl 7-aminoheptanoate **3f** (206 mg, 1.05 mmol, 3.0 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (132 mg, 0.35 mmol, 1.0 eq.) and sodium cyanoborohydride (69 mg, 1.10 mmol, 3.1 eq.) in methanol (4 mL) with Molecular Sieves (3Å), compound **20f** was isolated as a white solid (62 mg, 34%).

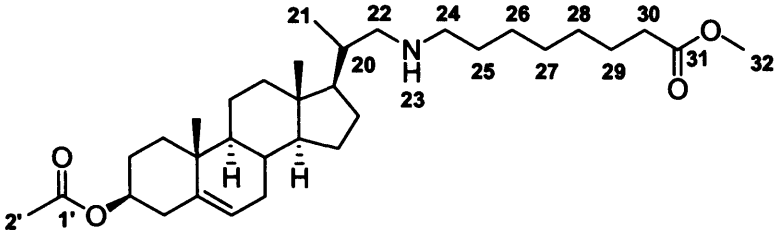
2- Following the **general procedure D** starting from triethylamine (42 µL, 0.30 mmol, 1.2 eq), methyl 7-aminoheptanoate **3f** (57 mg, 0.29 mmol, 1.1 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (95 mg, 0.26 mmol, 1.0 eq.) and

mL), compound **20f** was isolated as a white solid (77 mg, 51%).

4- Following the **general procedure D** starting from triethylamine (30 µ
0.22 mmol, 1.5 eq.), methyl 7-aminoheptanoate **3f** (38 mg, 0.19 mmol, 1.3 e
3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (55 mg, 0.15 mmol, 1.0 eq.) a
triacetoxy sodium borohydride (50 mg, 0.24 mmol, 1.6 eq.) in methanol (2n
for 24h, compound **20f** was isolated as a white solid (42 mg, 55%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.62 (s, 3H, H^{18}), 0.92 (d, 3H, H^{21}, J = 6.
0.95 (s, 3H, H^{19}), 1.96 (s, 3H, $H^{2'}$), 0.80 to 2.00 (m, 27H, H^1, H^2, H^7, H^8,
H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}), 2.22 (m, 5H, H^4, H^{29} a
H^{22}), 2.50 (m, 3H, H^{24} and $H^{22'}$), 3.60 (s, 3H, H^{31}), 4.53 (m, 1H, H^3), 5.30 (
1H, H^6);
^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 (C
34.4 (C^{29}), 36.7 (C^{20}), 37.0 (C^{10}), 38.5 (C^4), 42.9 (C^{13}), 50.5 (C^{24}), 51.9 (C
55.8 (C^{22}), 21.4, 24.8, 25.3, 27.5, 28.2, 28.4, 29.5, 30.1, 32.3, 32.3, 37.4, 4(
50.4, 54.7, 56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C
C^{28}), 74.4 (C^3), 123.0 (C^6), 140.1 (C^5), 171.0 ($C^{1'}$), 174.7 (C^{30});
Mp = 72-73 °C; **R_f** = 0.28 ($CHCl_3$/MeOH: 90/10);
Anal.Calcd. for $C_{32}H_{53}NO_4 \cdot 0.2H_2O$: % Theory: C, 74.00; H, 10.36; N, 2.
% Found: C, 74.06; H, 10.47; N, 2.72 ;
LRMS, m/z (ES^+ mode): 516.5 ($[M+H]^+$, 100%);
HRMS (ES^+ mode), **Calculated for $C_{32}H_{54}NO_4$ ($[M+H]^+$):** 516.4053; **Foun**
516.4064 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl octanoate) amine (20g)



$C_{33}H_{55}NO_4$
MW = 529.79 g.mol^{-1}

1- Following the **general procedure D** starting from triethylamine (103 µL, 0.74 mmol, 2.1 eq), methyl 8-aminooctanoate **3g** (152 mg, 0.73 mmol, 2.0 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (133 mg, 0.36 mmol, 1.0 eq.) and sodium cyanoborohydride (50 mg, 0.80 mmol, 2.2 eq.) in methanol (4 mL) for 48h, compound **20g** was isolated as a white solid (121 mg, 64%).

2- Following the **general procedure D** starting from triethylamine (50 µL, 0.36 mmol, 1.2 eq.), methyl 8-aminooctanoate **3g** (72 mg, 0.34 mmol, 1.1 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (110 mg, 0.30 mmol, 1.0 eq.) and sodium cyanoborohydride (0.30 mL, 0.30 mmol, 1.0 eq.) in methanol (2 mL), compound **20g** was isolated as a white solid (80 mg, 51%).

3- Following the **general procedure D** starting from triethylamine (24 µL, 0.17 mmol, 1.2 eq.), methyl 8-aminooctanoate **3g** (37 mg, 0.18 mmol, 1.3 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (51 mg, 0.14 mmol, 1.0 eq.) and triacetoxy sodium borohydride (50 mg, 0.24 mmol, 1.7 eq.) in methanol (1.5 mL) for 24h, compound **20g** was isolated as a white solid (51 mg, 71%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.75 (s, 3H, H^{18}), 1.04 (d, 3H, H^{21}, J = 6.3), 1.07 (s, 3H, H^{19}), 2.09 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 29H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}, H^{29}), 2.36 (m, 5H, H^4, H^{30} and H^{22}), 2.65 (m, 3H, H^{24} and $H^{22'}$), 3.72 (s, 3H, H^{32}), 4.65 (m, 1H, H^3), 5.43 (m, 1H, H^6);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 34.5 (C^{30}), 36.7 (C^{20}), 37.0 (C^{10}), 38.5 (C^{4}), 42.9 (C^{13}), 50.5 (C^{24}), 51.9 (C^{32}), 55.7 (C^{22}), 21.4, 24.8, 25.3, 27.6, 28.2, 28.4, 29.5, 29.6, 30.0, 32.3, 32.3, 37.4, 40.1, 50.4, 54.7, 56.9 (C^{1}, C^{2}, C^{7}, C^{8}, C^{9}, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}, C^{29}), 74.4 (C^{3}), 123.0 (C^{6}), 140.1 (C^{5}), 171.0 ($C^{1'}$), 174.8(C^{31});

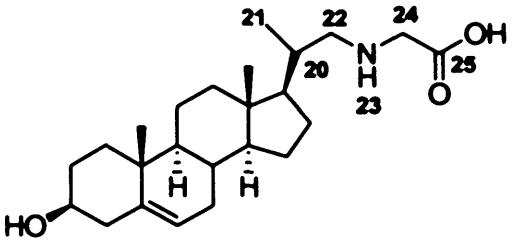
Mp = 67-68 °C; **R_f** = 0.33 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{33}H_{55}NO_4 \cdot 0.3H_2O$: % Theory: C, 74.06; H, 10.47; N, 2.62; **% Found:** C, 73.93; H, 10.49; N, 2.62 ;

LRMS, m/z (ES^+ mode): 530.5 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{33}H_{56}NO_4$ ($[M+H]^+$):** 530.4209; **Found:** 530.4193 .

3β-ol-23,24-bisnor-5-en-22-(ethanoic acid) amine (24a)



$C_{24}H_{39}NO_3$
MW = 389.57 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl ethanoate) amine **20a** (65 mg, 0.15 mmol, 1.0 eq.) and sodium hydroxide (1.6 mL, C = 1M, 1.6 mmol, 10.7 eq.) in THF (5 mL), compound **24a** was isolated as a white solid (48 mg, 84%).

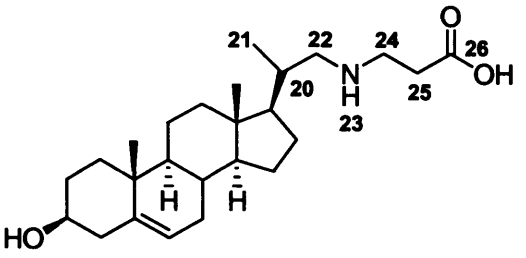
Mp = 213-215 °C; **R_f** = 0.04 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{24}H_{39}NO_3 \cdot 1.2H_2O$: % Theory: C, 70.10; H, 10.15; N, 3.41; **% Found:** C, 70.00; H, 10.27; N, 3.30 ;

LRMS, m/z (Cl^+ mode): 390.4 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{24}H_{40}NO_3$ ($[M+H]^+$):** 390.3003; **Found:** 390.3006 .

3β-ol-23,24-bisnor-5-en-22-(propanoic acid) amine (24b)

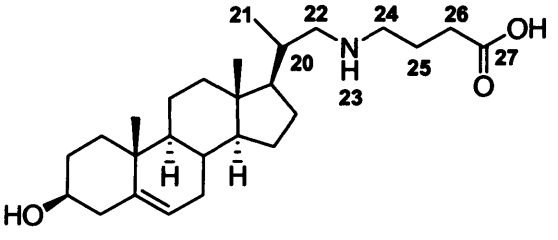


$C_{25}H_{41}NO_3$
MW = 403.60 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl propanoate) amine **20b** (72 mg, 0.16 mmol, 1.0 eq.) and sodium hydroxide (1.9 mL, C = 1M, 1.9 mmol, 11.9 eq.) in THF (5 mL), compound **24b** was isolated as a white solid (37 mg, 59%).

Mp = 246-247 °C; $\mathbf{R_f}$ = 0.04 ($CHCl_3$/MeOH: 90/10);
Anal.Calcd. for $C_{25}H_{41}NO_3$•2.7H_2O: % Theory: C, 66.40; H, 10.34; N, 3.10; **% Found:** C, 66.47; H, 10.14; N, 2.92 ;
LRMS, m/z (Cl^+ mode): 404.4 ($[M+H]^+$, 39%) ;
HRMS (ES^+ mode), Calculated for $C_{25}H_{42}NO_3$ ($[M+H]^+$): 404.3159; Found: 404.3157 .

3β-ol-23,24-bisnor-5-en-22-(butanoic acid) amine (24c)

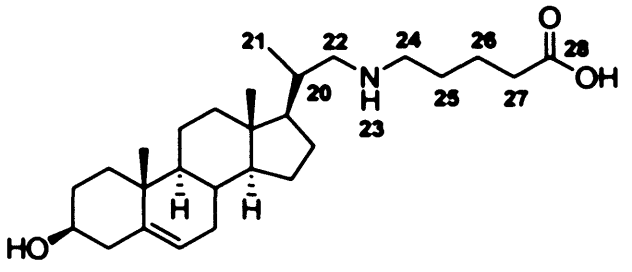


$C_{26}H_{43}NO_3$
MW = 417.62 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl butanoate) amine **20c** (56 mg, 0.12 mmol, 1.0 eq.) and sodium hydroxide (1.2 mL, C = 1M, 1.2 mmol, 10.0 eq.) in THF (4 mL), compound **24c** was isolated as a white solid (42 mg, 86%).

^{1}H NMR (300MHz, DMSO): δ 0.64 (s, 3H, H^{18}), 0.83 (d, 3H, H^{21}, J = 6.6), 0.92 (s, 3H, H^{19}), 0.80 to 2.20 (m, 23H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}), 2.55 (m, 2H, H^{26}), 2.83 (m, 1H, H^{22}), 3.03 (m, 2H, $^{22'}$H and ^{24}H), 3.40 (m, 2H, H^3 and $^{24'}$H), 5.23 (m, 1H, H^6), 4.67 (m, 1H, OH);
^{13}C NMR (75MHz, DMSO): δ 12.0 (C^{18}), 17.5 (C^{21}), 19.5 (C^{19}), 25.0 (C^{25}), 33.9 (C^{26}), 34.0 (C^{20}), 36.4 (C^{10}), 39.4 (C^4), 42.4 (C^{13}), 47.9 (C^{24}), 52.8 (C^{22}), 21.0, 24.7, 28.6, 31.7, 31.7, 31.8, 37.3, 42.5, 49.9, 53.4, 56.3, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 70.3 (C^3), 120.7 (C^6), 141.6 (C^5), 174.8 (C^{27});
Mp = 180-181 °C; **R_f** = 0.08 ($CHCl_3$/MeOH: 90/10);
Anal.Calcd. for $C_{26}H_{43}NO_3$•1.6HCl: % Theory: C, 65.61; H, 9.44; N, 2.94; **% Found:** C, 65.56; H, 9.36; N, 2.79 ;
LRMS, m/z (ES^+ mode): 418.4 ($[M+H]^+$, 100%), 440.4 ($[M+Na]^+$, 18%);
HRMS (ES^+ mode), **Calculated for $C_{26}H_{44}NO_3$ ($[M+H]^+$):** 418.3321; **Found:** 418.3326 .

3β-ol-23,24-bisnor-5-en-22-(pentanoic acid) amine (24d)



$C_{27}H_{45}NO_3$
MW = 431.65 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl pentanoate) amine **20d** (67 mg, 0.14 mmol, 1.0 eq.) and sodium hydroxide (1.4 mL, C = 1M, 1.4 mmol, 10.0 eq.) in THF (4 mL), compound **24d** was isolated as a white solid (49 mg, 83%).

^{1}H NMR (300MHz, DMSO): δ 0.66 (s, 3H, H^{18}), 0.79 (d, 3H, H^{21}, J = 6.5), 0.94 (s, 3H, H^{19}), 0.80 to 2.20 (m, 25H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}), 2.52 (m, 4H, H^{22} and H^{27}), 2.88 (m, 2H, H^{24}), 3.40 (m, 1H, H^3), 4.54 (d, 1H, OH, J = 4.3), 5.26 (m, 1H, H^6);

^{13}C NMR (75MHz, DMSO): δ 12.0 (C^{18}), 17.5 (C^{21}), 19.5 (C^{19}), 33.8 (C^{27}), 34.0 (C^{20}), 36.4 (C^{10}), 39.4 (C^{4}), 42.4 (C^{13}), 47.8 (C^{24}), 52.9 (C^{22}), 21.0, 24.3, 24.7, 27.0, 28.6, 31.7, 31.7, 31.8, 37.3, 42.5, 49.9, 53.4, 56.3, (C^{1}, C^{2}, C^{7}, C^{8}, C^{9}, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}), 70.3 (C^{3}), 120.8 (C^{6}), 141.5 (C^{5}), 174.7 (C^{28});

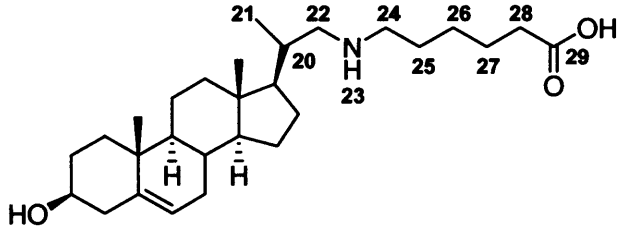
Mp = 215-217 °C; **R_f** = 0.06 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{27}H_{45}NO_3$•1.3HCl: % Theory: C, 67.69; H, 9.74; N, 2.92; **% Found:** C, 67.76; H, 9.51; N, 2.86 ;

LRMS, m/z (ES^+ mode): 432.4 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{27}H_{46}NO_3$ ($[M+H]^+$):** 432.3478; **Found:** 432.3481 .

3β-ol-23,24-bisnor-5-en-22-(hexanoic acid) amine (24e)



$C_{28}H_{47}NO_3$
MW = 445.68 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl hexanoate) amine **20e** (64 mg, 0.13 mmol, 1.0 eq.) and sodium hydroxide (1.3 mL, C = 1M, 1.3 mmol, 10.2 eq.) in THF (4 mL), compound **24e** was isolated as a white solid (49 mg, 86%).

^{1}H NMR (300MHz, DMSO): δ 0.65 (s, 3H, H^{18}), 0.90 (s, 3H, H^{19}), 1.00 (d, 3H, H^{21}, J = 6.5), 0.80 to 2.10 (m, 27H, H^{1}, H^{2}, H^{4}, H^{7}, H^{8}, H^{9}, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.18 (t, 2H, H^{28}, J = 7.2), 2.54 (m, 1H, H^{22}), 2.80 (m, 3H, $H^{22'}$ and H^{24}), 3.20 (m, 1H, H^{3}), 5.20 (m, 1H, H^{6}), 8.34 (m, 2H, H^{23} and OH), 12.02 (s, 1H, COOH);

^{13}C NMR (75MHz, DMSO): δ 12.0 (C^{18}), 17.5 (C^{21}), 19.5 (C^{19}), 33.8 (C^{28}), 34.0 (C^{20}), 36.4 (C^{10}), 39.4 (C^{4}), 42.4 (C^{13}), 47.8 (C^{24}), 52.8 (C^{22}), 21.0, 24.2,

24.6, 25.8, 27.5, 28.4, 31.7, 31.7, 31.8, 37.3, 42.5, 49.9, 53.4, 56.3, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}), 70.3 (C^3), 120.7 (C^6), 141.6 (C^5), 174.8 (C^{29});

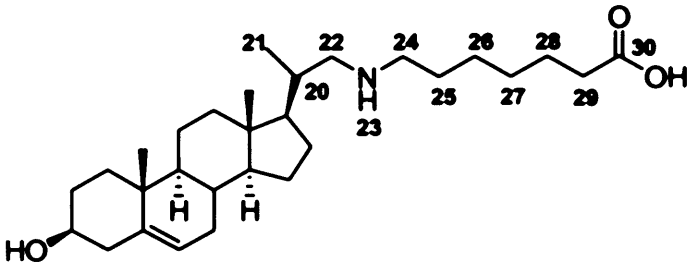
Mp = 218-220 °C; R_f = 0.04 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{28}H_{47}NO_3$•1.0HCl: % Theory: C, 69.75; H, 10.03; N, 2.91; **% Found:** C, 69.83; H, 10.19; N, 2.85 ;

LRMS, m/z (ES^+ mode): 446.5 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{28}H_{48}NO_3$ ($[M+H]^+$):** 446.3634; **Found:** 446.3633 .

3β-ol-23,24-bisnor-5-en-22-(heptanoic acid) amine (24f)



$C_{29}H_{49}NO_3$
MW = 459.70 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl heptanoate) amine **20f** (63 mg, 0.12 mmol, 1.0 eq.) and sodium hydroxide (1.4 mL, C = 1M, 1.4 mmol, 11.7 eq.) in THF (4 mL), compound **24f** was isolated as a white solid (48 mg, 86%).

^{1}H NMR (300MHz, DMSO): δ 0.66 (s, 3H, H^{18}), 0.93 (s, 3H, H^{19}), 1.02 (d, 3H, H^{21}, J = 6.3), 0.80 to 2.10 (m, 29H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}), 2.20 (t, 2H, H^{29}, J = 7.3), 2.59 (m, 1H, H^{22}), 2.83 (m, 3H, $H^{22'}$ and H^{24}), 3.25 (m, 1H, H^3), 5.25 (m, 1H, H^6), 8.40 (m, 2H, H^{23} and OH), 12.02 (s, 1H, COOH);

^{13}C NMR (75MHz, DMSO): δ 12.0 (C^{18}), 17.5 (C^{21}), 19.5 (C^{19}), 33.8 (C^{29}), 34.0 (C^{20}), 36.4 (C^{10}), 39.4 (C^4), 42.4 (C^{13}), 47.8 (C^{24}), 52.8 (C^{22}), 21.0, 24.2, 24.6, 25.3, 26.1, 27.6, 28.3, 31.7, 31.8, 31.8, 37.3, 42.5, 49.9, 53.4, 56.3, (C^1,

C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}), 70.3 (C^3), 120.7 (C^6), 141.6 (C^5), 174.8 (C^{30});

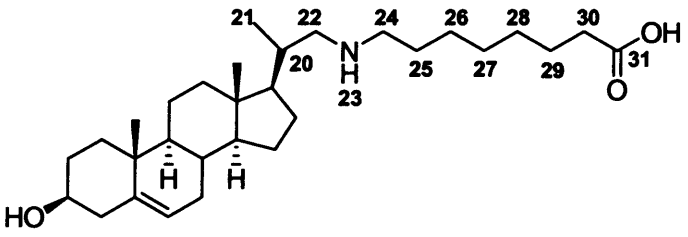
Mp = 257-259 °C; $\mathbf{R_f}$ = 0.04 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{29}H_{49}NO_3$•2.4HCl: % Theory: C, 63.65; H, 9.47; N, 2.56; **% Found:** C, 63.48; H, 9.46; N, 2.4 ;

LRMS, m/z (ES^+ mode): 460.5 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{29}H_{50}NO_3$ ($[M+H]^+$):** 460.3791; **Found:** 460.3796 .

3β-ol-23,24-bisnor-5-en-22-(octanoic acid) amine (24g)



$C_{30}H_{51}NO_3$
MW = 473.73 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-(methyl octanoate) amine **20g** (38 mg, 0.07 mmol, 1.0 eq.) and sodium hydroxide (1.0 mL, C = 1M, 1.0 mmol, 13.9 eq.) in THF (2 mL), compound **24g** was isolated as a white solid (34 mg, 100%).

