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Original article

Synthesis of oxysterols and nitrogenous sterols with antileishmanial and trypanocidal activities

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Abstract

Two sterol families have been synthesized: the first one is nitrogenous sterols containing amino, *N*-hydroxyimino or cyano group and the second one is oxysterols such as ketosterol and hydroxysterols. These compounds were then evaluated in vitro against *Leishmania donovani* promastigotes and *Trypanosoma brucei brucei* trypomastigotes. The most active compounds against *L. donovani* promastigotes were 7β-aminomethylcholesterol and 7α,β-aminocholesterol (IC_{50} in a range from 1 to 3 μM, pentamidine: 2.8 μM). These compounds were active on intramacrophage amastigotes with IC_{50} of 1.3 μM. Such an activity justifies further in vivo antileishmanial evaluation. Against *T. b. brucei*, (24R, S)-24-hydroxy-24-methylcholesterol (MEC, 12.5 μM) was the most active compound from these series.

Keywords: Antileishmanial; Trypanocidal; Nitrogenous sterols; Oxysterols

1. Introduction

Leishmaniases are parasitic diseases due to several species of unicellular parasite in the genus *Leishmania* that is endemic in several parts of the world. They are a complication of AIDS in both the developing world and industrialized world [1]. The parasite is transmitted to some mammals and humans by the bite of an insect vector, the female phlebotome sandfly. Since about 50 years, the antimonials sodium stibogluconate (Pentostam®, Glaxo Wellcome, UK) and meglumine antimoniate (Glucantime®, Aventis, France) are the first-line treatment for leishmaniases. Amphotericin B (AmB) and pentamidine, as alternatives to antimony are considered to cause serious and irreversible toxic effects [2]. The antifungal agent AmB, a polyene macrolide antibiotic [3], has long been recognized as a powerful antileishmanial drug [4], and it is the most active antileishmanial agent in use. Its activity results from the specific target of AmB at the level of sterols, mainly ergosterol, which is found in the membrane of *Leishmania* genus and fungi [5]. However, sometimes the high level of toxicity forbids its clinical uses. Largely liposomal AmB developed as Ambisome® gave interesting results by reducing the renal toxicity [6]. Other new approaches have been proposed including the use of other parenteral and non-parenteral agents such as the aminoglycoside, aminosidine (topical application) or oral agents. Oral administration has the advantage of reducing socio-economic difficulties that are present in endemic areas where health facilities are lacking. Since 4 years, hexadecylphosphocholine (HePC, miltefosine), an antineoplastic agent, has been identified as the first effective oral treatment in visceral infection [7]. This newly developed drug is a real advance in antileishmanial chemotherapy. However, the possibility to obtain miltefosine-resistant parasites by drug pressure is an indicator of a potential appearance of miltefosine-resistant cases in the field [8]. This situation justifies the research for new drugs because new therapeutic agents are urgently

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required. One target of interest is sterol biosynthesis. The main sterol found in mammalian cells is cholesterol, while the pathogens which cause Chagas disease and leishmaniasis (*Trypanosoma cruzi* and species of *Leishmania*, respectively), synthesize ergosterol and related 24-alkylated sterol [9,10]. These sterols have differences in their biosynthetic pathway that are attractive for drug design. The ergosterol is thought to have two roles within these kinetoplastid parasites; it has a structural role in the cell membrane and may have a "sparking" or " hormonal" role similar to that seen in yeast. In *Leishmania* sp. the case is less certain.

Leishmania parasites have a strict requirement for specific endogenous sterols for survival and growth and they cannot use the abundant supply of cholesterol present in their mammalian hosts [11]. Therefore, the inhibitory biosynthesis of *Leishmania* sterols was proposed as a source of potential targets for therapy [12]. It has been shown that various antifungal agents as sterols biosynthesis inhibitors have been described to have clinically useful antileishmanial properties [13–15]. For example, inhibitors of 14α-demethylase, sterol 24-methyltransferase, Δ^8-Δ^7-sterol isomerase and Δ^{14}-sterol reductase have been shown as having anti-parasitic activity [16,17]. Thus, a large number of inhibitors of enzymes from the sterol pathway have been studied for their in vitro and in vivo antileishmanial properties such as azoles and azasterols [18–20].

Concerning *Trypanosomatidae*, the bloodstream forms of *Trypanosoma brucei brucei* differs from other trypanosomes as it contains predominantly cholesterol and apparently suppresses de novo synthesis of C-28-sterols; *T. brucei* assimilates host cholesterol from serum lipoproteins to meet its sterol requirement [21].

The rationale of the present study is based on the fact that modified sterols cause a depletion of normal sterols and an accumulation of abnormal amounts of sterol precursors with cytostatic or cytotoxic consequences [22].

In the frame of our work, we have focused on a study of