^{1}H NMR (300MHz, DMSO): δ 0.68 (s, 3H, H^{18}), 0.95 (s, 3H, H^{19}), 1.03 (d, 3H, H^{21}, J = 6.2), 0.80 to 2.10 (m, 31H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}, H^{29}), 2.20 (t, 2H, H^{30}, J = 7.3), 2.60 (m, 1H, H^{22}), 2.86 (m, 3H, $H^{22'}$ and H^{24}), 3.29 (m, 1H, H^3), 4.62 (d, 1H, OH, J = 4.3), 5.27 (m, 1H, H^6), 8.32 (m, 1H, H^{23}), 12.03 (s, 1H, COOH);

^{13}C NMR (75MHz, DMSO): δ 12.0 (C^{18}), 17.5 (C^{21}), 19.5 (C^{19}), 34.0 (C^{20} and C^{30}), 36.4 (C^{10}), 39.4 (C^4), 42.4 (C^{13}), 47.9 (C^{24}), 52.8 (C^{22}), 21.0, 24.2, 24.7, 25.5, 26.2, 27.6, 28.6, 28.7, 31.7, 31.7, 31.8, 37.3, 42.5, 49.9, 53.4, 56.3, (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}, C^{29}), 70.3 (C^3), 120.7 (C^6), 141.6 (C^5), 174.8 (C^{31});

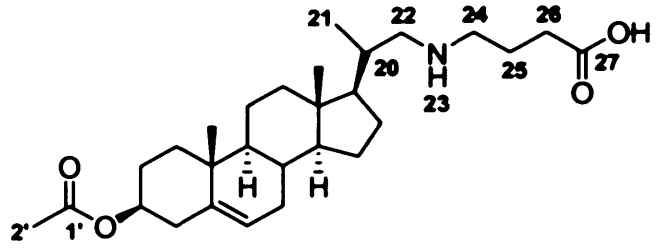
Mp = 208-210 °C; $\mathbf{R_f}$ = 0.05 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{30}H_{51}NO_3 \cdot 2.4HCl$: % Theory: C, 64.20; H, 9.59; N, 2.50; **% Found:** C, 64.20; H, 9.71; N, 2.39 ;

LRMS, m/z (ES^+ mode): 474.5 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{30}H_{52}NO_3$ ($[M+H]^+$):** 474.3947; **Found:** 474.3951 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(butanoic acid) amine (19c)



$C_{28}H_{45}NO_4$
MW = 459.66 g.mol^{-1}

Following the **general procedure D** starting from 4-aminobutanoic acid (38 mg, 0.37 mmol, 1.2 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (110 mg, 0.30 mmol, 1.0 eq.) and sodium cyanoborohydride (0.30 mL, 0.30 mmol, 1.0 eq.) in methanol (4 mL), compound **19c** was isolated as a white solid (85 mg, 63%).

^{1}H NMR (300MHz, MeOH): δ 0.84 (s, 3H, H^{18}), 1.11 (s, 3H, H^{19}), 1.19 (d, 3H, H^{21}, J = 6.5), 2.04 (s, 3H, $H^{2'}$), 1.0 to 2.10 (m, 21H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}), 2.38 (d, 2H, H^4, J= 7.1), 2.47 (t, 2H, H^{26}, J= 6.6), 2.80 (t, 1H, H^{22}, J= 11.0), 3.06 (m, 2H, $H^{22'}$ and H^{24}), 3.33 (m, 1H, $H^{24'}$), 4.60 (m, 1H, H^3), 5.44 (m, 1H, H^6);

^{13}C NMR (75MHz, MeOH): δ 12.6 (C^{18}), 17.9 (C^{21}), 20.1 (C^{19}), 22.5 ($C^{2'}$), 36.3 (C^{26}), 38.0 (C^{20}), 38.1 (C^{10}), 39.5 (C^4), 44.2 (C^{13}), 51.9 (C^{24}), 55.2 (C^{22}), 21.6, 23.5, 23.5, 29.2, 29.2, 33.3, 33.6, 38.6, 41.2, 50.7, 54.9, 58.2 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}), 75.8 (C^3), 123.9 (C^6), 141.4 (C^5), 172.8 ($C^{1'}$), 181.0 (C^{27});

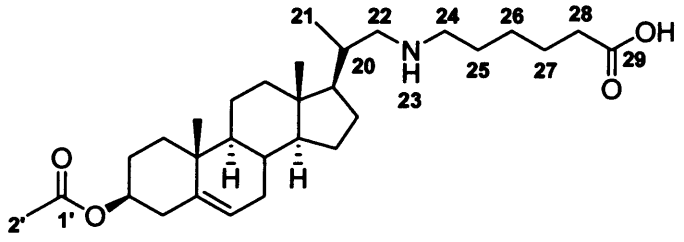
Mp = 160-161 °C; **R_f** = 0.14 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{28}H_{45}NO_4 \cdot 1.1H_2O$: % Theory: C, 70.14; H, 9.92; N, 2.92; **% Found:** C, 70.20; H, 9.83; N, 3.06 ;

LRMS, m/z (ES^+ mode): 482.3 ($[M+Na]^+$, 100%); 460.4 ($[M+H]^+$, 14%);

HRMS (ES^+ mode), **Calculated for $C_{28}H_{45}NO_4Na$ ($[M+Na]^+$)**: 482.3246; **Found:** 482.3249 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(hexanoic acid) amine (19e)



$C_{30}H_{49}NO_4$
MW = 487.71 g.mol^{-1}

Following the **general procedure D** starting from 6-aminohexanoic acid (75 mg, 0.57 mmol, 2.0 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (103 mg, 0.28 mmol, 1.0 eq.) and sodium cyanoborohydride (34 mg, 0.54 mmol, 1.9 eq.) in methanol (4 mL), compound **19e** was isolated as a white solid (116 mg, 86%).

^{1}H NMR (300MHz, MeOH): δ 0.85 (s, 3H, H^{18}), 1.12 (s, 3H, H^{19}), 1.17 (d, 3H, H^{21}, J = 6.5), 2.07 (s, 3H, $H^{2'}$), 0.9 to 2.10 (m, 25H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.28 (t, 2H, H^{28}, J= 7.1), 2.38 (d, 2H, H^4, J= 7.2), 2.79 (t, 1H, H^{22}, J= 11.4), 3.02 (m, 2H, $H^{22'}$ and H^{24}), 3.33 (m, 1H, $H^{24'}$), 4.55 (m, 1H, H^3), 5.45 (m, 1H, H^6);

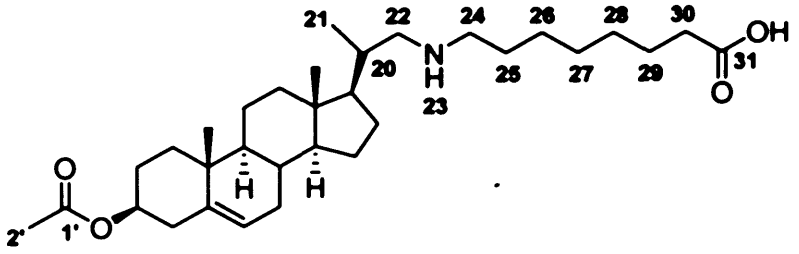
^{13}C NMR (75MHz, MeOH): δ 11.8 (C^{18}), 16.9 (C^{21}), 19.3 (C^{19}), 21.7 ($C^{2'}$), 35.4 (C^{28}), 37.3 (C^{20}), 37.8 (C^{10}), 38.7 (C^4), 43.4 (C^{13}), 51.0 (C^{24}), 54.5 (C^{22}), 20.8, 21.7, 25.8, 26.7, 28.3, 28.3, 32.5, 32.7, 32.7, 36.9, 40.1, 50.0, 53.6, 57.4 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}), 75.0 (C^3), 123.1 (C^6), 140.6 (C^5), 172.0 ($C^{1'}$), 180.3 (C^{29});

Mp = 176-178 °C; **R_f** = 0.14 ($CHCl_3$/MeOH: 90/10);

LRMS, m/z (ES^+ mode): 488.2 ($[M+H]^+$, 100%); 510.2 ($[M+Na]^+$, 68%);

HRMS (ES^+ mode), **Calculated for $C_{30}H_{50}NO_4$ ($[M+H]^+$)**: 488.3740; **Found:** 488.3753 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(octanoic acid) amine (19g)

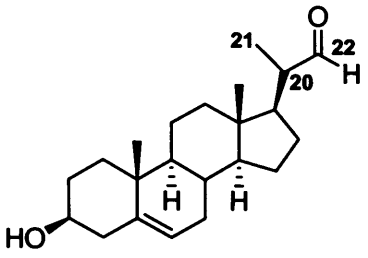


$C_{32}H_{53}NO_4$
MW = 515.77 g.mol^{-1}

Following the **general procedure D** starting from 8-aminooctanoic acid (46 mg, 0.29 mmol, 1.0 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (103 mg, 0.28 mmol, 1.0 eq.) and sodium cyanoborohydride (0.40 mL, 0.40 mmol, 1.4 eq.) in methanol (4 mL) for 48h, compound **19g** was isolated as a white solid (117 mg, 82%).

^{1}H NMR (300MHz, MeOH): δ 0.82 (s, 3H, H^{18}), 1.10 (s, 3H, H^{19}), 1.16 (d, 3H, H^{21}, J= 6.5), 2.06 (s, 3H, $H^{2'}$), 1.0 to 2.1 (m, 29H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}, H^{29}), 2.31 (t, 2H, H^{30}, J= 7.1), 2.34 (d, 2H, H^4, J= 6.9), 2.79 (t, 1H, H^{22}, J= 11.21), 3.05 (m, 2H, $H^{22'}$ and H^{24}), 3.31 (m, 1H, $H^{24'}$), 4.44 (m, 1H, H^3), 5.43 (m, 1H, H^6);
^{13}C NMR (75MHz, MeOH): δ 12.9 (C^{18}), 17.4 (C^{21}), 20.1 (C^{19}), 22.5 ($C^{2'}$), 36.0 (C^{30}), 38.1 (C^{10}), 38.1 (C^{20}), 38.6 (C^4), 43.8 (C^{13}), 49.9 (C^{24}), 51.8 (C^{22}), 21.6, 25.3, 25.3, 26.5, 27.4, 27.8, 29.2, 30.2, 30.3, 33.5, 33.5, 38.6, 39.5, 50.2, 55.9, 58.3 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{25}, C^{26}, C^{27}, C^{28}, C^{29}), 75.8 (C^3), 123.9 (C^6), 141.4 (C^5), 172.8 ($C^{1'}$), 179.0 (C^{31});
Mp = 129-131 °C; **R_f** = 0.26 ($CHCl_3$/MeOH: 90/10);
LRMS, m/z (ES^+ mode): 516.5 ($[M+H]^+$, 100%); 538.5 ($[M+Na]^+$, 78%);
HRMS (ES^+ mode), **Calculated for $C_{32}H_{54}NO_4$ ($[M+H]^+$)**: 516.4053; **Found**: 516.4048 .

3β-ol-23,24-bisnor-chol-5-en-22-al (25)



$C_{22}H_{34}O_2$
MW = 330.50 g.mol^{-1}

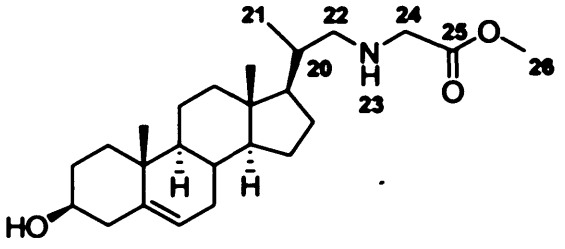
1- Following the **general procedure C** for 96h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (266 mg, 0.71 mmol, 1.0 eq.) and sodium hydroxide (3.5 mL, C = 2M, 7.0 mmol, 9.9 eq.) in THF (20 mL), compound **25** was isolated as a white solid (62 mg, 26%).

2- Following the **general procedure C** for 24h starting from 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (415 mg, 1.11 mmol, 1.0 eq.) and potassium carbonate (542 mg, 3.92 mmol, 3.5 eq.) in MeOH / H_2O (30 / 10 ml), compound **25** was isolated as a white solid (346 mg, 94%).

^{1}H NMR (300MHz, MeOD): δ 0.72 and 0.76 (2s, 3H, H^{18}), 1.02 and 1.04 (2s, 3H, H^{19}), 1.06 and 1.16(2d, 3H, H^{21}, J= 6.8 and J= 6.8), 2.31 (m, 3H, H^4 and H^{20}), 0.90 to 2.10 (m, 18H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}), 3.54 (m, 1H, H^3), 5.37 (m, 1H, H^6), 9.57 and 9.60 (2d, 1H, H^{22}, J= 5.0 J= 3.2);
^{13}C NMR (125MHz, MeOH): δ 12.2 and 12.8 (C^{18}), 13.5 and 13.6 (C^{21}), 19.4 and 19.4 (C^{19}), 36.5 and 36.6 (C^{10}), 42.1 and 42.3 (C^4), 43.0 (C^{13}), 48.8 (C^{20}), 20.8 and 21.0, 23.9 and 24.7, 26.5, 27.0, 31.7 and 31.8, 31.8 and 31.9, 37.3, 38.5 and 39.5, 49.5 and 50.1, 51.0 and 52.0, 56.0 and 56.2 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 71.8 (C^3), 121.4 and 121.5 (C^6), 140.8 and 140.9 (C^5), 205.1 and 205.8 (C^{22});
Mp = 152-153 °C; **R_f** = 0.77 ($CHCl_3$/MeOH: 90/10);
Anal.Calcd. for $C_{22}H_{34}O_2$*0.5HCl: % Theory: C, 75.77; H, 9.97 **% Found:** C, 75.62; H, 9.95 ;
LRMS, m/z (ES^+ mode): 331.4 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{37}H_{52}N_2O_6Na$ ($[M+Na]^+$)**: 331.3153; **Found**: 331.3159 .

3β-ol-23,24-bisnor-chol-5-en-22-(methyl ethanoate) amine (26a)



$C_{25}H_{41}NO_3$
MW = 403.60 g.mol^{-1}

Following the **general procedure D** starting from triethylamine (110 µL, 0.79 mmol, 3.2 eq), methyl 2-aminoethanoate **3a** (78 mg, 0.62 mmol, 2.5 eq.), 3β-ol-23,24-bisnor-chol-5-en-22-al **25** (82 mg, 0.25 mmol, 1.0 eq.) and sodium cyanoborohydride (0.5 mL, 0.5 mmol, 2.0 eq.) in methanol (6 mL), compound **26a** was obtained pure (48 mg, 48%).

^{1}H NMR (500MHz, MeOH): δ 0.77 (s, 3H, H^{18}), 0.97 (d, 3H, H^{21}, J = 6.6), 1.05 (s, 3H, H^{19}), 0.90 to 2.10 (m, 18H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}), 2.25 (d, 2H, H^4, J= 5.0), 2.34 (m, 1H, H^{20}), 2.63 (dd, 1H, H^{22}, J= 2.8; J= 11.4), 2.86 (dd, 1H, $H^{22'}$, J= 3.1; J= 11.3), 3.40 (m, 3H, H^3 and H^{24}), 3.74 (s, 3H, H^{26}), 5.36 (m, 1H, H^6);

^{13}C NMR (125MHz, MeOH, 25°C): δ 12.4 and 12.6 (C^{18}), 17.8 and 18.2 (C^{21}), 19.9 (C^{19}), 37.7 (C^{10}), 43.1 (C^4), 43.5 and 43.7 (C^{13}), 51.3 and 51.3 (C^{24}), 52.2 and 52.3 (C^{26}), 55.4 and 56.1 (C^{22}), 22.2, 25.2 and 25.4, 28.7, 29.0, 33.0 and 33.0, 33.3 and 33.3, 36.8, 38.6, 40.8 and 41.1, 51.8, 55.5 and 55.7, 58.1 and 58.1 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}), 72.5 (C^3), 122.4 (C^6), 142.3 (C^5), 173.9 and 173.9 (C^{25});

^{13}C NMR (125MHz, MeOH, 40°C): δ 12.4 and 12.7 (C^{18}), 17.8 and 18.2 (C^{21}), 19.9 (C^{19}), 37.7 (C^{10}), 43.1 (C^4), 43.5 and 43.7 (C^{13}), 51.4 and 51.5 (C^{24}), 51.8 (C^{26}), 55.7 and 56.2 (C^{22}), 22.2, 25.2 and 25.4, 28.6, 29.0, 33.0 and 33.0, 33.3 and 33.3, 36.8, 38.6, 40.8 and 41.1, 51.8, 55.6 and 55.6, 58.1 and 58.1 (C^1,

C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}), 72.5 (C^3), 122.4 (C^6), 142.4 (C^5), 174.1 (C^{25});

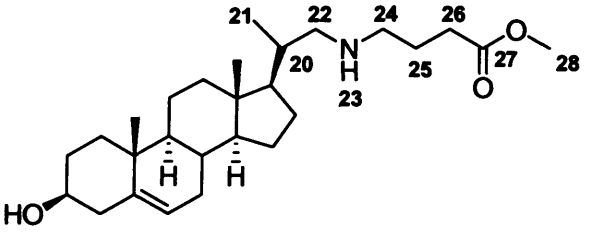
R_f = 0.51 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{25}H_{41}NO_3 \cdot 0.6H_2O$: % Theory: C, 72.46; H, 10.26; N, 3.38; **% Found:** C, 72.53; H, 10.13; N, 3.34 ;

LRMS, m/z (ES^+ mode): 404.3 ($[M+H]^+$, 100%)

HRMS (ES^+ mode), **Calculated for $C_{25}H_{42}NO_3$ ($[M+H]^+$):** 404.3165; **Found:** 404.3163 .

3β-ol-23,24-bisnor-chol-5-en-22-(methyl butanoate) amine (26c)



$C_{27}H_{45}NO_3$
MW = 431.65 g.mol^{-1}

Following the **general procedure D** starting from triethylamine (120 µL, 0.86 mmol, 2.3 eq.), methyl 4-aminobutanoate **3c** (111 mg, 0.72 mmol, 2.0 eq.), 3β-ol-23,24-bisnor-chol-5-en-22-al **25** (123 mg, 0.37 mmol, 1.0 eq.) and sodium cyanoborohydride (0.6 mL, 0.60 mmol, 1.6 eq.) in methanol (8 mL), compound **26c** was obtained pure (99 mg, 61%) .

^{1}H NMR (500MHz, MeOH): δ 0.65 (s, 3H, H^{18}), 0.87 (d, 3H, H^{21}, J = 6.5), 0.90 (s, 3H, H^{19}), 0.90 to 2.20 (m, 23H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}), 2.34 (t, 2H, H^{26}, J= 7.1), 2.51 (t, 1H, H^{22}, J= 11.1), 2.81 (m, 3H, $H^{22'}$ and H^{24}), 3.27 (m, 1H, H^3), 3.56 (s, 3H, H^{28}), 5.22 (m, 1H, H^6);

^{13}C NMR (125MHz, MeOH): δ 12.3 and 12.6 (C^{18}), 17.2 and 17.6 (C^{21}), 19.9 (C^{19}), 32.3 (C^{26}), 37.7 (C^{10}), 43.0 (C^4), 43.4 and 43.8 (C^{13}), 49.3 (C^{24}), 52.3 (C^{28}), 54.6 and 55.2 (C^{22}), 22.1, 23.1 and 23.3, 25.1 and 25.4, 28.6, 28.9, 32.9 and 33.0, 33.3 and 33.3, 35.0 and 36.2, 38.6, 40.7 and 41.0, 51.6 and 52.3, 55.2

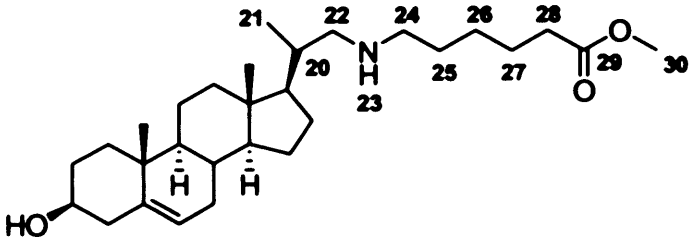
and 55.6, 57.9 and 58.0 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}, C^{25}), 72.4 (C^3), 122.3 (C^6), 142.3 and 142.3 (C^5), 175.0 and 175.0 (C^{27});

R_f = 0.35 ($CHCl_3$/MeOH: 90/10);

LRMS, m/z (ES^+ mode): 432.4 ($[M+H]^+$, 100%)

HRMS (ES^+ mode), **Calculated for $C_{27}H_{46}NO_3$ ($[M+H]^+$)**: 432.3478; **Found:** 432.3470 .

3β-ol-23,24-bisnor-chol-5-en-22-(methyl hexanoate) amine (26e)



$C_{29}H_{49}NO_3$
MW = 459.70 g.mol^{-1}

Following the **general procedure D** starting from triethylamine (150 µL, 1.08 mmol, 2.5 eq.), methyl 6-aminohexanoate **3e** (151 mg, 0.83 mmol, 1.9 eq.), 3β-ol-23,24-bisnor-chol-5-en-22-al **25** (147 mg, 0.44 mmol, 1.0 eq.) and sodium cyanoborohydride (0.6 mL, 0.60 mmol, 1.4 eq.) in methanol (9 mL), compound **26e** was obtained pure (144 mg, 71%).

^{1}H NMR (500MHz, MeOH): δ 0.65 (s, 3H, H^{18}), 0.88 (d, 3H, H^{21}, J = 6.6), 0.90 (s, 3H, H^{19}), 0.80 to 2.20 (m, 27H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.24 (t, 2H, H^{28}, J= 7.3), 2.55 (t, 1H, H^{22}, J= 11.4), 2.83 (m, 3H, $H^{22'}$ and H^{24}), 3.27 (m, 1H, H^3), 3.53 (s, 3H, H^{30}), 5.22 (m, 1H, H^6);

^{13}C NMR (125MHz, MeOH): δ 12.3 and 12.6 (C^{18}), 17.2 and 17.5 (C^{21}), 19.9 (C^{19}), 34.4 and 34.8 (C^{28}), 37.7 (C^{10}), 43.1 (C^4), 43.4 and 43.8 (C^{13}), 49.7 (C^{24}), 52.1 (C^{30}), 54.3 and 55.0 (C^{22}), 22.2, 25.0 and 25.4, 25.4 and 25.5, 27.2 and 27.2, 27.2 and 27.3, 28.6, 28.9, 32.9 and 33.0, 33.2 and 33.3, 36.0, 38.6, 40.7 and 41.0, 51.6 and 52.1, 55.1 and 55.6, 57.9 and 58.0 (C^1, C^2, C^7, C^8, C^9, C^{11},

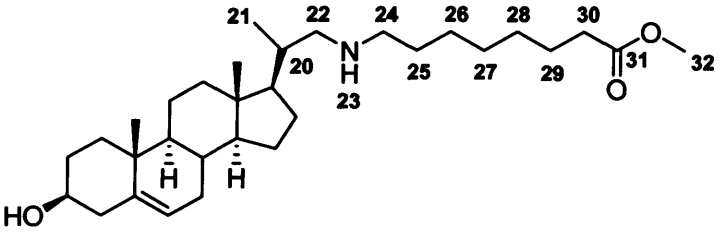
C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}, C^{25}, C^{26}, C^{27}), 72.4 (C^3), 122.4 (C^6), 142.3 (C^5), 175.7 (C^{29});

Mp = 135-136 °C; $\mathbf{R_f}$ = 0.43 ($CHCl_3$/MeOH: 90/10);

LRMS, m/z (ES^+ mode): 460.4 ($[M+H]^+$, 100%) ;

HRMS (ES^+ mode), **Calculated for $C_{29}H_{50}NO_3$ ($[M+H]^+$):** 460.3791; **Found:** 460.3799 .

3β-ol-23,24-bisnor-chol-5-en-22-(methyl octanoate) amine (26g)



$C_{31}H_{53}NO_3$
MW = 487.76 g.mol^{-1}

Following the **general procedure D** starting from triethylamine (140 µL, 1.01 mmol, 2.4 eq.), methyl 8-aminooctanoate **3g** (154 mg, 0.73 mmol, 1.7 eq.), 3β-ol-23,24-bisnor-chol-5-en-22-al **25** (138 mg, 0.42 mmol, 1.0 eq.) and sodium cyanoborohydride (0.6 mL, 0.60 mmol, 1.4 eq.) in methanol (9 mL), compound **26g** was obtained pure (136 mg, 67%).

^{1}H NMR (500MHz, MeOH): δ 0.65 (s, 3H, H^{18}), 0.88 (d, 3H, H^{21}, J = 6.6), 0.90 (s, 3H, H^{19}), 0.80 to 2.10 (m, 31H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}, H^{28}, H^{29}), 2.20 (t, 2H, H^{30}, J= 7.3), 2.55 (t, 1H, H^{22}, J= 11.1), 2.82 (m, 3H, $H^{22'}$ and H^{24}), 3.26 (m, 1H, H^3), 3.52 (s, 3H, H^{32}), 5.21 (m, 1H, H^6);

^{13}C NMR (125MHz, MeOH): δ 12.3 and 12.6 (C^{18}), 17.1 and 17.5 (C^{21}), 19.9 (C^{19}), 34.7 and 34.8 (C^{30}), 37.7 (C^{10}), 43.0 (C^4), 43.4 and 43.8 (C^{13}), 49.8 (C^{24}), 52.0 (C^{32}), 54.3 and 55.0 (C^{22}), 22.2 and 22.2, 25.0 and 25.3, 25.3 and 25.9, 27.4 and 27.5, 27.5, 28.6, 28.9, 29.9 and 29.9, 29.9, 32.9 and 33.0, 33.2 and 33.3, 36.0, 38.6, 40.7 and 41.0, 51.6 and 52.0, 55.0 and 55.6, 57.9 and 57.9 (C^1,

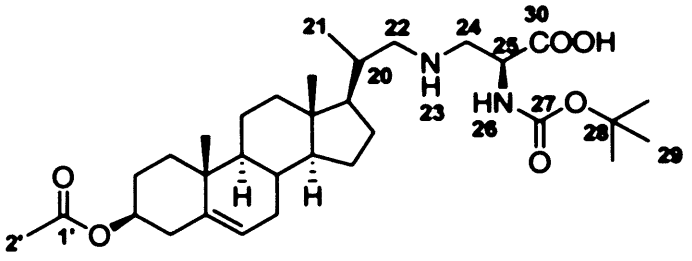
C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}, C^{25}, C^{26}, C^{27}, C^{28}, C^{29}), 72.4 (C^3), 122.4 (C^6), 142.3 and 142.3 (C^5), 176.0 (C^{31});

Mp = 143-144 °C; $\mathbf{R_f}$ = 0.43 ($CHCl_3$/MeOH: 90/10);

LRMS, m/z (ES^+ mode): 488.4 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{31}H_{54}NO_3$ ($[M+H]^+$)**: 488.4104; **Found:** 488.4109 .

3β-acetoxy-23,24-bisnor-5-en-22-(3-(2-Boc-amino)propanoic acid) amine (27)



$C_{32}H_{52}N_2O_6$
MW = 560.77 g.mol^{-1}

Following the **general procedure D** starting from Boc-Dap-OH (104 mg, 0.51 mmol, 1.3 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (146 mg, 0.39 mmol, 1.0 eq.) and sodium cyanoborohydride (0.5 mL, 0.5 mmol, 1.3 eq.) in methanol (6 mL) for 24h, compound **27** was isolated as a white solid (170 mg, 77%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.77 (s, 3H, H^{18}), 1.07 (s, 3H, H^{19}), 1.10 (d, 3H, H^{21}, J= 6.5), 1.49 (s, 9H, H^{30}) , 2.09 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 18H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}), 2.37 (d, 2H, H^4, J= 6.8), 2.80 (m, 1H, H^{22}), 3.16 (m, 1H, H^{24}), 3.33 (m, 1H, $H^{22'}$), 3.46 (m, 1H, $H^{24'}$), 4.13 (m, 1H, H^{25}), 4.66 (m, 1H, H^3), 5.43 (m, 1H, H^6), 6.13 (d, 1H, H^{26}, J= 12.6);

^{13}C NMR (75MHz, $CDCl_3$): δ 12.3 (C^{18}), 17.5 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 28.8 (C^{30}), 34.5 (C^{20}), 37.0 (C^{10}), 38.5 (C^4), 42.6 (C^{13}), 50.7 (C^{24}), 53.2 (C^{25}), 56.8 (C^{22}), 21.4, 24.7, 28.1, 28.3, 32.2, 33.4, 37.4, 40.0, 50.2, 53.9, 56.8 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.3 (C^3), 80.3 (C^{29}), 122.9 (C^6), 140.1 (C^5), 157.0 (C^{27}), 171.0 ($C^{1'}$), 173.4 (C^{31});

Mp = 171-173 °C; $\mathbf{R_f}$ = 0.39 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{32}H_{52}N_2O_6 \cdot 1.1H_2O$: % Theory: C, 66.20; H, 9.41; N, 4.83;

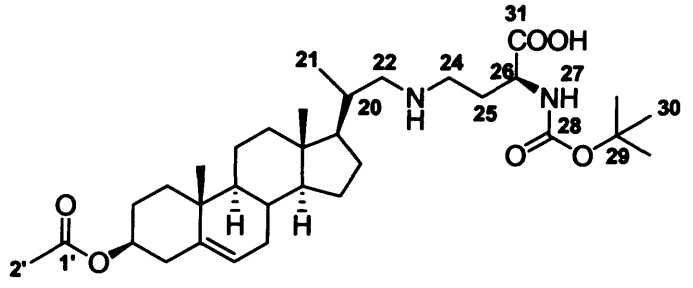
% Found: C, 66.25; H, 9.20; N, 4.77 ;

LRMS, m/z (ES^+ mode): 583.3 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{32}H_{52}N_2O_6Na$ ($[M+Na]^+$):** 583.3723;

Found: 583.3717 .

3β-acetoxy-23,24-bisnor-5-en-22-(4-(3-Boc-amino)butanoic acid) amine (28)



$C_{33}H_{54}N_2O_6$
MW = 574.79 g.mol^{-1}

Following the **general procedure D** starting from Boc-Dab-OH (138 mg, 0.63 mmol, 1.2 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (204 mg, 0.55 mmol, 1.0 eq.) and sodium cyanoborohydride (0.6 mL, 0.6 mmol, 1.1 eq.) in methanol (10 mL), compound **28** was isolated as a white solid (253 mg, 80%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.77 (s, 3H, H^{18}), 1.07 (s, 3H, H^{19}), 1.14 (d, 3H, H^{21}, J= 6.6), 1.49 (s, 9H, H^{30}) , 2.10 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 21H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}), 2.38 (d, 2H, H^4), 2.58 (m, 1H, H^{22}), 2.90 (m, 1H, H^{24}), 3.10 (m, 1H, $H^{22'}$), 3.21 (m, 1H, $H^{24'}$), 3.90 (m, 1H, H^{26}), 4.65 (m, 1H, H^3), 5.41 (m, 1H, H^6), 6. 80 (m, 1H, H^{27});

^{13}C NMR (75MHz, $CDCl_3$): δ 12.3 (C^{18}), 17.6 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 28.8 (C^{30}), 33.5 (C^{25}), 34.9 (C^{20}), 37.0 (C^{10}), 38.5 (C^4), 42.6 (C^{13}), 50.3 (C^{24}), 54.2 (C^{26}), 56.9 (C^{22}), 21.3, 24.7, 27.8, 28.1, 32.1, 32.2, 37.4, 39.9, 50.2, 53.8, 56.8 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 74.3 (C^3), 80.1 (C^{29}), 122.8 (C^6), 140.1 (C^5), 157.1 (C^{28}), 171.0 ($C^{1'}$), 176.6(C^{31});

Mp = 168-170 °C; R_f = 0.38 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{33}H_{54}N_2O_6 \cdot 1.3H_2O$: % Theory: C, 66.26; H, 9.54 N, 4.68;

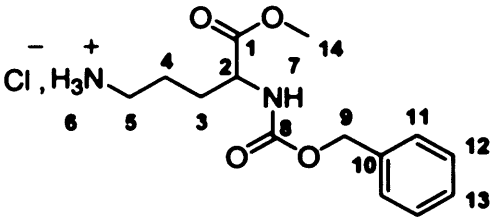
% Found: C, 66.30; H, 9.22; N, 4.62 ;

LRMS, m/z (ES^+ mode): 597.3 ($[M+Na]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{33}H_{54}N_2O_6Na$ ($[M+Na]^+$):** 597.3880;

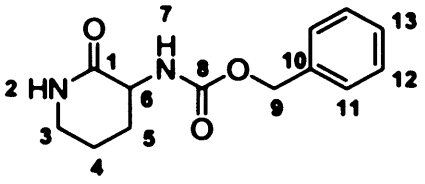
Found: 597.3867 .

Z-Orn-OMe (29)



$C_{14}H_{21}N_2O_4Cl$
MW = 316.78

1- Following the **general procedure A** starting from thionyl chloride (1.0 mL, 13.71 mmol, 3.3 eq.) and Z-Orn-OH (1.098 g, 4.13 mmol, 1.0 eq.) in methanol (6 mL, 152.44 mmol, 36.9 eq.). The crude was purified by column chromatography ($CHCl_3$/MeOH/TEA: 89/10/1) and the byproduct **30** was obtained and identified (389 mg, 38%).



$C_{13}H_{16}N_2O_3$
MW = 248.28

^{1}H NMR (300MHz, MeOH): δ 1.90 (m, 3H, H^4 and H^5), 2.17 (m, 1H, $H^{5'}$), 3.32 (m, 2H, H^3), 4.10 (m, 1H, H^6), 5.11 (s, 2H, H^9), 7.40 (m, 5H, H^{11}, H^{12} and H^{13});

^{13}C NMR (75MHz, MeOH): δ 22.8 (C^4), 29.5 (C^5), 43.2 (C^3), 52.8 (C^6), 68.0 (C^9), 129.3, 129.4 and 129.9 (C^{11}, C^{12} and C^{13}), 138.7 (C^{10}), 159.0 (C^8), 173.7 (C^1);

R_f = 0.59 (CH_3Cl/MeOH/TEA: 89/10/1);

LRMS, m/z (ES+ mode): 519.0 ($[2M+Na]^+$, 100%), 271.1 ($[M+Na]^+$, 40%).

2- Following the **general procedure A** starting from thionyl chloride (1.0 mL, 13.71 mmol, 3.5 eq.) and Z-Orn-OH (1.036 g, 3.89 mmol, 1.0 eq.) in methanol (10 mL, 254.07 mmol, 65.3 eq.). The crude was purified by column chromatography ($CHCl_3$/MeOH: 90/10) and afford the product **29** pure (920 mg, 75%).

^{1}H NMR (300MHz, MeOH): δ 1.74 (m, 3H, H^3 and H^4), 1.94 (m, 1H, $H^{3'}$), 2.96 (s, 3H, H^{14}), 4.22 (dd, 1H, H^2, J= 4.9, J= 8.1), 5.11 (s, 2H, H^9), 7.36 (m, 5H, H^{11}, H^{12} and H^{13});

^{13}C NMR (75MHz, MeOH): δ 25.5 (C^3), 29.9 (C^4), 40.6 (C^5), 53.2 (C^{14}), 55.3 (C^2), 68.2 (C^9), 129.2 and 129.9 (C^{11} and C^{13}), 129.5 (C^{12}), 138.((C^{10}), 159.& (C^8), 174.3 (C^1);

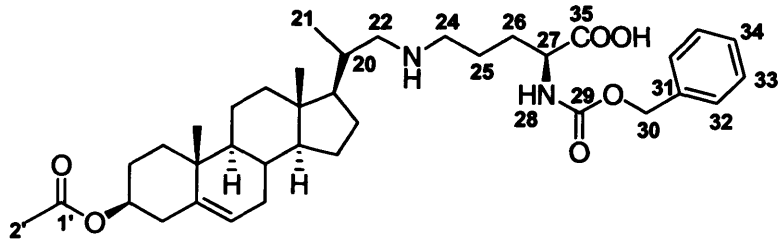
Mp = 139-141°C; **R_f** = 0.15 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{14}H_{21}N_2O_4Cl$•0.3H_2O: % Theory: C, 52.19; H, 6.76; N, 8.69; Cl, 11.00 **% Found:** C, 52.23; H, 6.66; N, 8.63; Cl, 10.92 ;

LRMS, m/z (ES+ mode): 281.3 ($[M-Cl]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{14}H_{21}N_2O_4$ ($[M-Cl]^+$):** 281.1501; Found: 281.1512 .

3β-acetoxy-23,24-bisnor-5-en-22-(5-(2-Z-amino)pentanoic acid) amine (31)



$C_{37}H_{54}N_2O_6$
MW = 622.83 g.mol^{-1}

Following the **general procedure D** starting from Z-Orn-OH (184 mg, 0.69 mmol, 1.4 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (190 mg, 0.51 mmol, 1.0 eq.) and sodium cyanoborohydride (0.63 mL, 0.63 mmol, 1.2 eq.) in methanol (4 mL), compound **31** was isolated as a white solid (182 mg, 57%).

^{1}H NMR (300MHz, $CDCl_3$): δ 0.72 (s, 3H, H^{18}), 1.06 (s, 3H, H^{19}), 2.09 (s, 3H, $H^{2'}$), 0.80 to 2.10 (m, 26H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{21}, H^{25}, H^{26}), 2.37 (d, 2H, H^4, J = 6.9), 2.54 (m, 1H, H^{22}), 2.83 (m, 2H, H^{24} and $H^{22'}$), 3.16 (m, 1H, $H^{24'}$), 4.23 (m, 1H, H^{27}), 4.64 (m, 1H, H^3), 5.15 (m, 2H, H^{30}), 5.42 (m, 1H, H^6), 7.32 (m, 5H, H^{32}, H^{33}, H^{34});

^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 17.8 (C^{21}), 19.7 (C^{19}), 21.8 ($C^{2'}$), 37.0 (C^{10}), 38.5 (C^4), 43.0 (C^{13}), 53.3 (C^{24}), 55.7 (C^{22}), 56.8 (C^{27}), 21.4, 24.3, 24.6, 27.9, 28.2, 30.1, 32.2, 32.2, 32.9, 37.4, 39.9, 50.3, 54.4, 56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}, C^{25}, C^{26}), 66.9 (C^{30}), 74.3 (C^3), 122.8 (C^6), 127.7, 128.5, 128.9 (C^{32}, C^{33}, C^{34}), 137.0 (C^{31}), 140.1 (C^5), 155.9 (C^{29}), 171.0 ($C^{1'}$), 175.9(C^{35});

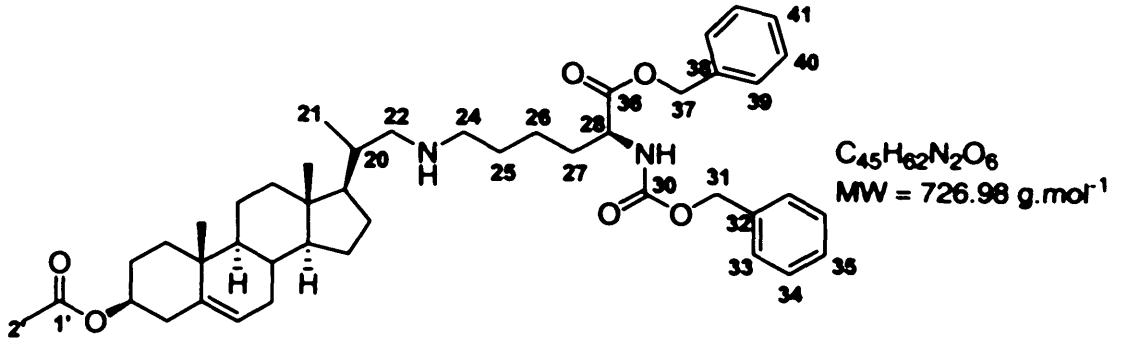
Mp = 181-182 °C; **R_f** = 0.40 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{37}H_{54}N_2O_6 \cdot 1.8H_2O$: % Theory: C, 67.82; H, 8.86; N, 4.28; **% Found:** C, 67.48; H, 8.46; N, 4.29 ;

LRMS, m/z (ES^+ mode): 645.3 ($[M+Na]^+$, 100%), 623.3 ($[M+H]^+$, 59%);

HRMS (ES^+ mode), **Calculated for $C_{37}H_{54}N_2O_6Na$ ($[M+Na]^+$):** 645.3880; **Found:** 645.3887 .

3β-acetoxy-23,24-bisnor-5-en-22-(6-(5-Z-amino)benzyl hexanoate) amine (33)



$C_{45}H_{62}N_2O_6$
MW = 726.98 g.mol^{-1}

Following the **general procedure D** starting from triethylamine (140 µL, 1.01 mmol, 1.7 eq), Z-Lys-OBzl (436 mg, 0.82 mmol, 1.4 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (215 mg, 0.58 mmol, 1.0 eq.) and sodium

cyanoborohydride (0.80 mL, 0.80 mmol, 1.4 eq.) in methanol (10 mL), compounds **33** (97 mg, 23%) and **34** (34 mg, 09%) were isolated as two oils.

^{1}H NMR (300MHz, $CDCl_3$): δ 0.75 (s, 3H, H^{18}), 1.05 (d, 3H, H^{21}, J = 6.6), 1.07 (s, 3H, H^{19}),2.09 (s, 3H, $H^{2'}$), 0.90 to 2.10 (m, 25H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.37 (m, 3H, H^4 and H^{22}), 2.61 (m, 3H, $H^{22'}$ and H^{24}), 4.47 (m, 1H, H^{28}), 4.66 (m, 1H, H^3), 5.16 (s, 2H, H^{31}), 5.22 (d, 2H, H^{37}, J = 4.9), 5.42 (m, 1H, H^6), 5.47 (m, 1H, H^{23}), 7.40 (m, 10H, H^{33}, H^{34}, H^{35}, H^{39}, H^{40}, H^{41});

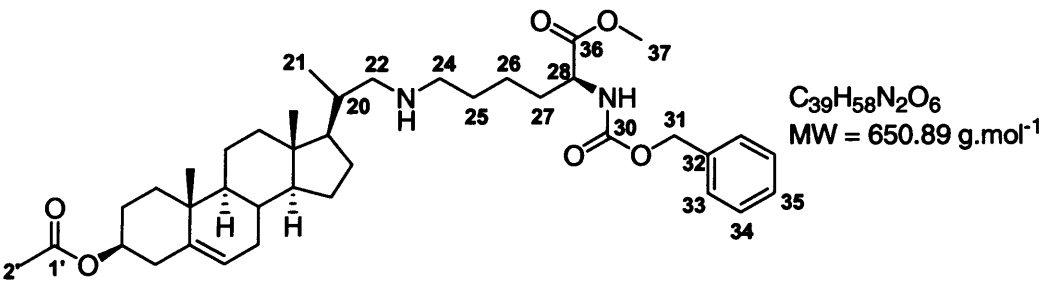
^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 37.0 (C^{10}), 38.5 (C^4), 42.9 (C^{13}), 50.0 (C^{24}), 54.6 (C^{28}), 55.7 (C^{22}), 21.4, 23.3, 24.8, 28.2, 28.4, 29.3, 32.3, 32.3, 32.9, 36.6, 37.4, 40.1, 50.4, 54.3, 56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}, C^{25}, C^{26}, C^{27}), 67.4 (C^{31}), 67.5 (C^{37}), 74.4 (C^3), 123.0 (C^6), 128.5, 128.6, 128.7, 128.9, 129.0, 129.0 (C^{33}, C^{34}, C^{35}, C^{39}, C^{40}, C^{41}), 135.7 (C^{38}), 136.7 (C^{32}), 140.1 (C^5), 156.3 (C^{30}), 171.0 ($C^{1'}$), 172.7(C^{36});

R_f = 0.45 ($CHCl_3$/MeOH: 90/10);

LRMS, m/z (ES^+ mode): 727.5 ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{45}H_{63}N_2O_6$ ($[M+H]^+$):** 727.4681; **Found:** 727.4684 .

3β-acetoxy-23,24-bisnor-5-en-22-(6-(5-Z-amino)methyl hexanoate) amine (34)



$C_{39}H_{58}N_2O_6$
MW = 650.89 g.mol^{-1}

^{1}H NMR (300MHz, $CDCl_3$): δ 0.74 (s, 3H, H^{18}), 1.03 (d, 3H, H^{21}, J= 6.4),1.07 (s, 3H, H^{19}),2.08 (s, 3H, $H^{2'}$), 0.90 to 2.20 (m, 25H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.32 (m, 3H, H^4 and H^{22}), 2.62 (m, 3H, $H^{22'}$ and H^{24}), 3.79 (s, 3H, H^{37}), 4.42 (m, 1H, H^{28}), 4.65 (m, 1H, H^3), 5.16 (s, 2H, H^{31}), 5.38 (m, 1H, H^{23}), 5.42 (m, 1H, H^6), 7.40 (s, 5H, H^{33}, H^{34}, H^{35});

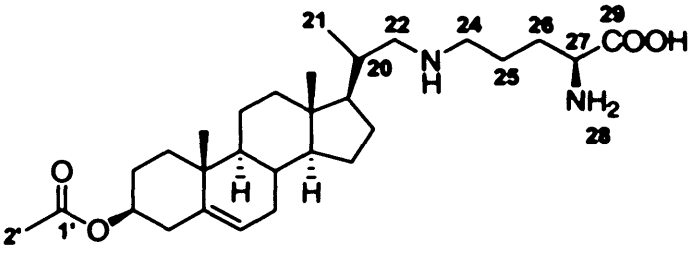
^{13}C NMR (75MHz, $CDCl_3$): δ 12.4 (C^{18}), 18.2 (C^{21}), 19.7 (C^{19}), 21.9 ($C^{2'}$), 37.0 (C^{10}), 38.5 (C^4), 42.9 (C^{13}), 50.4 (C^{24}), 52.8 (C^{37}), 54.7 (C^{28}), 55.9 (C^{22}), 21.4, 23.4, 24.8, 28.2, 28.4, 30.0, 32.3, 32.3, 33.0, 36.9, 37.4, 40.1, 50.3, 54.3, 56.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}, C^{25}, C^{26}, C^{27}), 67.4 (C^{31}), 74.4 (C^3), 123.0 (C^6), 128.5, 129.0, 129.0 (C^{33}, C^{34}, C^{35}), 136.7 (C^{32}), 140.1 (C^5), 156.3 (C^{30}), 171.0 ($C^{1'}$), 173.4 (C^{36});

R_f = 0.36 ($CHCl_3$/MeOH: 90/10);

LRMS, m/z (ES^+ mode): 651.5 ($[M+H]^+$, 100%);

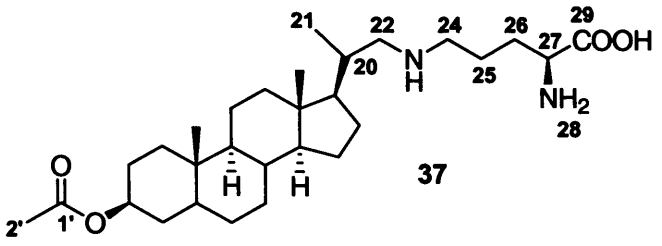
HRMS (ES^+ mode), **Calculated for $C_{39}H_{59}N_2O_6$ ($[M+H]^+$):** 651.4368; **Found:** 651.4367 .

Attempted synthesis of 3β-acetoxy-23,24-bisnor-5-en-22-(5-(2-amino)pentanoic acid) amine (36)



$C_{29}H_{48}N_2O_4$
MW = 488.70 g.mol^{-1}

1- 3β-acetoxy-23,24-bisnor-5-en-22-(5-(4-Z-amino) pentanoic acid) amine **31** (61 mg, 0.10 mmol, 1.0 eq.) and Pd/C 10% (426 mg) were dissolved in DCM (10mL). The resulting solution was put under hydrogen and stirred overnight. The solution was then filtered on celite with MeOH (150 mL). A mixture (39 mg) of the compound **36** and the byproduct **37** was recovered.



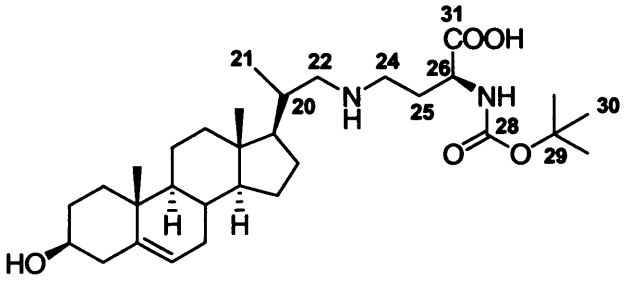
37

$C_{29}H_{50}N_2O_4$
MW = 490.72 g.mol^{-1}

LRMS, m/z (ES^+ mode): 489.4 ($[M+H]^+$, 100%) and 491.4 ($[M+H]^+$, 84%); **HRMS** (ES^+ mode), **Calculated for $C_{29}H_{49}N_2O_4$ ($[M+H]^+$):** 489.3692; **Found:** 489.3695 .

2- 3β-acetoxy-23,24-bisnor-5-en-22-(5-(4-Z-amino) pentanoic acid) amine **31** (74 mg, 0.12 mmol, 1.0 eq.) and Pd/C 5% (200 mg) were dissolved in DCM (10mL). The resulting solution was put under hydrogen and stirred 7h. The solution was then filtered on celite with MeOH (150 mL). A mixture (23 mg) of the compound **36** and starting material **31** was recovered which could not be purified by column chromatography.

3β-ol-23,24-bisnor-5-en-22-(4-(2-Boc-amino)butanoic acid) amine (38)



$C_{31}H_{52}N_2O_5$
MW = 532.75 g.mol^{-1}

Following the **general procedure C** for 48h starting from 3β-acetoxy-23,24-bisnor-5-en-22-(5-(2-Boc-amino) butanoic acid) amine **28** (98 mg, 0.17 mmol, 1.0 eq.) and potassium carbonate (82 mg, 0.59 mmol, 3.5 eq.) in MeOH/H_2O (6 mL / 2 mL), compound **38** was isolated as a white solid (64 mg, 70%).

^{1}H NMR (500MHz, MeOH): δ 0.80 (s, 3H, H^{18}), 1.05 (s, 3H, H^{19}), 1.06 (d, 3H, H^{21}, J = 7.4), 1.48 (s, 9H, H^{30}), 0.90 to 2.30 (m, 23H, H^1, H^2, H^4, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}), 2.75 (m, 1H, H^{22}), 3.12 (m, 3H, $H^{22'}$ and H^{24}), 3.41 (m, 1H, H^3), 3.99 (m, 1H, H^{26}), 5.37 (m, 1H, H^6);

^{13}C NMR (125MHz, MeOH): δ 12.3 and 12.6 (C^{18}), 17.2 and 17.6 (C^{21}), 19.9 (C^{19}), 28.7 (C^{30}), 31.3 (C^{25}), 37.7 (C^{10}), 43.0 (C^4), 43.4 and 43.8 (C^{13}), 47.3 and 47.7 (C^{24}), 54.0 (C^{26}), 54.8 (C^{22}), 22.2 and 22.2, 25.0 and 25.3, 28.4, 28.8, 32.3 and 32.9, 33.2 and 33.3, 35.9, 38.6, 40.5 and 40.9, 51.6 and 51.6, 55.0 and 55.3, 57.9 and 57.9 (C^1, C^2; C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}), 72.4 (C^3), 80.8 (C^{29}), 122.3 and 122.3 (C^6), 142.3 and 142.3 (C^5), 158.1 (C^{28}), 175.5 and 177.1 (C^{31});

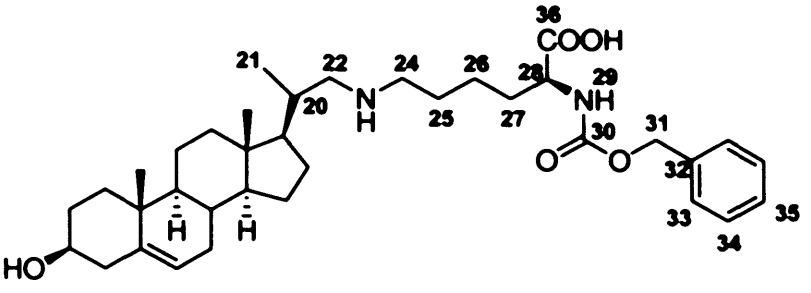
Mp = 243-244 °C; **R_f** = 0.29 ($CHCl_3$/MeOH: 90/10);

Anal.Calcd. for $C_{31}H_{52}N_2O_5$•1.1HCl: % Theory: C, 65.00; H, 9.34; N, 4.89; **% Found:** C, 65.05; H, 9.38; N, 4.62 ;

LRMS, m/z (ES^+ mode): 555.4 ($[M+Na]^+$, 100%), 533.5 ($[M+H]^+$, 22%);

HRMS (ES^+ mode), **Calculated for $C_{31}H_{52}N_2O_5Na$ ($[M+Na]^+$):** 555.3774; **Found:** 555.3782 .

3β-ol-23,24-bisnor-5-en-22-(6-(2-Z-amino)hexanoic acid) amine (39)



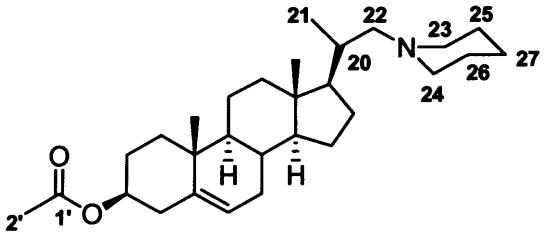
$C_{36}H_{54}N_2O_5$
MW = 594.82 g.mol^{-1}

Following the **general procedure C** for 48h starting from a mixture 3β-acetoxy-23,24-bisnor-chol-5-en-22-(6-(5-Z-amino)benzyl hexanoate) amine **33** and 3β-acetoxy-23,24-bisnor-chol-5-en-22-(6-(5-Z-amino)methyl hexanoate) amine **34** (286 mg, 0.12 mmol, 1.0 eq.), and sodium hydroxide ((5.1 mL, C =

1M, 5.1 mmol) in THF (15 mL), compound **39** was isolated as a white solid (141 mg, 23%, two steps).

^{1}H NMR (500MHz, MeOH): δ 0.69 (s, 3H, H^{18}), 0.95 (s, 3H, H^{19}), 1.03 (d, 3H, H^{21}, J= 6.4), 0.8 to 2.00 (m, 25H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.15 (m, 2H, H^4), 2.62 (t, 1H, H^{22}, J= 11.1), 2.88 (m, 3H, $H^{22'}$ and H^{24}), 3.30 (m, 1H, H^3), 3.95 (t, 1H, H^{28}, J= 5.9), 4.99 (s, 2H, H^{31}), 5.26 (m, 1H, H^6), 7.26 (m, 5H, H^{33}, H^{34} and H^{35});
^{13}C NMR (125MHz, MeOH): δ 12.3 and 12.6 (C^{18}), 17.0 and 17.4 (C^{21}), 19.9 (C^{19}), 35.7 (C^{10}), 43.0 (C^4 and C^{13}), 49.3 (C^{24}), 54.0 and 54.7 (C^{22}), 58.0 (C^{28}), 67.5 (C^{31}), 22.2 , 23.5, 25.0 and 25.3, 26.5, 28.5, 28.8, 32.3 and 32.9, 32.9 and 33.3, 33.3, 35.8, 38.5, 40.9, 51.6, 54.9 and 55.5, 57.0 and 57.9 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}, C^{20}, C^{25}, C^{26}, C^{27}), 72.4 (C^3), 122.3 (C^6), 128.8 (C^{33}), 129.0 (C^{35}), 129.5 (C^{34}), 138.4 (C^5), 142.3 (C^{32}), 158.1 (C^{30}), 178.0 (C^{36});
Mp = 217-218 °C; **R_f** = 0.09 ($CHCl_3$/MeOH: 90/10);
LRMS, m/z (ES^+ mode): 595.4 ($[M+H]^+$, 100%), 617.4 ($[M+Na]^+$, 88%);
HRMS (ES^+ mode), **Calculated for $C_{36}H_{55}N_2O_5$ ($[M+H]^+$):** 595.4111;
Found: 595.4109 .

3β-acetoxy-23,24-bisnor-chol-5-en-22-(piperidyl) amine (40)



$C_{29}H_{47}NO_2$
MW = 441.69 g.mol^{-1}

1- Following the **general procedure D** starting from triethylamine (0.28 mL, 2.01 mmol, 1.8 eq), piperidine (0.16 mL, 1.61 mmol, 1.5 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (407 mg, 1.09 mmol, 1.0 eq.) and sodium

cyanoborohydride (1.6 mL, 1.60 mmol, 1.5 eq.) in methanol (15 mL) for 24h, compound **40** was isolated as a white solid (182 mg, 38%).

2- Following the **general procedure D** starting from piperidine (0.15 mL, 1.51 mmol, 1.6 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (358 mg, 0.96 mmol, 1.0 eq.) and sodium cyanoborohydride (1.4 mL, 1.40 mmol, 1.5 eq.) in methanol (15 mL), for 24h, compound **40** was isolated as a white solid (100 mg, 24%).

3- Following the **general procedure D** starting from acetic acid (0.10 mL, 1.74 mmol, 1.4 eq), piperidine (0.2 mL, 2.01 mmol, 1.6 eq.), 3β-acetoxy-23,24-bisnor-chol-5-en-22-al **23** (450 mg, 1.21 mmol, 1.0 eq.) and sodium cyanoborohydride (1.8 mL, 1.80 mmol, 1.5 eq.) in methanol (15 mL) overnight, compound **40** was isolated as a white solid (282 mg, 53%).

^{1}H NMR (500MHz, $CDCl_3$): δ 0.64 (s, 3H, H^{18}), 0.95 (s, 3H, H^{19}), 1.00 (d, 3H, H^{21}, J = 6.3), 1.97 (s, 3H, $H^{2'}$), 0.70 to 2.10 (m, 25H, H^1, H^2, H^7, H^8, H^9, H^{11}, H^{12}, H^{14}, H^{15}, H^{16}, H^{17}, H^{20}, H^{25}, H^{26}, H^{27}), 2.29 (m, 6H, H^4, H^{23} and H^{24}), 2.53 (m, 2H, H^{22}), 4.52 (m, 1H, H^3), 5.30 (m, 1H, H^6).

^{13}C NMR (125MHz, $CDCl_3$): δ 11.9 (C^{18}), 18.1 (C^{21}), 19.3 (C^{19}), 21.4 ($C^{2'}$), 25.0 (C^{27}), 33.8 (C^{20}), 36.6 (C^{10}), 38.1 (C^4), 42.7 (C^{13}), 55.0 (C^{23} and C^{24}), 21.0, 24.4, 27.5, 27.8, 31.9, 31.9, 37.0, 39.6, 50.0, 56.2, 56.5 (C^1, C^2, C^7, C^8, C^9, C^{11}, C^{12}, C^{14}, C^{15}, C^{16}, C^{17}), 65.0 (C^{22}), 74.0 (C^3), 122.5 (C^6), 139.7 (C^5), 170.5 ($C^{1'}$).

Mp = 164-165 °C; **R_f** = 0.37 ($CHCl_3$/MeOH: 90/10);

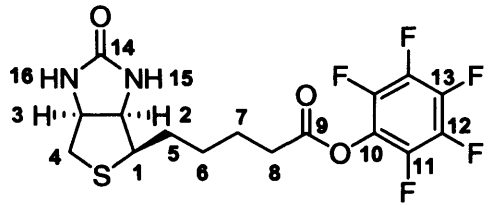
Anal.Calcd. for $C_{29}H_{47}NO_2 \cdot 0.8H_2O$: % Theory: C, 76.37; H, 10.74; N, 3.07; **% Found:** C, 76.37; H, 10.70; N, 3.14 ;

LRMS, m/z (Cl^+ mode): ($[M+H]^+$, 100%);

HRMS (ES^+ mode), **Calculated for $C_{29}H_{48}NO_2$ ($[M+H]^+$)**: 442.3680; **Found**: 442.3677 .

IX.5 Proteomic approach (Chapter VII)

9-(pentafluorophenyl) ester biotin (41)



$C_{16}H_{15}F_5N_2O_3S$
MW = 410.36 g.mol^{-1}

1- Biotin (0.5 g, 2.05 mmol, 1.0 eq.), PFP (427 mg, 2.32 mmol, 1.1 eq.) and EDC (1.179 g, 6.15 mmol, 3.0 eq.) were solubilised in dry DMF (22 mL) and the resulting solution was stirred at room temperature overnight. The solution was then poured into water (20 mL) and extracted with chloroform (40 mL). The chloroform layer was washed four times with water (20 mL), dried with magnesium sulfate, filtered and the solvents removed from the filtrate under reduced pressure. The crude was purified by column chromatography over silica gel using $CHCl_3$/MeOH (100%:0% → 80%:20%). The solvents were removed from the collected fractions under reduced pressure to yield compound **41** as a silvery/white solid (308mg, 37%).

2- Biotin (1.020 g, 4.17 mmol, 1.0 eq.), PFP (837 mg, 4.55 mmol, 1.1 eq.) and DCC (2.510 g, 12.20 mmol, 3.0 eq.) were solubilised in dry DMF (40 mL) and the resulting solution was stirred at room temperature for three hours. The solution was then poured into water (50 mL) and extracted with chloroform (60 mL). The chloroform layer was washed three times with water (50, 100 and 100 mL), dried with magnesium sulfate, filtered and the solvents removed from the filtrate under reduced pressure. The crude product was purified by column chromatography over silica gel using $CHCl_3$/MeOH (100%:0% → 80%:20%). The solvents were removed from the collected fractions under reduced pressure to yield **41** as a white solid (1.052g, 61%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.63 (quint, 2H, H^6, J = 7.8), 1.81 (m, 2H, H^5), 1.90 (quint, 2H, H^7, J = 7.0), 2.77 (t, 2H, H^8, J = 7.3), 2.78 (dd, 1H, H^{4a}, ^2J = 11.3, ^3J = 16.6), 3.01 (dd, 1H, H^{4b}, ^2J = 12.8, ^3J= 5.0), 3.24 (m, 1H, H^1), 4.41 (m, 1H, H^2), 4.59 (m, 1H, H^3), 4.86 (s, 1H, H^{16}), 5.25 (s, 1H, H^{15});

^{13}C NMR (75MHz, $CDCl_3$): δ 25.0, 28.6, 30.1 (C^5, C^6, C^7), 33.4 (C^8), 41.0 (C^4), 55.6 (C^1), 60.5, 62.4 (C^2, C^3), 114.1 (C^{10}), 139.8, 139.9, 140.3 (C^{11}, C^{12}, C^{13}), 163.4 (C^{14}), 169.8 (C^9);

^{19}F NMR (282MHZ, $CDCl_3$): δ 153.2, 158.3, 162.6 (F^{11}, F^{12}, F^{13});

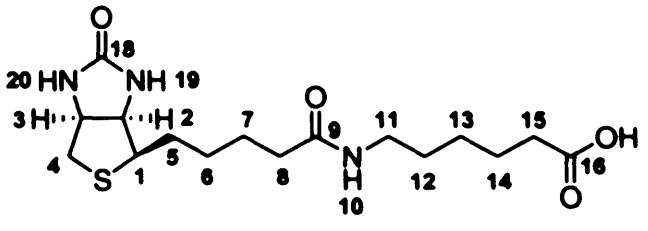
Mp = 184-186 °C; **R_f** = 0.38 ($CHCl_3$/MeOH: 90/10);

Anal. Calcd. for $C_{16}H_{15}F_5N_2O_3S$: % Theory: C, 46.83; H, 3.68; N, 6.83; S, 7.81; **% Found:** C, 47.00; H, 3.87; N, 6.65; S, 7.89 ;

LRMS, m/z (ES^+ mode): 433.0 ([M+Na^+], 100%);

HRMS (ES^+ mode), **Calculated for $C_{16}H_{15}F_5N_2O_3SNa$ ([M+Na^+]):** 433.0621; **Found:** 433.0611 .

9-(6-hexanoic acid) amide biotin (42)



$C_{16}H_{27}N_3O_4S$
MW = 357.47 g.mol^{-1}

Et_3N (0.24 mL, 1.75 mmol, 7.0 eq.) was added to a solution of **41** (103 mg, 0.25 mmol, 1.0 eq.) and 6-aminocaproic acid (98 mg, 0.75 mmol, 3.0 eq.) in dry DMF (5 mL). The mixture was left stirring at room temperature overnight. The solution was extracted using a mixture of water (20 mL) and hydrochloric acid (1.5 mL) and chloroform (40 mL). The water layer was placed under reduced pressure to yield a white solid. Water (20 mL) was added to the crude product and a solid was recovered by filtration. The solid was dried under reduced pressure to yield **42** as a white solid (47 mg, 52%).

^{1}H NMR (300 MHz, DMSO): δ 1.2 to 1.8 (m, 12H, H^5, H^6, H^7, H^{12}, H^{13}, H^{14}), 2.05 (t, 2H, H^8, J = 7.3), 2.19 (t, 2H, H^{15}, J = 7.4), 2.54 (dd, 1H, H^{4a}, ^2J = 12.5), 2.83 (dd, ^{1}H, H^{4b}, ^2J = 12.4, ^3J = 5.1), 3.01 (q, 2H, H^{11}, J = 6.5), 3.11 (m, 1H, H^1), 4.14 (m, 1H, H^3), 4.31 (m, 1H, H^2) 6.38, 6.44 (2s, 2H, H^{19}, H^{20}), 7.76 (t, 1H, H^{10}, J = 5.4);

^{13}C NMR (75MHz, DMSO): δ 24.6, 25.7, 26.3, 28.4, 28.6, 29.3 (C^5, C^6, C^7, C^{12}, C^{13}, C^{14}), 34.0 (C^8), 35.6 (C^{15}), 38.6 (C^4), 40.2 (C^{11}), 55.8 (C^1), 59.5 (C^2), 61.4 (C^3), 163.0 (C^{18}), 172.1 (C^9), 174.8 (C^{16});

Mp = 205-206 °C; **R*f*:** 0.08 in $CHCl_3$:MeOH (90:10);

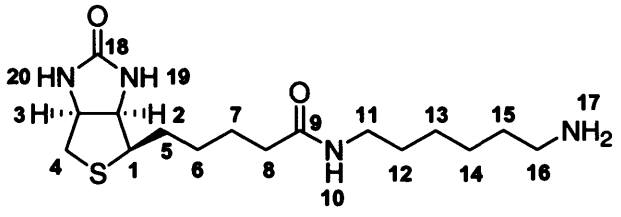
Anal. Calc. For $C_{16}H_{27}N_3O_4S$•0.9HCl: C 49.24, H 7.21, N 10.77, S 8.22; **Found:** C 49.38, H 7.13, N 10.70, S 8.26 ;

LRMS, m/z (ES^+ mode): 380.2 ([M+Na^+], 100%], 402.2 ([M-H+2Na^+], 16%];

HRMS (ES^+ mode) **Calculated for $C_{16}H_{27}N_3O_4SNa$ ([M+Na^+])**: 380.1620;

Found: 380.1628 .

9-(6-aminohexyl) amide biotin (44)



$C_{16}H_{30}N_4O_2S$
MW = 342.50 g.mol^{-1}

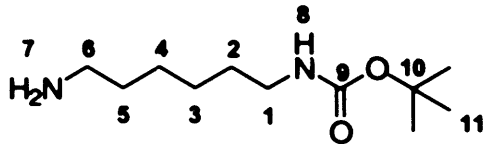
1- In flask **(A)**, 9-(pentafluorophenyl) ester biotin **41** (99 mg, 0.24 mmol, 1.0 eq.) was solubilised in dry DMF (3 mL). In flask **(B)**, hexamethylenediamine (87 mg, 0.75 mmol, 3.1 eq.) was solubilised in dry DMF (2 mL). Triethylamine (0.12 mL, 0.875 mmol, 3.6 eq.) was then added to flask **(B)** and the resulting solution was stirred at room temperature for 30 minutes. The content of flask (B) was then added drop wise to flask **(A)** and the resulting solution was stirred at room temperature overnight. The solution was extracted with water (20 mL) and chloroform (30 mL). The water layer was placed under reduced pressure. The crude was purified by column chromatography over silica gel using $MeOH/CHCl_3/NH_4OH$ (10%:90%:1% → 30%:70%:1%). The solvents were

removed from the collected fractions under reduced pressure to yield **44** as a white solid (4mg, 5%).

LRMS (ES^+ mode): m/z = 343.1 [$(M+H^+)$, 37%]

2- In flask **(A)**, 9-(pentafluorophenyl) ester biotin **41** (316 mg, 0.77 mmol, 1.0 .eq) was solubalised in dry DMF (25 mL). In flask **(B)**, hexamethylenediamine (281 mg, 2.87 mmol, 3.1 eq.) was solubalised in dry DMF (10 mL). Triethylamine (0.39 mL, 2.77 mmol, 3.6 eq.) was then added to flask **(B)** and the resulting solution was stirred at room temperature for 30 minutes. The content of flak **(B)** was then added drop wise to flak **(A)** and the resulting solution was stirred at room temperature overnight. The following morning the solution was poured into water (80 mL) and extracted with chloroform (70 mL). The water layer was washed once with chloroform (50 mL), filtered and the solvent removed from the filtrate under reduced pressure to yield **44** crude as a white solid (429 mg, 162%). The crude was directly used in the next step.

6-(aminohexyl)*tert*-butanoate ester amine (47)



$C_{11}H_{24}N_2O_2$
MW = 216.32 g.mol^{-1}

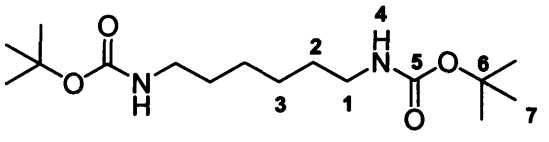
1- In flask **(A)**, hexamethylenediamine (215 mg, 1.85 mmol, 1.0 eq.) was solubilised in dry DCM (2 mL). In flak **(B)**, $(Boc)_2O$ (340 mg, 1.56 mmol, 0.84 eq.) was solubilised in dry DCM (3 mL). The content of flask **(B)** was then added drop wise to flask **(A)** and the resulting solution was stirred at room temperature overnight. The following morning the solution was poured into 20 mL of water/ice and extracted with ether (15 mL). The ether layer was washed twice with water (15 mL) followed by saturated solution of sodium chloride (15 mL). The ether layer was then dried with magnesium sulfate, filtered and the solvents removed from the filtrate under reduced pressure. The crude was

purified by column chromatography over silica gel using $CHCl_3$/MeOH (100%:0%→99%:1%) followed by $CHCl_3$/MeOH/NH_4OH (82%:15%:3%). The solvents were removed from the collected fractions under reduced pressure to yield **47** as a white solid (14 mg, 4%) and 1,6-di-*tert*-butanoate ester-1,6-diamino hexane **48** (88 mg, 18%).

2- In flask **(A),** hexamethylenediamine (429 mg, 3.69 mmol, 1.0 eq.) was solubilised in dry DCM (4 mL). In flak **(B),** $(Boc)_2O$ (172 mg, 0.79 mmol, 0.2 eq.) was solubilised in dry DCM (6 mL). The content of flask **(B)** was then added drop wise to flask **(A)** and the resulting solution was stirred at room temperature overnight. The following morning MeOH (10 mL) was added. Excess Boc and DCM were removed from the solution under reduced pressure. The crude product was purified by column chromatography over silica gel using $CHCl_3$/MeOH (100%:0%→97%:3%) followed by $CHCl_3$/MeOH/NH_4OH (82%:15%:3%). The solvents were removed from the collected fractions under reduced pressure to yield **47** as a white solid (77 mg, 45%).

^{1}H NMR (300 MHz, MeOH): δ 1.4 to 1.6 (m, 17H, H^2, H^3, H^4, H^5, H^{11}), 2.72 (t, 2H, H^1, J = 7.1), 3.11 (t, 2H, H6, J = 6.9);
^{13}C NMR (75MHz, MeOH): δ 30.6, 30.6, 33.9, 36.6 (C^2, C^3, C^4, C^5), 31.8 (C^{11}), 44.2 (C^6), 45.4 (C^1), 82.7 (C^{10}), 161.5 (C^9);
R*f* = 0.10 ($CHCl_3$/MeOH: 90:10);
LRMS, m/z (ES^+ mode): 217.1 ([M+H^+], 100%), 433.0 ([2M+H^+], 13%).

1,6-di-*tert*-butanoate ester-1,6-diamino hexane (48)



$C_{16}H_{32}N_2O_4$
MW = 316.44 g.mol^{-1}

^{1}H NMR (300 MHz,$CDCl_3$): δ 1.39 (m, 4H, H^3), 1.50 (m, 22H, H^2 and H^7), 3.14 (m, 4H, H^1), 4.57 (m, 2H, H^4);

^{13}C NMR (75MHz, $CDCl_3$): δ 26.8 (C^3), 28.9 (C^7), 30.4 (C^2), 40.8 (C^1), 79.5 (C^6), 156.4 (C^5);

LRMS, m/z (ES^+ mode): 339.1 ([M+Na^+), 100%), 654.9 ([2M+Na^+), 67%).

CHAPTER X

BIOLOGICAL EXPERIMENTAL

X.1 Materials and methods for the Northern blotting of the *T.b. brucei* SMT mRNA

X.1.1 Materials

Buffer / Solution	**Composition**
SSC 20x	NaCl 3M, sodium citrate 0.3 M (pH 7.0).
Hybridization solution	Formamide 50%, Denhardt solution 5x, SSC 5x, Herring sperm 1%, EDTA 5 mM (pH 8.0).
Denhardt solution 50x	Ficoll 400 10g/l, polyvinilpyrrolidone 10g/l, whey bovine albumin 10g/l. Sterilized for filtration.
Washing solution for DNA or RNA filtration	SSC 2x, SDS 0.1% (p/v).
Solution for the deshybridization of the filter of *nylon* with RNA.	Tris-HCl (pH 8.0) 5 mM, EDTA 2 mM, Denhardt solution 0.1x.
MOPS 10x. Solution for the electrophorase of RNA	MOPS 0.2 M, pH 5.5-7, anhydrous sodium acetate 0.05 M, EDTA 0.01 M. Sterilized for filtration.
Loading buffer for RNA	Formamide 48 % (v/v), MOPS 1x, formaldehyde (37 %) 6.41 % (v/v), EtBr 0.7 mg/ml, blue of de bromophenol saturated 5.3 % (p/v), sterile glycerol 5.3 %.

Table X.1: Solutions used for the mRNA isolation and the Northern blot

X.1.2 Methods

X.1.2.1 Culture and cell manipulation

Freeze and thaw of the parasites: the cells were stocked at -80°C in liquid nitrogen in an aliquot of 1mL culture with 10% (v/v) glycerol. 2 mL tubes were

used for freezing from *Sarstedt*. The freezing took place when the culture of the parasite was in the logarithmic phase of growth ($1x10^6$ parasites/mL for the bsf, $5x10^6$ parasites/mL for the procyclic form). The defreezing was achieved at 37°C; the aliquot were centrifuged at 2500x g for 10 minutes, in order to remove glycerol. The cells were then resuspended in 2-5 mL of the appropriate culture solution.

***T.b. brucei* procyclic form culture (29-13):** the procyclic form was cultivated at 28°C in a SDM-79 solution completed by 10% (v/v) of inactivated bovine whey and Hening. For maintaining the culture of the parasites when the ultimate logarithmic phase of the growth curve was reached, 1 mL of the 6-$7x10^6$ parasites/mL solution was introduced in a fresh 5 mL culture solution. Ventilated sterile bottles of 25 or 50 mL were used.

***T.b. brucei* bsf culture (S16):** the bsf was cultivated at 37°C in an HMI-9 solution completed with 10% (v/v) of inactivated bovine whey. For maintaining the culture of the parasites when the ultimate logarithmic phase of the growth curve was reached, 50 µL of the 15-$20x10^6$ parasites/mL solution was introduced in a fresh 5 mL culture solution. Non ventilated sterile bottles of 25 or 50 mL were used.

X.1.2.2 Molecular biology techniques

RNA isolation (Invitrogen, Trizo reagent): both forms of parasites were cultivated until the optimum concentration:

- $1.1x10^6$ parasites/mL for procyclic;
- $6.1x10^6$ parasites/mL for bsf.

The solutions were concentrated and then homogenised by adding Trizol reagent (1mL). The separation was achieved by incubating for 5 minutes at 15-30°C, adding $CHCl_3$ (0.2mL for each Trizol mL added), incubating for 3 minutes at 15-30°C and concentrated for 15 minutes at 12000 rpm at 4°C. In

order to precipitate the RNA, isopropanol was added (0.5mL for each Trizol mL added) to the aqueous layer, incubated for 10 minutes at 15-30°C and centrifuged for 10 minutes at 12000 rpm at 4°C. The pellet was washed with ethanol 75% (1mL for each Trizol ml added) and centrifuged for 5 minutes at 7500 rpm at 4°C. The pellet was almost dried and then dissolved in RNase-free water.

RNA electrophoresis: a 1% denatured agarose gel with a 1x MOPS buffer and formaldehyde (37%) 2M were used. The electrophoresis was run at room temperature for 4 hours at 100 volts. The RNA was isolated using the protocol for the *QuickPrep Micro mRNA Purification Kit*. Each sample of RNA-poly A+ was resuspended in water and quantified. Six µL of loading buffer was mixed with 3 µL of DNA solution containing the sample. It was denatured at 65°C for 10 minutes, put in ice, and then loaded onto the gel. The gel was exposed to UV light to visualise the RNA bands.

RNA transfer to a nylon filter (Northern blot): the method used was the same as for a Southern blot[171], based on the transfer of denatured RNA from an agarose gel to a nylon membrane (Hybond-N® de Amersham). Then, the filter was incubated with the radioactive probe which then hybridises with its complementary strand.

Radioactive marking of DNA - radioactive probe synthesis: the method used was the *random priming* method. The final volume was 20 µL with:

- 4 µL of *T.b. brucei* SMT DNA (50-200 ng expected);
- 1.3 µL of each deoxyribonucleoside triphosphate dTTP, dATP and dGTP (0.030mM);
- 1 µL of Klenow enzyme (2 units);
- 5 mL of dCTP (50 µCi de [α-^{32}P]).

The reaction was carried out for 2 hours at 37°C, and stopped with 5 µL of EDTA 0.2M. The mixture was purified by column chromatography (1 mL Sephadex G-50), and DNA eluted with 100 µL of double-distilled water (DDW). Two µL of this solution was used in the scintillation counter to determine the number of cpm.

RNA hybridisation with the radioactive probe: the nylon filter were hybridised with a radioactive probe already prepared (cf: radioactive probe synthesis). The hybridisation was carried out at 42°C overnight, adding 10^6 cpm DNA marking for each hybridisation solution mL. The filter was first washed with a 10x SSC / 1% SDS solution for 30 minutes at rt, then by a prewarmed 1x SSC / 0.5% SDS solution for 45 minutes at 42°C, and finally by a prewarmed 0.1x SSC / 0.2% SDS solution for 30 minutes at 42°C. The filter was dried at rt and a photo taken with an X-OMAT®AR (Kodak) linked to a Dupont amplifier screen. The filter could be stored at -80°C until being visualised.

X.2 Materials and methods for the cloning, over expression, purification and enzyme assays of the *T.b. brucei* SMT enzyme

X.2.1 Materials

X.2.1.1 Bacteria

XL1-Blue: *Rec A1, end A1, gyr A96, thi-1, hdsR17, supE44, relA1, lac* [F'*proAB lacI*^{q} *ZDM15 Tn10* (Tet^r)].

BL21(DE3): F$^-$dcm, ompT, hsdS($r_B^-m_B^-$) gal λ (DE3).

BL21 (DE3) pLysS: F$^-$ dcm, ompT, hsdS ($r_B^-m_B^-$) gal λ (DE3) [pLysS Cam^r].

X.2.1.2 Buffers / Solutions

Buffer / Solution	Composition
LB (Luria Bertani)	10 g/L of tryptone, 5 g/L of yeast extract and 5 g/L of sodium chloride.
PBS (saline phosphate buffer)	NaCl 0.14 M, KCl 2.6 mM, NaH_2PO_4 10 mM, KH_2PO_4 1.2 mM (pH 7.2).
TAE 1x	Tris-Acetate 0.04 M, EDTA 1 mM (pH 8.0)
Loading buffer 6x for DNA	Blue of bromophenol 0.25 % (p/v), xylene cyanol 0.25 % (p/v), glycerol 30 % (v/v)
Loading buffer 4x for the proteins on the SDS-PAGE gels	Tris-HCl 0.27 M, SDS 12 % (p/v), glycerol 40 % (v/v), 2-mercaptoethanol 20 % (v/v)
Solution to dye gels with proteins	Blue of Commassie R250, methanol 25 %, acetic acid 10 %
Washing solution for filter with proteins	PBS 1x or TBS 1x, Tween 20 0.1% (v/v).
Protein locking solution.	BSA 0.1 % (p/v), skimmed-milk 5 % (p/v), Tween 20 0.1 % (p/v) in PBS 1x or TBS 1x.
Gel SDS-PAGE 15%, *Separating gel* (Acrylamide-Bis Acrylamide (30:0.8)).	15% buffer 3 M Tris pH 8.8, 10% SDS, 10% ammonium persulfate, TEMED 7.5%); *Stacking gel* (Acrylamide-BisAcrylamide (30:0.8) 4% buffer 2.5M Tris pH 6.8, 10% SDS, 10% ammonium persulfate, TEMED 5%).
8X Phosphate buffer pH 7.4	1.42g of $Na_2HPO_4.2H_2O$, 1.11g of $NaH_2PO_4;H_2O$ and 23.38g of NaCl in 90mL of DDW; adjust pH to 7.4, complete to 100mL with DDW and filter through a 0.45µm filter.

2M Imidazole pH 7.4	13.62g of imidazole, 90mL of DDW, adjust pH to 7.4, complete to 100mL with DDW and filter through a 0.45μm filter.
0.1M $NiSO_4$	2.63g of $NiSO_4.6H_2O$, complete to 100mL with DDW and filtered through a 0.45μm filter.
Protein Wash Solution	0.5mL of Tris-HCl 50 mM pH 7.4, 200μL of $MgCl_2$ 0.1M, 1.41μL of β-mercapto. 14.2M, 20μL of 0.5 EDTA 1mM pH 7.4, 1.5mL of glycerol 87% and 6.37mL of DDW.
SMT activity measure buffer.	Tris-HCl 50 mM pH 7.4, $MgCl_2$ 2 mM, CHAPS 4mM, Tween 80 0.5% (v/v), and proteases inhibitors

Table X.2: Solutions used for the cloning, over expression, purification and enzyme assays of the *T.b. brucei* enzyme

X.2.1.3 Antibiotics / Compounds

Ampicilin (100 μg/mL) (Roche Molecular Biochemicals): used to select the bacteria that contained the plasmid with the β-lactamase gene. The product of this gene gave resistance to the antibiotic.

Kanamycin (30 μg/mL): used to select the bacteria that had the pET28a system (Novagen), and for the over expression of the protein in *E. coli*. The plasmid pET28a contains the gene *kan*, which was responsible for resistance to Kanamycin when over expressed.

Chloramphenicol (34μg/mL): the bacteria *E. coli* BL21(DE3)pLysS have a plasmid resistant to chloramphenicol. This antibiotic was used to save this phenotype.

Isopropyl-β-D-tio-galactopyranoside (IPTG, 0.25 mM) (Roche): was added to the solution of the transfected bacteria with the plasmid containing the gene *lac*. The IPTG inactivated the product of the gene *lacI* (repressor of the *lac* operator) and induced the expression of the gene *lacZ* that codes the β-galactosidase.

5-Bromo-4-chloro-3-indoyl-β-D-galactosidase (Xgal, 0.417 mg/ml) (Roche): this compound was recognised by β-galactosidase, giving a blue color to the colony if present. Therefore Xgal allowed the selection of positive or negative recombinant bacteria.

X.2.1.4 Oligonucleotides

Name	Sequence (5' to 3')
TbSMT-1	GGAATTCCATATGTCGGCCGGATCTCGT
TbSMT-2	CGGGATCCTTAGCACGACAGCTCTTCCCC
SP6promoter	ATTTAGGTGACACTATA
T7promoter	AATACGACTCACTATAG
T7terminator	GCTAGTTATTGCTCAGC

Table X.3: Oligonucleotides

X.1.1.5 Vectors

pGEM-T (Promega): a vector of 3000 bp that comes from the vector pGEM®-5Zf(+) that was first digested with the endonuclease which cleaves the *Eco*RV restriction site and after addition of the deoxythymidine (T) residue at the 3' position of the insertion site. This was used as an amplification vector for cloning DNA fragments obtained from PCR.

pET-28a (+) (Novagen): a expression vector of 5369 bp in which *LmSMT* or *TbbSMT* genes were cloned, allowing LmSMT and TbbSMT proteins to be over expressed in *E. coli*.

X.2.2 Methods

Polymerisation Chain reaction (PCR): this method allowed large scale production of specific DNA from a complete DNA sample. The reaction was carried out in a final volume of 100 µL:

- 1 µL of DNA;
- 3.6 µL of the oligonucleotide TbSMT-1 (5' site);
- 3.8 µL of the oligonucleotide TbSMT-2 (reverse complementary 3'site);
- 4 µL of dNTP;
- 5 µL of Taq buffer polymerase 1x;
- 0.5 µL (2.5 units) of Taq polymerase.

The initial general conditions were a denaturation cycle at 95°C for 5 minutes, then 30 cycles of:

- Denaturation at 94°C for 1 minute;
- Annealation at 55°C for 1 minute;
- Extension at 72°C for 1 minute;

And finally an extension cycle at 72°C for 10 minutes.

The products of the PCR were analysed by electrophoresis with an agarose gel, and positive samples were purified by the Promega kit *Wizard® DNA Clean-Up System.*

DNA ligation reaction: this ligation using DNA fragments and plasmid vectors was carried out with the T4 DNA ligase (gibco BRL). The ligation in the pGEM-T® vector was carried out as followed:

- 5 µL of buffer 2x;
- 1 µL of the pGEM-T vector;
- 1 µL T4 DNA ligase;
- 3 µL of DNA fragments (from the PCR, 450 ng).

The ligation in pET28a was achieved using buffer 5x, T4 DNA ligase, pET28a, DNA fragments and water in the concentrations discussed in Section VIII.3.1. The ligation with pGEM-T was carried out for 2 hours at rt whereas the ligation onto pET28a was carried out overnight at 16°C. In both cases, the enzyme was inactivated at 65°C for 10 minutes, and the mixture used for transfection in electrocompetent cells, either BL21(DE3) pLys or BL21(DE3).

Thermal shock method: this method was used to transfect the *E. coli* cells with plasmids that present a replication origin and a resistance gene to a specific antibiotic. An aliquot of 100 µL of cells treated with $CaCl_2$ was mixed with 10-100 ng of plasmid and kept in ice for 30 minutes. The cells were then exposed to a thermal shock (45 seconds at 42°C), and incubated in ice for 2 minutes. After the transfection, the cells recovered at 37°C in 0.9 mL of LB solution without antibiotic for 1 hour at 160 rpm. The cells were finally spread on a semisolid culture with the antibiotic.

Electroporation: one 40 µL aliquot of electrocompetent cells was mixed with 1µL of DNA plasmid (1 ng/µL) and transferred to an electroporation bowl previously put in ice. The bacteria were transfected using the following conditions: 2.45 kV; 125 Ω; 12.25 kV/cm, with a pulse of 2.8 to 3.2 ms. The cells were recovered with 960 µL of LB solution without antibiotic for 1 hour at 37°C and under 160 rpm shaking. A 100 µL aliquot of the transfected cells was spread on a semisolid culture with the antibiotic.

DNA extraction from *E. coli*: for the low scale extraction of DNA, the isolation protocol of the *kit Wizard® Plus Minipreps DNA Purification System.* (Promega) was used. For larger scale extraction, the method from *Current protocols in Molecular Biology*[171], and the *kit Concert™ Nucleic acid purification system* (Gibco BRL) were used.

DNA digestion with restriction endonucleases: the digesting reaction was carried out in a 30 µL volume. The buffer used was the optimum for each

enzyme as suggested by the company. The reaction was quenched with 6 µL of loading buffer 6x.

DNA electrophoresis: the electrophoresis with agarose gels allowed the separation of DNA fragments according to their molecular weight. After the electrophoresis, the gel was dyed with ethydium bromide (0.5 µg/mL), the later goes between the two strands of DNA allowing the observation of the different bands with an UV lamp. The gel was prepared with 0.8 to 1.2% of agarose in a TAE 1x solution. The samples were run in the same buffer using a 50-100V voltage for 1 to 4 hours. The molecular weight marks were obtained after digestion of λ and ϕ174 DNA with respectively the endonucleases *HINd*III and *Hae*III. The DNA fragments obtained from PCR after digestion with the restriction enzymes were recovered from the gels by filtration through glass wool.

Sequencing: the DNA sequences were determined by the technical service of the Instituto de Parasitologia y Biomedicina "Lopez-Neyra". The sequencing after ligation in pGEM-T was carried out with the preparation of two solutions:

4.8 µL of DNA + 1.2 µL of T7 promoter

and

4.9 µL of DNA + 1.1 µL of SP6 promoter

The sequencing after ligation in pET28a was carried out with the preparation of two solutions:

4.8 µL of DNA + 1.2 µL of T7 promoter

and

4.8 µL of DNA + 1.2 µL of T7 terminator

Induction of the *T.b. brucei* SMT protein with IPTG: the *E.coli* BL21(DE3) cells were transfected with pET28a-TbbSMT plasmid. To induce the expression of TbbSMT protein, a single colony was inoculated into 10mL of LB medium containing Kanamycin (30 µg/mL) and was incubated at 37°C at

150 rpm until the OD_{600} was about 1. From this culture, 50 mL were inoculated and incubated overnight in similar conditions. 40 mL were then added to 1 litre of medium, and incubated at 19°C and 200 rpm until an OD_{600} of 0.6. The over expression of TbbSMT was then induced by adding IPTG 1mM and incubating for 4 hours. The cells were harvested by centrifugation at 4800 rpm for 10 minutes at 4°C in 50 mL aliquots and stored at -80°C until needed.

Purification of the *T.b. brucei* SMT protein:

- HisTrap™ Column preparation: wash the column with 10mL of DDW, then load it with 5mL of 0.1M $NiSO_4$ solution, and finally wash with 5mL of DDW,
- The following elution solutions were prepared:

$C_{Imi.}$ (mM)	Buffer 8X (mL)	Chaps 0.1M (mL)	Glycerol 87% (mL)	$MgCl_2$ 0.1M (µL)	β-Mercapto. (µL)	Imidazole 2M (µL)	DDW (mL)
10	3	2.4	2.76	480	16.8	120	15.22
20	1.25	1	1.15	200	7	100	6.29
40	1.25	1	1.15	200	7	200	6.19
60	1.25	1	1.15	200	7	300	6.09
100	1.25	1	1.15	200	7	500	5.89
150	1.25	1	1.15	200	7	750	5.64
300	1.25	1	1.15	200	7	1500	4.89
500	1.25	1	1.15	200	7	2500	3.89
1000	1.25	1	1.15	200	7	5000	1.39

Table X.4: Solutions of imidazole used for the elution of the protein (all solutions had a final pH of 7.4 and were filtered with a 0.45µm filter.

Preparation of the protein solution: a solution of 1.25mL of 8X Phosphate buffer (pH 7.4), 1mL of CHAPS (0.1M), 1.15mL of glycerol (87%), 200µL of $MgCl_2$ (0.1M), 7µL of β-mercaptoethanol (14.2M), 50µL of Imidazole (2M), protease inhibitors (Leupeptin, Aprotinin, Phenantrolin, Trypsin inhibitor,

Benzamidine and Phenylmethylsulfonyl fluoride) and 5.93mL of DDW was prepared, the pH adjusted to 7.4 and then filtered. 5mL of that solution was added to the frozen protein and the cells were disrupted by sonication (8 times, 30 seconds, duty cycle 50%). The mixture was centrifuged at 13000 rpm for 30 minutes at 4°C to obtain the soluble fraction. The concentration of the protein was measured. The final concentration after dilution with DDW was 1.5 mg/mL.

- The column was equilibrated with 10mL of imidazole solution (10mM);
- The sample was eluted in the column;
- 5mL of elution buffer was used first and 1mL fractions were recovered;
- Samples with protein were washed with the Protein Wash Solution;
- The column was washed with 10mL of Imidazole solution (10mM);
- The column was cleaned for further use: nickel ions were removed by washing with 10mL of a solution containing sodium Phosphate (20mM), NaCl (0.5M), EDTA (0.05M) which pH had been adjusted to 7.4; the proteins that precipitated were removed by filling the column with 1M NaOH solution for 2h. Finally, the column was washed with a solution containing buffer and DDW until pH of the flow-through reaches 7.

Over expression of the *Leishmania major* protein

The method to over express the *L. major* SMT enzyme was established in the laboratory by the Dr. M.C. Jimenez Jimenez[173]. The construction of the plasmid pET28-LmSMT (Figure X.1) was obtained amplifying the coding sequence of *Leishmania major* SMT gene and cloning it in pET28a vector (Novagen). The ligation was performed with the restriction sites *Nde*I and *Bam*HI. The DNA sequence was checked to see if the Taq DNA polymerase did not introduce any erroneous nucleotides.

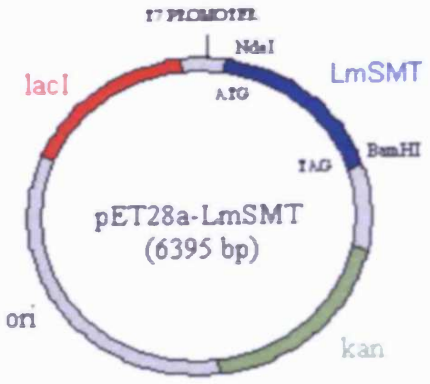


Figure X.1: Plasmid pET28a-LmSMT

Assays of SMT activity inhibition produced by azasterols, total protein extracts from *E. coli* BL21 (DE3) pLysS transformed with pET28a-LmSMT plasmid (which confers resistance to kanamycin) were used. This strain possesses an additional plasmid which confers resistance to chloramphenicol, and the fusion protein HisLmSMT was over expressed when induced with IPTG 1mM for 4 hours at 19°C. The expression was analysed with SDS-PAGE denatured gel running an electrophoresis. The soluble extract was directly used for the enzymatic assays as explain for the *T.b. brucei* protein next paragraph.

Over expression of the *T.b. brucei* protein and enzyme assays

Preparation of the soluble extract: 1mg of total protein was resuspended in a buffer containing Tris-HCl (50mM, pH 7.4), $MgCl_2$ (2mM), CHAPS (4mM), Tween 80 0.5% (v/v) with protease inhibitors. To obtain the protein extract, the cells were disrupted by sonication (3 times, 30 seconds, duty cycle 50%). The resulted solution was centrifuged at 12000 rpm for 30 minutes at 4°C to obtain the soluble fraction, which contained the active form of the enzyme.

SMT activities assays: the final volume of the SMT activities assays were 600 µL which contained 1.7 µg/µL of protein extract obtained from the above protocol. The enzyme substrates used in the assay were desmosterol (100µM) and ^{14}C-*S*-adenosyl-$_L$-methionine (200µM, 600000 dpm per reaction, 5dpm/pmol). Desmosterol was dissolved in chloroform, which must be

evaporated before adding the rest of the components. The inhibitor was re-suspended at first in a minimal volume of its corresponding solvent and later added to the reaction mixture as an aqueous dilution. The reaction solution also contained the buffer Tris-HCl (50 mM), $MgCl_2$ (2mM), CHAPS (4mM), Tween 80 0.5% (v/v) and DDW if necessary. The reaction was started with the protein extract. Incubation was performed at 30°C for 45 minutes, and was stopped with 500µL of KOH 10% dissolved in 10% (v/v) of DDW and 90% (v/v) of methanol. To quantify the efficiency of the extraction, an internal standard consisting of an isotopic mixture of ^{3}H-cholesterol (3 mg, 30000 dpm per reaction) was added. The methylated sterol product was extracted 3 times with 1 mL of hexane, mixed for 20 seconds in a vortex mixer. The resulting organic layer was washed once with Tris-HCl (50mM) buffer to remove the ^{14}C-*S*-adenosyl-$_L$-methionine not incorporated. 1 mL of the organic layer was added to 10 mL of hydrofluoric acid and the radioactivity measured in a scintillation counter.

X.3 Materials and methods for the electron microscopy

X.3.1 Materials

Buffers / Solutions	Composition
Karnovsky fixing solution.	Glutaraldehyde 0.5 %, Formaldehyde 4 % in a Cacodilate buffer 0.1 M, pH 7.4

Table X.5: Solution used for fixing the parasites samples growth with inhibitors

X.3.2 Methods

Fixing preparation: the samples were centrifuged for 20 minutes at 3000 rpm at 4°C, washed with 10 mL of a PBS solution, centrifuged for 20 minutes 3000 rpm at 4°C, washed with 5 mL of a PBS solution and centrifuged for 15

minutes at3000 rpm at 4°C. The Karnovsky fixing solution was prepared as follow:

- 1.5 mL of Cacodilate buffer;
- 0.3 mL of glutaraldehyde;
- 1.2 mL of double distilled water.

1.5 mL of the Karnovsky fixing solution was added to each sample which was then stored at 4°C.

Appendix 1

Screening Protocols: Experimental procedure and references reported in this appendix have been written by Dr S. L. Croft, London School of Hygiene and Tropical Medicine, London (UK).

A.1 *In Vitro* Assays

A.1.1 Compound preparation

Stock solutions of the test compounds, plus control drugs, were prepared at a concentration of 20mg/ml in DMSO (Sigma, UK). Two times concentration 3-fold serial dilutions, in triplicate, of the compounds, were prepared in 96-well plates (Falcon, Life Technologies, UK).

A.1.2 *Leishmania donovani*

Peritoneal exudate macrophages were harvested from CD1 mice, 24 hours after starch induction. After washing the macrophages, they 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 hours. *Leishmania donovani* L82 amastigotes were harvested from an infected Golden hamster spleen and were used to infect the macrophages at a ratio of 5 parasites: 1 macrophage. Infected cells were left for a further 24 hours and then exposed to drug for a total of 5 days, with the overlay being replaced on day 3[193].The top concentration for the test compounds was 30μg/mL and all concentrations were carried out in quadruplicate. The ED_{50} value for the positive control drug, Pentostam©, is usually 3-8μgSb^V/mL. On day 5 the overlay was removed, the slides fixed (100% methanol) and stained (10% Giemsa, 10 minutes) before being evaluated microscopically. ED_{50} (ED_{90}) values were calculated using Msx*l*fit.

A.1.3 *Trypanosoma cruzi*

Murine (CD1) peritoneal macrophages were harvested 24 hours after starch induction. 100μL was dispensed into 96-well plates at a concentration of 4x 10^5mL. After 24 hours the cells were infected with *Trypanosoma cruzi* Tulahuan LAC-Z trypomastigotes, harvested from L6 feeder layer cultures. Between 24 to 48 hours later the infected cells were exposed to drug for 3 days. 50μL of 500μM CPRG:1% nonidet P-40 was added to each well. The plates were read after 2-5 hours, λ570 ED_{50} (ED_{90}) values were calculated using Msx*l*fit[194]. L6 fibroblasts were also used as host cells.

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[193] Neal, R. A.; Croft, S. L. An *in Vitro* System for Determining the Activity of Compounds Against the Intracellular Amastigote Form of *Leishmania donovani*. *J. Antimicrob. Chemother.* **1984**, *14*, 463-475.

[194] Buckner, F. S.; Verlinde, C.; LaFlamme, A. C.; vanVoorhis, W. C. Efficient Technique for Screening Drugs for Activity Against *Trypanosoma cruzi* Using Parasites Expressing β-Galactosidase. *Antimicrob. Agents Chemother.* **1996**, *40*, 2592-2597.

A.1.4 *Trypanosoma brucei spp.*

Trypanosoma brucei rhodesiense STIB900 blood stream form (bsf) trypomastigotes were maintained in HMI-18 medium[195] with 15% heat-inactivated foetal calf serum (HIFCS) [Harlan-SeraLab, UK] at 37°C, 5%, CO_2/air mixture. Trypomastigotes were washed and resuspended in fresh medium at a concentration of $2x10^5$mL. 100µL was added to the drug dilutions. The top concentration for the test compounds was 30µg/mL. The ED_{50} for Pentamidine is usually between 1.0 and 0.1pg/mL. Plates were incubated for 72 hours at 37°C, 5% CO_2. At 72 hours the plates were assessed microscopically before Alamar Blue was added[196] Plates were read after 5-6 hours on a Gemini Fluorescent plate reader (Softmax Pro. 3.1.1, Molecular Devices, UK) at EX/EM 530/585nm with a filter cut-off at 550nm. ED_{50} values were calculated with Ms*xl*fit (IDBS, UK)

A.1.5 *Plasmodium falciparum*

Plasmodium falciparum 3D7 cultures were mantained in RPMI 1640 medium (Sigma, UK) 37°C, 5% CO_2 in 5% hematocrit. Synchronised ring stage cultures were prepared at 1% parasitemia and 50µL added per well, the top test drug final concentration being 30µg/mL. After 24 hours incubation, 37°C, 5% CO_2 5µl (^{3}H)-hypoxanthine was added (0.2µCi/well) [Desjardins *et al.*, 1979, O'Neill *et al.*, 1985] and plates were shaken for 1 minute and then incubated for 48 hours. The plates were freeze/thawed rapidly, harvested and dried. (^{3}H)-hypoxanthine uptake was measured using a microbeta counter (Wallac 1450). ED_{50} values were calculated as before.

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[195] Hirumi, H.; Hirumi, K. Continuous Cultivation of Trypanosoma-Brucei Blood Stream Forms in a Medium Containing a Low Concentration of Serum-Protein without Feeder Cell-Layers. *J. Parasitol.* **1989**, *75*, 985-989.

[196] Raz, B.; Iten, M.; GretherBuhler, Y.; Kaminsky, R.; Brun, R. The Alamar Blue(R) assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. *Acta Trop.* **1997**, *68*, 139-147.

A.1.6 Cytotoxicity Assay

- Plates seeded with 100µL KB cells @ 4 x 10^4/mL, RPMI 1640 + 10% HIFCS. Incubated at 37°C,5% CO_2 for 24 hrs;
- Overlay removed and replaced by test drugs in fresh medium @ 300, 30, 3 and 0.3µg/mL. Positive control drug is Podophyllotoxin (Sigma, UK). Dilutions in triplicate. Plate incubated for a further 72hrs, at 37°C, 5% CO_2;
- Wells assessed microscopically for cell growth. Overlay removed and wells washed with PBS (pH7.0) x 3. 100µL PBS + 10µL AlamarBlue™ added per well and plates incubated for 2-4 hours (37°C,5% CO_2) before reading at EX/EM 530/585nm (cut-off 550nm) in a Gemini plate reader;
- ED_{50} ($ED_{(90)}$) values calculated c.f. blanks and untreated controls.

A.2 *In Vivo* Assay: *Trypanosoma brucei spp.*

A.2.1 Parasite and animal strains

- Female BALB/c mice (pathogen free), 18-20g;
- *T. b. rhodesiense* STIB900 blood stream forms (bsf) either from culture or from a passage animal.

A.2.2 General experimental procedure

Mice were infected with 5 x 10^4 bsf i.p. After 24 hours, dosing commenced and continued for 4 consecutive days. Subsequently, the mice were monitored 3 times a day and the number of days survived recorded. The end of the experiment was on day 60 post- infection. Parasitemia may also be monitored via tail blood.

A.1.4 *Trypanosoma brucei spp.*

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- Wells assessed microscopically for cell growth. Overlay removed and wells washed with PBS (pH7.0) x 3. 100µL PBS + 10µL AlamarBlue™ added per well and plates incubated for 2-4 hours (37°C,5% CO_2) before reading at EX/EM 530/585nm (cut-off 550nm) in a Gemini plate reader;
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A.2.2 General experimental procedure

Mice were infected with 5 x 10^4 bsf i.p. After 24 hours, dosing commenced and continued for 4 consecutive days. Subsequently, the mice were monitored 3 times a day and the number of days survived recorded. The end of the experiment was on day 60 post- infection. Parasitemia may also be monitored via tail blood.

A.2.3 Preparation and administration of compounds

Compounds were prepared in 10% DMSO/ddH_2O for i.p. injection unless otherwise specified. A dose of 50mg/kg/day was used for the primary screen. The secondary screen would be a repeat experiment at a range of doses i.p. plus one group receiving oral treatment (p.o.) at 50mg/kg/day. Tertiary screening would involve oral treatment over a dose range. Positive control drug used is pentamidine at 10mg/kg x 1 day i.p., or Melarsoprol 3mg/kg i.p. x 1.

Appendix 2

Bioorganic & Medicinal Chemistry

Preparation of Transition-State Analogues of Sterol 24-Methyl Transferase as Potential Anti-Parasitics

Preparation of Transition-State Analogues of Sterol 24-Methyl Transferase as Potential Anti-Parasitics

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c. London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, UK
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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 was 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 *Trypanosoma cruzi*, but no inhibition of the enzyme. In addition, some of the compounds had antiproliferative activity against the blood stream forms of *Trypanosoma brucei rhodesiense*, which causes African trypanosomiasis.

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 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 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 together with 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 sterol 24-methyltransferase (24-SMT), which has no direct counter-part in mammalian sterol biosynthesis pathways. The proposed mechanism of action of the enzyme is shown in Figure 1.[10-18] 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** (Figure 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.

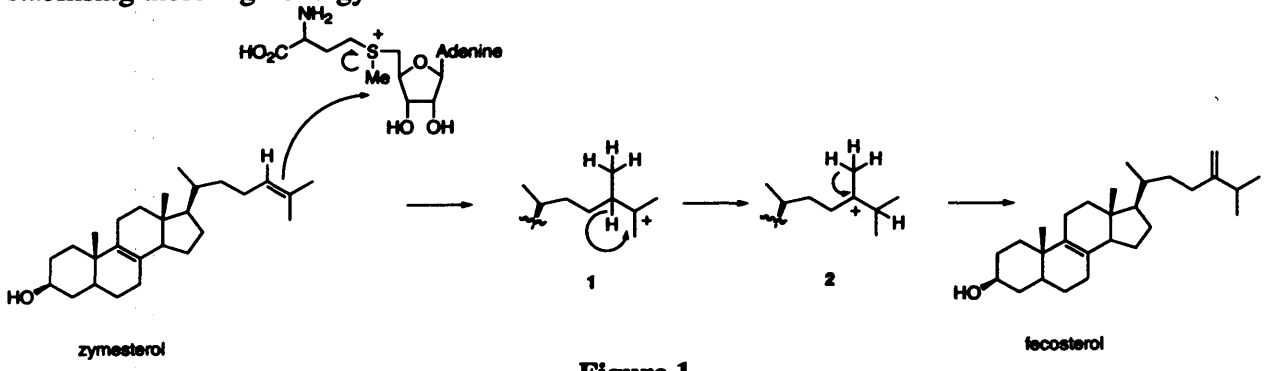


Figure 1

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

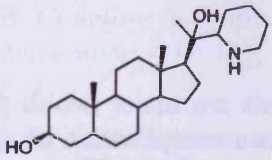


Figure 2

In this article we describe a strategy in which we have prepared mimics of the Transition State (TS). In the Transition State, there is a molecule of sterol interacting with a molecule of SAM. The binding of these two substrates appears to be independent from 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 (Figure 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 which do not have good drug-like properties. Therefore fragments of the SAM moiety were coupled to the sterol.

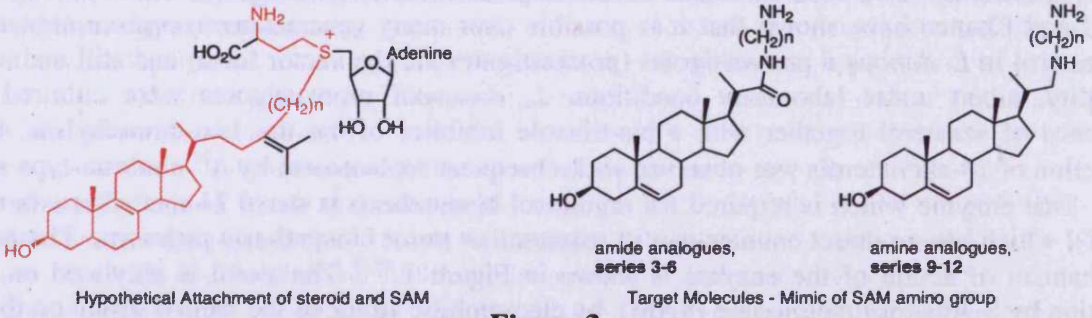


Figure 3

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

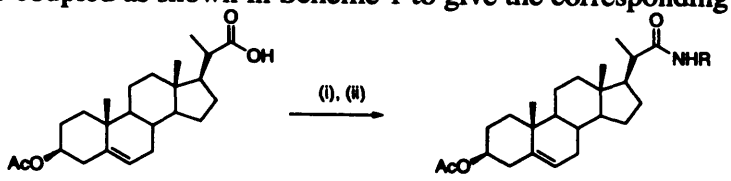
We have recently reported the results of the biological assays for series **9-12** (amine analogues).[23] In this paper we describe the preparation of these compounds, the synthesis and biological assays for series **3-6** (amide analogues), and a comparison of the two series.

Chemistry

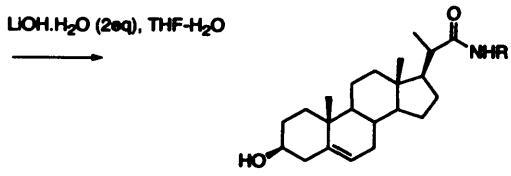
Preparation of Amides

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.

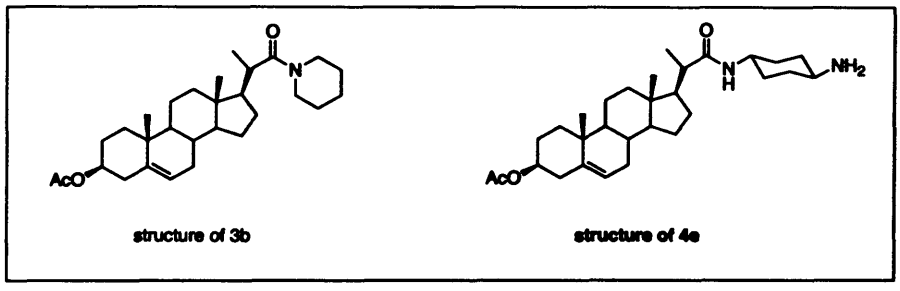
The starting material was the commericially available 3β-acetoxy-5-cholenic acid. Several methods for coupling the sterol to the amines was investigated. The best method appeared to be using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and HOBt. Amines were coupled as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



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

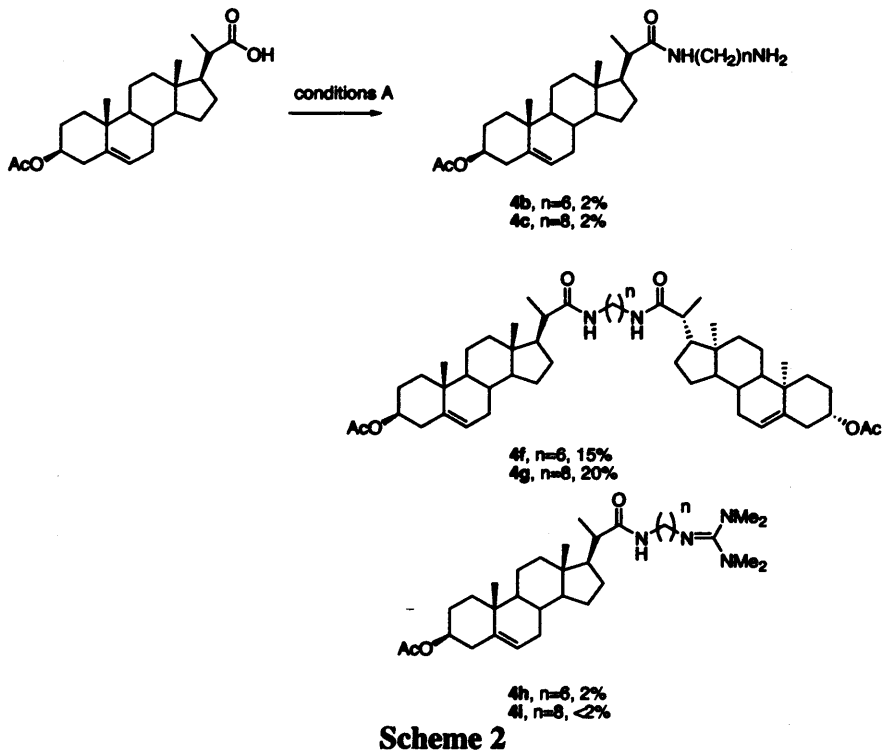


5a, R = Bu, 97%
5b, R = N-piperidyl, 70%
5c, R = CH_2Ph, 65%
6a, R = $(CH_2)_4NH_2$, 95%
6b, R = $(CH_2)_6NH_2$, 90%
6c, R = $(CH_2)_8NH_2$, 75%
6d, R = $(CH_2)_{10}NH_2$, 90%
6e, R = 1,4-*trans*-$(C_6H_{10})NH_2$, 70%



Scheme 1:
Procedure A: (i) DIPEA (3eq.), TBTU (2eq.), HOBT (2eq.), DMF, r.t. 30mins. (ii) R'R''NH (2eq.), r.t.
Procedure B: (i) DIPEA (3eq.), TBTU (1.1eq.), HOBT (1.1eq.), DMF, r.t. 30mins. (ii) $H_2N(CH_2)_nNH_2$ (4eq.), r.t.

However when 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]



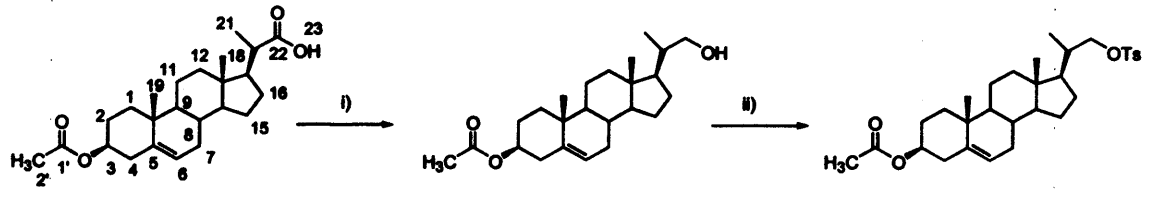
Scheme 2

In order to circumvent this problem, the method was modified to give procedure B. The amount of TBTU was reduced to 1 equivalent which should avoid coupling of the terminal amino group with excess TBTU to give the 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 dimmers. 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]

Preparation of Amines

The starting point for the synthesis was the commercially available sterol 3β-acetoxy-5-cholenic acid. This was reduced to the 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.



7 **8**

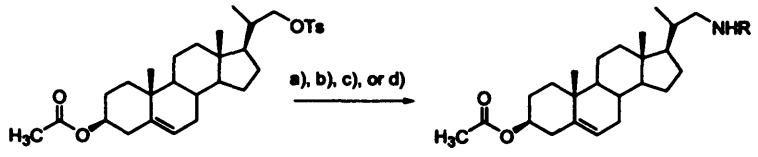
i) $BH_3.SMe_2$ (1M CH_2Cl_2) (1eq.), THF, -10°C→ 0°C, 8h, 57%; ii) TsCl (2eq.), pyridine: DCM (1: 1), 0°C→4°C, 24 h, 90%

Scheme 3

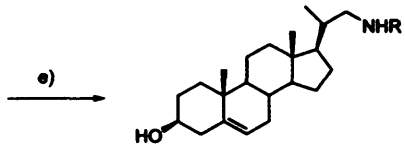
Synthesis of the azasterols was conducted by coupling the tosyl sterol **8** with amines varying in the nature and length of the side chain. Initially it was decided to prepare some simple amines to

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 excess of the amine (10-20 equivalents) and diisopropylethylamine (DIPEA) in DMF.[29] Secondly, the displacement was carried out by using a smaller excess of the amine (3-4 equivalents), an inorganic base (potassium carbonate) and the phase transfer catalyst tetrabutylammonium iodide in DMF.[30]



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)

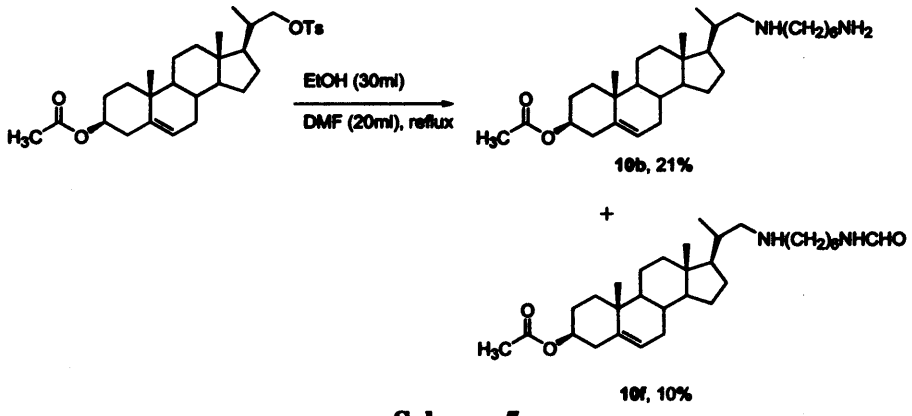


11a, R = butyl, 78%
11b, R = piperidyl, 84%
12c, R = $(CH_2)_8NH_2$, 70%
12d, R = $(CH_2)_{10}NH_2$, 93%
12e, R = 1,4-*trans*-$(C_6H_{10})NH_2$, 60%

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

Scheme 4

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 tosyl **2** in ethanol, 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 stemming from the reaction of the free terminal amino group in the side chain of the sterol with DMF (Scheme 5).



Scheme 5

Analogues **5a-6e** with a free hydroxyl group in position 3β of the sterol were prepared by hydrolysis of the corresponding acetates using lithium hydroxide (Scheme 4).[32]

Biology

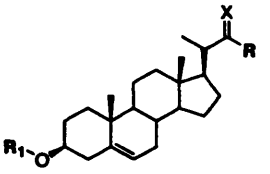
It has been proposed by Nes[10] that inhibitors of 24-SMT must have a free 3β-OH for binding to the active site. The compounds protected with an acetate at the 3β-OH were also assayed for activity, as the acetate group will probably be cleaved by esterases *in vitro* or *in vivo* to yield the active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

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 conducted using *E. coli* cell-free extracts containing soluble protein as the enzyme source. Essentially none of the amide compounds, either with the 3β-OH protected as the acetate (compounds **3** and **4**) or those with the acetate removed (compounds **5** and **6**) showed significant inhibition of the enzyme. For the amine 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.

It is interesting to compare compounds **5a** and **5b** with **11a** and **11b**. These indicate 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 a 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.

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]

Sterol Composition

For 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 (eg 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 amides (**5a-b**) showed little effect on sterol composition at 1μM 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 sterols) which is consistent with inhibition of 24-SMT in the intact parasites.

Table 2: Effects of Inhibitors on the free sterol composition of *Leishmania mexicana* promastigotes

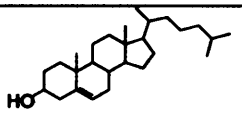
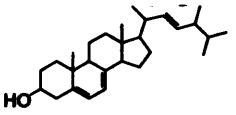
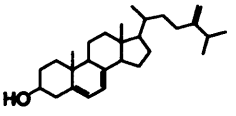
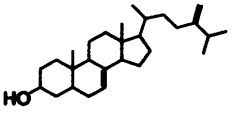
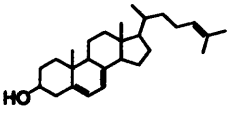
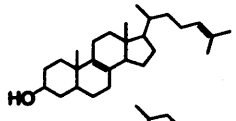
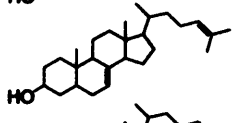
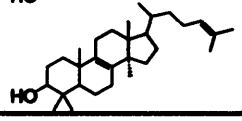
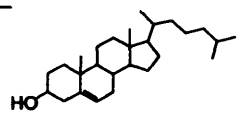
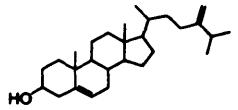
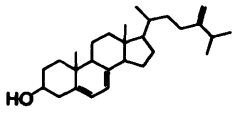
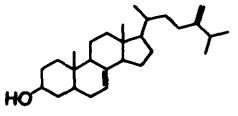
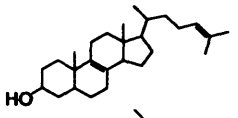
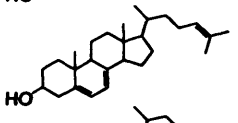
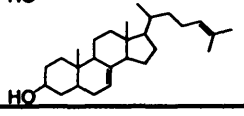
No			**5a**	**5b**	**5c**
Concentration (μM)		0	1	1	1
Cholesterol (exogenous)		9.8	13.1	10.9	11.3
Ergosterol		6.7	7.6	3.3	6.1
Ergosta-5,7,24(24^1)-trien-3β-ol (5-dehydroepisterol)		73.0	66.0	72.2	48.8
Ergosta-7,24(24^1)-dien-3β-ol (episterol)		8.3	8.5	10.0	5.7
Cholesta-5,7,24-trien-3β-ol		<1	1.2	<1	19.0
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

Table 3: The effects of compounds **AZA**, **9a** and **11a** on the free sterol composition of *L. amazonensis* promastigotes[a].[23]

No		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]

In vitro antiproliferative 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 lifecycles 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, this is the intracellular amastigote form. Compounds were also evaluated against the related parasite *Trypanosoma brucei rhodesiense* as the trypomastigote form. In this organism, ergosterol biosynthesis is reported in the vector stage of the parasite,[33] but sequestration of sterols via LDL receptors is thought to occur in the blood stream form trypomastigote.

Amide Series (3-6)

For the amide 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 **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, with 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 of less than 10μM.

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.

Amine Series (9-12)

The activity of the amine series of compounds contrasts with that of the amides. For the amines, those with a simple alkyl substituent (series **9** and **11**) showed generally strong anti-parasitic activity. There were changes to 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} 0.2μM) and compound **9b** against *L. donovani* (ED_{50} 2.5μM).

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.

Discussion

Simple amide 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 the 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 the 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 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 un-expected. According to the literature,[33-35] whilst the vector form of the parasite biosynthesises ergosterol in a similar way to *Leishmania* and *T. cruzi*, in the bloodstream form of *T. b. rhodesiense* is unable to biosynthesise sterols *de novo* but takes up cholesterol via LDL receptors. The activity of these compounds against blood stream form *T. b. rhodesiense* could imply that erogsterol biosynthesis is still important in the blood stream 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 amine 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 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 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 acetate 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.

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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. I.R. 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.

Procedure A: A solution of 3β-acetoxy-5-cholenic acid (1.00g, 2.57mmol), DIPEA (0.998g, 7.72mmol), TBTU (1.653g, 5.14mmol) and HOBt (0.695g, 5.14mmol) in dry DMF (35ml) 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-butylamine (3a). 0.802g, 70%; mp 183-185°C; R_f = 0.44 (40% EtOAc/Hexane); IR (KBr) 3323 (CONHst), 2940 (CH), 1732 (CH_3CO), 1645 (CONH), 1543 (NH), 1458 (CH_2), 1371(CH_3), 1248 (C-O), 1039 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.73 (3H, s, 18-CH_3), 0.97 (3H, t, *J7*, 27-CH_3), 1.05 (3H, s, 19-CH_3), 1.22 (3H, d, *J7*, 21-CH_3), 1.27-2.04 (23H, 9×CH_2, 5×CH), 2.07 (3H, s, 2'-CH_3), 2.36 (2H, d, *J7*, 4-CH_2), 3.26 (2H, m, 24-CH_2), 4.64 (1H, m, 3-CH), 5.41 (1H, d, *J5*, 6-CH), 5.49 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-piperidylamine (3b). 0.948g, 81%; R_f = 0.42 (40% EtOAc/Hexane); IR (KBr) 2931 (CH), 1729 (CH_3CO), 1634 (CONH), 1434 (CH_2), 1368 (CH_3), 1247 (C-O), 1120, 1037 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.80 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 1.20 (3H, d, *J7*, 21-CH_3), 1.26-2.06 (24H, 10×CH_2,4×CH), 2.11 (3H, s, 2'-CH_3), 2.40 (2H, d, *J7*, 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, *J5*, 6-CH). ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-benzylamine (3c). 0.988g, 80%; mp 186-187°C; R_f = 0.5 (40% EtOAc/Hexane); IR (KBr) 3336 (CONHst), 2936 (CH), 1731 (CH_3CO), 1646 (CONH), 1534 (NH), 1451 (CH_2), 1370 (CH_3), 1247 (C-O), 1037, 739, 696 (ArCH) cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.78 (3H, s, 18-CH_3), 1.11 (3H, s, 19-CH_3), 1.32 (3H, d, *J7*, 21-CH_3), 1.35-2.06 (19H, 7×CH_2, 5×CH), 2.12 (3H, s, 2'-CH_3), 2.42 (2H, d, *J7*, 4-CH_2), 4.52 (2H, dd, *J5*, *J5*, 24-CH_2), 4.70 (1H, m, 3-CH), 5.47 (1H, d, *J5*, 6-CH), 5.90 (1H, 23-NH), 7.41 (5H, m, 26-CH, 27-CH, 28-CH, 29-CH, 30-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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 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.

Procedure B: A solution of of 3β-acetoxy-5-cholenic acid (0.5g, 1.28mmol), DIPEA (0.67ml, 3.86mmol), TBTU (0.454g, 1.4mmol) and HOBt (0.191g, 1.4mmol) in dry DMF (30ml) was stirred at room temperature for 30 min. The mixture was added drop-wise over 60 minutes to a solution of diamine (4eq.) in dry DMF (20ml) 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,4)-diaminobutyl (4a). 0.321g, 55% yield; mp 171-173°C; R_f = 0.16 ($CHCl_3$:MeOH:NH_4OH , 85:10:5); IR (KBr) 3299 (CONHst), 2935 (CH), 1731 (CH_3CO), 1643 (CONH), 1552 (NH), 1444 (CH_2), 1371 (CH_3), 1247 (C-Ost), 1039, 632 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.72 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.21 (3H, d, *J7*, 21-CH_3), 1.27-2.00 (23H: 9×CH_2, 5×CH), 2.07 (3H, s, 2'-CH_3), 2.35 (2H, d, *J7*, 4-CH_2), 2.76 (2H, t, *J7*, 27-CH_2), 3.27 (2H, m, 24-CH_2), 4.64 (1H, m, 3-CH), 5.40 (1H, d, *J5*, 6-CH), 5.87 (1H, t, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{28}H_{46}N_2O_3.1.02H_2O$: C, 70.5; H, 10.2; N, 5.9; Found: C, 70.5; H, 9.9; N, 5.7.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-1,6-diaminohexyl (4b). 0.374g, 60% yield; mp 148-149°C; R_f = 0.26 ($CHCl_3$:MeOH:NH_4OH , 85:10:5); IR (KBr) 3302 (CONHst), 2936 (CH), 1731 (CH_3CO), 1644 (CONH), 1547 (NH), 1462 (CH_2), 1371 (CH_3), 1247 (C-O), 1039 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.71 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 1.20 (3H, d, *J7*, 21-CH_3), 1.28-2.02 (27H: 11×CH_2, 5×CH), 2.05 (3H, s, 2'-CH_3), 2.34 (2H, d, *J7*, 4-CH_2), 2.70 (2H, t, *J7*, 29-CH_2), 3.24 (2H, m, 24-CH_2), 4.62 (1H, m, 3-CH), 5.39 (1H, d, *J5*, 6-CH), 5.53 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{30}H_{50}N_2O_3.0.72H_2O$: C, 72.1; H, 10.4; N, 5.6; Found: C, 72.1; H, 10.2; N, 5.3.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,8)-diaminooctyl (4c). 0.250g, 40% yield; mp 147-149°C; R_f = 0.38 ($CHCl_3$:MeOH:NH_4OH , 85:10:5); IR (KBr) 3302 (CONHst), 2929 (CH), 1728 (CH_3CO), 1643 (CONH), 1551 (NH), 1246 (C-O), 1038 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.78 (3H, s, 18-CH_3), 1.11 (3H, s, 19-CH_3), 1.18 (3H, d, *J7*, 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, *J7*, 4-CH_2), 2.77 (2H, t, *J7*, 31-CH_2), 3.28 (2H, m, 24-CH_2), 4.69 (1H, m, 3-CH), 5.47 (1H, d, *J5*, 6-CH), 5.53 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{32}H_{54}N_2O_3.1.01H_2O$: C, 72.1; H, 10.6; N, 5.3; Found: C, 72.1; H, 10.3; N, 4.8.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,10)-diaminodecyl (4d). 0.430g, 62% yield; mp 153-155°C; R_f = 0.44 ($CHCl_3$:MeOH:NH_4OH , 85:10:5); IR (KBr) 3289 (CONHst), 2927 (CH), 1728 (CH_3CO), 1642 (CONH), 1557 (NH), 1462 (CH_2), 1248 (C-Ost), 1036 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.67 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.16 (3H, d, *J7*, 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, *J7*, 4-CH_2), 2.65 (2H, t, *J7*, 33-CH_2), 3.20 (2H, m, 24-CH_2), 4.58 (1H, m, 3-CH), 5.35 (1H, d, *J4*, 6-CH), 5.44 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 for $C_{34}H_{59}N_2O_3$ ($[M+H]^+$): 543.4526; Found: 543.4522 ($M+H^+$). Anal. calcd. for $C_{34}H_{58}N_2O_3.0.42H_2O$: C, 74.2; H, 10.8; N, 5.1; Found: C, 74.2; H, 10.6; N, 4.8.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(trans-1,4)-diaminocyclohexyl (4e). 0.450g, 72% yield; mp 230-231°C; R_f = 0.26 ($CHCl_3$:MeOH:NH_4OH , 85:10:5); IR (KBr) 3319 (CONHst), 2939 (CH), 1729 (CH_3CO), 1643 (CONH), 1533 (NH), 1452 (CH_2), 1373 (CH_3), 1248 (C-Ost), 1037, 607 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.68 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.16 (3H, d, *J7*, 21-CH_3), 1.21-1.99 (27H: 11×CH_2, 5×CH), 2.04 (3H, s, 2'-CH_3), 2.32 (2H, d, *J7*, 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, *J4*, 6-CH), 5.66 (1H, d, *J8*, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{30}H_{48}N_2O_3.0.88H_2O$: C, 72.0; H, 10.0; N, 5.6; Found: C, 71.9; H, 9.8; N, 5.3

Bis-3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,6)-diaminohexyl (4f). Compound **4f** (0.163g, 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 (300MHz, $CDCl_3$): δ 0.81 (6H, s, 18-CH_3), 1.13 (6H, s, 19-CH_3), 1.30 (6H, d, *J7*, 21-CH_3), 1.36-2.12 (46H: 18×CH_2, 10×CH), 2.15 (6H, s, 2'-CH_3), 2.43 (4H, d, *J7*, 4-CH_2), 3.34 (4H, m, 24-CH_2), 4.68 (2H, m, 3-CH), 5.49 (2H, d, *J5*, 6-CH), 5.77 (2H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{54}H_{85}N_2O6$ ($[M+H]^+$): 857.6407; Found: 857.6415.

Bis-3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,8)-diaminooctyl (4g). Compound **4g** (0.241g, 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 (300MHz, $CDCl_3$): δ 0.64 (6H, s, 18-CH_3), 0.97 (6H, s, 19-CH_3), 1.13 (6H, d, *J7*, 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, *J7*, 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 (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,6)-diamino-6-N,N-dimethyl hexyl (4h). Compound **4h** (0.012g, 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 (300MHz, $CDCl_3$): δ 0.65 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.11 (3H, d, *J7*, 21-CH_3), 1.20-1.91 (27H: 11×CH_2, 5×CH), 1.98 (3H, s, 2'-CH_3), 2.26 (2H, d, *J7*, 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, *J5*, 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.

Procedure C:

3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-butylamine (5a). A mixture of **3a** (0.600g, 1.35mmol) and lithium hydroxide monohydrate (0.113g, 2.70mmol) in THF:H_2O (3:1, 32ml) 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** (0.562g, 97%) as a white solid: mp 202-203°C; R_f = 0.16 (40% EtOAc/Hexane); IR (KBr) 3287 (OH, CONHst), 2930 (CH), 1641 (CONH), 1551 (NH), 1458 (CH_2), 1369 (CH_3), 1068 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.83 (3H, s, 18-CH_3), 1.07 (3H, t, *J7*, 27-CH_3), 1.14 (3H, s, 19-CH_3), 1.32 (3H, d, *J7*, 21-CH_3), 1.37-2.37 (23H, 9×CH_2, 5×CH), 2.42 (2H, d, *J5*, 4-CH_2), 3.37 (2H, m, 24-CH_2), 3.66 (1H, m, 3-CH), 5.48 (1H, d, *J5*, 6-CH), 5.64 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-piperidylamine (5b). A mixture of **3b** (0.711g, 1.56mmol) and lithium hydroxide monohydrate (0.130g, 3.12mmol) in THF:H_2O (3:1, 40ml) 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 (CON), 1444 (CH_2), 1368 (CH_3), 1246 (C-OH), 1133, 1066, 1013, 955 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.76 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.18 (3H, d, *J7*, 21-CH_3), 1.26-2.33 (24H, 10×CH_2, 4×CH), 2.84 (1H, m, 20-CH), 2.32 (2H, d, *J5*, 4-CH_2), 3.58 (5H, m, 3-CH, 24-CH_2, 25-CH_2), 5.38 (1H, d, *J5*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{27}H_{43}NO_2.0.42H_2O$: C, 77.0; H, 10.5; N, 3.3; Found: C, 77.0; H, 10.4; N, 3.2

3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22--benzylamine (5c). A mixture of **3c** (0.787g, 1.65mmol) and lithium hydroxide monohydrate (0.138g, 3.29mmol) in THF:H_2O (3:1,

40ml) 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, CONHst), 2924 (CH), 1651 (CONH), 1521 (NH), 1456 (CH_2), 1346 (CH_3), 1209 (C-OH), 1069 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.73 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.27 (3H, d, *J7*, 21-CH_3), 1.30-2.32 (19H, 7×CH_2, 5×CH), 2.35 (2H, d, *J5*, 4-CH_2), 3.57 (1H, m, 3-CH), 4.47 (2H, dd, *J5*, *J5*, 24-CH_2), 5.40 (1H, d, *J5*, 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 (75MHz, $CDCl_3$): 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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,4)-diaminobutyl (6a). Compound **4a** (0.205g, 0.44mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6a** (0.180g, 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, CONH, NH_2), 2918 (CH), 1645 (CONH), 1549 (NH), 1452 (CH_2), 1372 (CH_3), 1058 cm^{-1}; ^1H NMR (300MHz, CD_3OD): δ 0.71 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 1.10 (3H, d, *J7*, 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, *J5*, 6-CH); ^{13}C NMR (75MHz, 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.

3β-Hydroxy -23,24-bisnor-chol-5-en-22-oxo-22-(1,6)-diaminohexyl (6b). Compound **4b** (0.165g, 0.33mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6b** (0.136g, 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, CONH, NH_2), 2935 (CH), 1645 (CONH), 1548 (NH), 1455 (CH_2), 1371 (CH_3), 1058 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.64 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 1.12 (3H, d, *J7*, 21-CH_3), 1.16-2.18 (27H: 11×CH_2, 5×CH), 2.23 (2H, d, *J3*, 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, *J5*, 6-CH), 5.46 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,8)-diaminooctyl (6c). A mixture of **4c** (0.100g, 0.19mmol) and lithium hydroxide monohydrate (0.016g, 0.38mmol) in THF:H_2O (3:1, 16ml) 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, CONH, NH_2), 2929 (CH),

1642 (CONH), 1548 (NH), 1057 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.72 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 1.20 (3H, d, *J7*, 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, *J7*, 31-CH_2), 3.24 (2H, m, 24-CH_2), 3.54 (1H, m, 3-CH), 5.36 (1H, d, *J5*, 6-CH), 5.68 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,10)-diaminodecyl (6d). Compound **4d** (0.200g, 0.36mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6d** (0.162g, 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, CONH, NH_2), 2928 (CH), 1642 (CONH), 1555 (NH), 1465 (CH_2), 1373 (CH_3), 1060 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.62 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.09 (3H, d, *J7*, 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 (1H, d, *J3*, 6-CH), 5.75 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(trans-1,4)-diaminocyclohexyl (6e). Compound **4e** (0.250g, 0.51mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6b** (0.162g, 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, CONH, NH_2), 2935 (CH), 1655 (CONH), 1508 (NH), 1457 (CH_2), 1372 (CH_3), 1202, 1071, 1005 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.61 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.08 (3H, d, *J7*, 21-CH_3), 1.13-2.14 (27H: 11×CH_2, 5×CH), 2.19 (2H, d, *J5*, 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, *J3*, 6-CH), 5.63 (1H, m, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-acetoxy-23,24-bisnor-chol-5-en-22-ol (7). A solution of borane-methyl sulfide complex (1M DCM) (38.61ml, 38.61mmol) was added drop-wise over 1 h. to a solution of 3β-acetoxy-5-cholenic acid (10.0g, 25.74mmol) in THF (100ml) and kept below 0°C by using a bath mixture of ice and sodium chloride. The reaction mixture was stirred for 8 hours. Then it was hydrolysed with water and potassium carbonate, filtered, diluted with diethylether, washed with sodium chloride (sat.), dried over sodium sulfate and concentrated. Chromatography over silica gel [MeOH/$CHCl_3$ (0→1%)] afforded compound **7** as a white solid (5.45g, 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_3COst), 1033, 741 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.65 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.01 (3H, d, *J7*, 21-CH_3), 1.98 (3H, s, 2'-CH_3), 1.05-1.97 (19H: 7×CH_2, 5×CH), 2.26 (2H, d, *J7*, 4-CH_2), 3.31 (1H, dd, *J3*, *J3*, 22-CH), 3.59 (1H, dd, *J3*, *J3*, 22'-CH), 4.55 (1H, m, 3-CH), 5.32 (1H, d, *J5*, 6-CH). ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-acetoxy-23,24-bisnor-chol-5-en-22-p-toluensulfonyloxy (8). Compound **7** (0.682g, 1.82mmol) was dissolved in pyridine:DCM (10ml:10ml) and cooled to 0°C. p-Toluensulfonylchloride (0.694g, 3.64mmol) 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 (sat.), dried over magnesium sulfate and the solvent removed to afford 0.844g (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 (300MHz, $CDCl_3$): δ 0.70 (3H, s, 18-CH_3), 0.95 (3H, d, *J7*, 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, *J7*, 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, *J5*, 6-CH), 7.40 (2H, d, *J8*, 28-CH, 29-CH), 7.82 (2H, d, *J5*, 26-CH, 27-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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_20] $^+$, 100), 298 ([M-AcO-H_20+H]$^+$, 55); MS (APCI $^-$) *m/z* (rel intensity) 171 (-OTs$^-$, 100).

3β-Acetoxy-23,24-bisnor-chol-5-en-22-butylamine (9a). A solution of **8** (0.383g, 0.72mmol), butylamine (0.72ml, 7.24mmol) and DIPEA (0.38ml, 2.17mmol) in DMF (10ml) 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 0.332g of crude. Chromatography over silica gel [MeOH/$CHCl_3$ (10%)] afforded 0.239g (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 (300MHz, $CDCl_3$): δ 0.78 (3H, s, 18-CH_3), 0.95 (3H, d, *J7*, 21-CH_3), 1.00 (3H, t, *J7*, 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, *J7*, 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, *J4*, 6-CH), 8.25 (1H, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{28}H_{47}NO_3.0.14H_2O$: C, 77.8; H, 11.0; N, 3.2; Found: C, 77.8; H, 11.1; N, 3.1

3β-Acetoxy-23,24-bisnor-chol-5-en-22-piperidyl amine (9b). To a solution of **8** (0.218g, 0.41mmol) in DMF (3ml) was added piperidine (0.12ml, 1.24mmol), potassium carbonate (0.085g, 6.18mmol) and tetrabutylammonium iodide, as the catalyst, (0.07g) and stirred at 100°C for 4 h. The reaction mixture was poured into water, extracted with ether, washed with NaCl (sat) and dried

over magnesium sulfate. Chromatography over silica gel [MeOH/$CHCl_3$ (1%)] afforded 0.083g (46%) of **9b** as a white solid: mp 155-157°C; R_f = 0.58 (20% MeOH/$CHCl_3$); IR (KBr) 2933 (CH), 1736 ($CH_3\underline{CO}$), 1443 (CH_2), 1368 (CH_3), 1247 ($CH_3\underline{CO}$st), 1034 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.74 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 1.10 (3H, d, *J7*, 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, *J4*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-benzyl amine (9c). To a solution of **8** (0.428g, 0.81mmol) in DMF (20ml) was added benzylamine (0.26ml, 2.43mmol), potassium carbonate (0.170g, 1.21mmol) and tetrabutylammonium iodide, as the catalyst, (0.14g) and stirred at 100°C for 4 h. The reaction mixture was poured into water, extracted with chloroform, washed with NaCl (sat) and dried over magnesium sulfate. Chromatography over silica gel [MeOH/$CHCl_3$ (10%)] afforded 0.183g (49%) of compound **9c** as a white solid: mp 132-134°C; R_f = 0.51 (10% MeOH/$CHCl_3$); ^1H NMR (300MHz, $CDCl_3$): δ 0.74 (3H, s, 18-CH_3), 0.90 (3H, s, 19-CH_3), 1.05 (H, d, *J7*, 21-CH_3), 1.09-2.07 (19H: 7×CH_2, 5×CH), 2.08 (3H, s, 2'-CH_3), 2.35 (2H, d, *J7*, 4-CH_2), 2.40 (1H, d, *J3*, 22-CH), 2.74 (1H, d, *J3*, 22'-CH), 3.78 (1H, d, *J13*, 24-CH), 3.89 (1H, d, *J13*, 24'-CH), 4.59 (1H, m, 3-CH), 5.42 (1H, d, *J4*, 6-CH), 7.29-7.39 (5H, CH aromatic), 8.31 (1H, 23-NH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,4-diaminobutyl (10a). To a solution of **8** (0.290g, 0.55mmol) in DMF (40ml) was added 1,4-diaminobutane (0.22ml, 2.19mmol), potassium carbonate (0.113g, 0.82mmol) and tetrabutylammonium iodide as the catalyst (0.10g), and stirred at 110°C for 4 h. The reaction mixture was poured into water, extracted with chloroform, washed with sodium chloride (sat) 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** (0.087g, 36% yield): mp 121-124°C; R_f = 0.27 (DCM:MeOH:NH_4OH, 85:10:5); ^1H NMR (300MHz, $CDCl_3$): 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, *J7*, 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, *J4*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,6-diaminohexyl (10b). Compound **8** (0.400g, 0.75mmol) and 1,6-diaminohexane (0.352g, 3.02mmol) were dissolved in ethanol (30ml) and DMF (15ml). 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** (0.074g, 20% yield) as a white solid: R_f = 0.27 (DCM:MeOH:NH_4OH, 85:10:5); ^1H NMR

(300MHz, $CDCl_3$): 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, *J8*, 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, *J4*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,8-diaminooctyl (10c). To a solution of **8** (0.370g, 0.69mmol) in DMF (30ml) was added 1,8-diaminooctane (0.403g, 2.79mmol), potassium carbonate (0.145g, 1.048mmol) and tetrabutylammonium iodide as the catalyst (0.07g) and stirred at 110°C for 4 h. The reaction mixture was poured into water, extracted with ether, washed with sodium chloride (sat) and dried over sodium sulfate. Chromatography over silica gel using firstly EtOAc and then MeOH/EtOAc (5%→10%, 5%Et_3N) as the eluent afforded a yellow solid, which it was identified as the diamine **10c** (0.130g, 37% yield): mp 132-133°C; R_f = 0.30 (DCM:MeOH:NH_4OH, 85:10:5); IR (KBr) 3286 (NHst), 2921 (CH), 1730 ($CH_3\underline{CO}$), 1470 (CH_2), 1372 (CH_3), 1259 (C-O), 1040, 802 cm^{-1}; ^1H NMR (300MHz, CD_3OD): δ 0.77 (3H, s, 18-CH_3), 1.04 (3H, d, *J7*, 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, *J7*, 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, *J5*, 6-CH); ^{13}C NMR (75MHz, 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 $C_{32}H_{56}N_2O_2.0.27H_2O$: C, 76.0; H, 11.3; N, 5.5; Found: C, 76.0; H, 11.2; N, 5.4.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,10-diaminodecyl (10d). 1,10-Diaminodecane (0.391g, 2.27mmol), potassium carbonate (0.117g, 0.85mmol) and tetrabutylammonium iodide, as the catalyst (0.117g), were added to a solution of **8** (0.300g, 0.56mmol) in DMF (40ml) and stirred at 110°C for 8 h. The reaction mixture was poured into water, extracted with chloroform, washed with sodium chloride (sat) 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** (0.141g, 47% yield): mp 131°C; R_f = 0.18 (MeOH:Et_3N:EtOAc, 30:5:65); ^1H NMR (300MHz, $CDCl_3$): δ 0.74 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 1.10 (3H, d, *J7*, 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, *J7*, 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, *J5*, 3-CH), 5.41 (1H, d, *J4*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,4-transdiaminocyclohexyl (10e). To a solution of **8** (0.400g, 0.75mmol) in DMF (30ml) was added *trans*-1,4-diaminocyclohexane (0.345g, 3.03mmol), potassium carbonate (0.157g, 3.03mmol) and tetrabutylammonium iodide as the

catalyst (0.05g) and stirred at 110°C for 3 days. The reaction mixture was poured into water, extracted with ether, washed with sodium chloride (sat) 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** (0.179g, 50% yield): mp 142-144°C; R_f = 0.24 (DCM:MeOH:NH_4OH, 85:10:5); IR (KBr) 2935 (CH), 1730 ($CH_3\underline{CO}$), 1455 (CH_2), 1370 (CH_3), 1250 (C-O), 1036 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.63 (3H, s, 18-CH_3), 0.92 (3H, d, *J7*, 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 (75MHz, $CDCl_3$): 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.

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 (0.037g, 10% yield): R_f = 0.43 (DCM:MeOH:NH_4OH, 85:10:5); ^1H NMR (300MHz, $CDCl_3$): 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, *J7*, 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, *J4*, 6-CH), 6.18 (1H, m, 30-NH), 8.19 (1H, s, 31-CHO); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-butylamine (11a). A mixture of **9a** (0.05g, 0.11mmol) and lithium hydroxide monohydrate (0.010g, 0.23mmol) in THF:H_2O (3:1, 16ml) 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 (NHst), 2955 (CH), 1458 (CH_2), 1376 (CH_3), 1260 (C-OH), 1064, 801 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$): δ 0.63 (3H, s, 18-CH_3), 0.87 (3H, d, *J7*, 21-CH_3), 0.93 (3H, s, 19-CH_3), 0.97 (3H, t, *J7*, 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 (75MHz, $CDCl_3$): δ 12.3 (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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-piperidyl amine (11b). A mixture of **9b** (0.140g, 0.32mmol) and lithium hydroxide monohydrate (0.040g, 0.95mmol) in THF:H_2O (3:1, 16ml) 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}; ^1H NMR (300MHz, $CDCl_3$): 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, *J5*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{27}H_{45}NO.0.32H_2O$: C, 80.0; H, 11.3; N, 3.5; Found: C, 80.0; H, 11.2; N, 3.4.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-1,8-diaminooctyl (12c). A mixture of **10c** (0.073g, 0.15mmol) and lithium hydroxide monohydrate (0.012g, 0.29mmol) in THF:H_2O (3:1, 16ml) 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 (0.061g). 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** (0.50g, 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}; ^{1}H NMR (300MHz, 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, *J5*, 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 $C_{30}H_{54}N_2O.1.5H_2O$: C, 74.2; H, 11.8; N, 5.8; Found: C, 73.9; H, 11.2; N, 5.3.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-1,10-diaminodecyl (12d). Diamine **10d** (0.057g, 0.11mmol) was hydrolysed as described in the synthesis of diamine **12c** using lithium hydroxide monohydrate (0.014g, 0.32mmol). The analogous procedure yielded diamine **12d** as a white solid (0.049g, 93%): R_f = 0.30 (DCM:MeOH:NH_4OH, 85:10:5); ^{1}H NMR (300MHz, $CDCl_3$): 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, *J4*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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.

3β-Hydroxy-23,24-bisnor-chol-5-en-22-1,4-transdiaminocyclohexyl (12e). Diamine **10e** (0.110g, 0.23mmol) was hydrolysed as described in the synthesis of diamine **12c** using lithium hydroxide monohydrate (0.029g, 0.70mmol). The analogous procedure yielded diamine **12e** as a white solid (0.060g, 60%): mp 173-175°C; R_f = 0.14 (DCM:MeOH:NH_4OH, 85:10:5); IR (KBr) 3260 (OH, NHst), 2930 (CH), 1452 (CH_2), 1375 (CH_3), 1066 cm^{-1}; ^{1}H NMR (300MHz, $CDCl_3$): 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, *J4*, 6-CH); ^{13}C NMR (75MHz, $CDCl_3$): 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 $C_{28}H_{48}N_2O.0.83H_2O$: C, 75.8; H, 11.3; N, 6.3; Found: C, 75.8; H, 11.1; N, 6.2.

Enzyme Assays

In assays of inhibition of 24-SMT, protein extracts from *E. coli* BL21 (DE3) pLysS/pET28a-HisLmSMT cells were used. Plasmid pET28a-HisLmSMT was obtained by cloning the entire coding sequence of the *Leishmania major SMT* gene in the pET28a vector (Novagen). *Leishmania major* recombinant SMT is produced as a His-tagged fusion protein and is over-expressed when induced with IPTG 1mM during 4 hours. Cells were disrupted by sonication in a buffer containing Tris-HCl 50mM pH 7.4, $MgCl_2$ 2mM, CHAPS 4mM, Tween 80 0.5% (v/v) and protease inhibitors (3 times, 30 seconds, duty cycle 50%). The sonicate was centrifuged at 12000 rpm for 30 minutes 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 50mM pH 7.4, $MgCl_2$ 2mM, CHAPS 4mM, Tween 80 0.5% (v/v), desmosterol 100 μM and ^{14}C-S-adenosyl-L-methionine 200 μM, 600000 dpm per reaction. The inhibitor was 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 minutes, and terminated with 0.5ml of KOH 10% dissolved in 80% (v/v) methanol. To quantify the efficiency of the extraction, ^{3}H-cholesterol (3 mg, 30000 dpm per reaction) was added as an internal standard. The methylated sterol product was extracted 3 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. 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.

Studies of lipid composition

L. mexicana amazonensis promastigotes were cultivated in LIT medium supplemented with lactabulmin and 10% fetal calf-serum (Gibco) (3) at 26°C, without agitation. The cultures were initiated with a cell density of 2.10^6 cells per ml and the drug was added at a cell density of 0.5-1.10^7 cells per ml. Cell densities were measured with an electronic particle counter (model ZBI; Coulter Electronics Inc., Hielah, Fla.) and by direct counting with a haemocytometer. Cell viability was followed by Trypan blue exclusion using light microscopy.

For the analysis of the effects of drugs on the lipid composition of promastigotes, total lipids from control and drug-treated cells were extracted and fractionated into neutral and polar lipid fractions by silicic acid column chromatography and gas-liquid chromatography.[36-39] 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 x 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 a 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.

In vitro Assays

L. donovani

Peritoneal exudate macrophages were harvested from CD1 mice, 24 hours 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 hours. *Leishmania 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 hours and then exposed to drug[40] for a total of 5 days, with the overlay being replaced on day 3.[41] The top concentration for the test compounds was 30μg/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 minutes) before being evaluated microscopically. ED_{50} (ED_{90}) values were calculated using Msx*l*fit. The ED_{50} value for the positive control drug, Pentostam©, is usually 3-8μgSb^V/ml.

T. cruzi

Murine (CD1) peritoneal macrophages were harvested 24 hours after starch induction. 100μl was dispensed into 96-well plates at a concentration of 4x 10^5/ml. After 24 hours the cells were infected with *Trypanosoma cruzi* Tulahuan LAC-Z trypomastigotes. 24 hours later the infected cells were exposed to drug[20] for 3 days. 50μl of 500μMCPRG:1% nonidet P-40 was added to each well. The plates were read after 2-5 hours, λ570.[42] ED_{50} (ED_{90}) values were calculated using Msx*l*fit. L6 fibroblasts are also used as host cells.

T. brucei

Trypanosoma brucei rhodesiense STIB900 bloodstream form (bsf) trypomastigotes were maintained in HMI-18 medium (Hirumi and Hirumi,1989) with 15% heat-inactivated foetal calf serum (HIFCS) [Harlan-SeraLab, UK] at 37°C, 5%, CO_2/air mixture. Trypomastigotes were washed and resuspended in fresh medium at a concentration of 2x10^5/ml. 100μl was added to the drug dilutions. The top concentration for the test compounds was 30μg/ml. The ED_{50} for pentamidine is usually between 1.0 and 0.1ng/ml. Plates were incubated for 72 hours at 37°C, 5% CO_2. At 72 hours the plates were assessed microscopically before Alamar Blue was added (Raz *et al.*, 1997). Plates were read after 5-6 hours on a Gemini Fluorescent plate reader (Softmax Pro. 3.1.1, Molecular Devices, UK) at EX/EM 530/585nm with a filter cut-off at 550nm. ED_{50} values were calculated with Msx*l*fit (IDBS, UK)

Cytotoxicity against mammalian cells

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

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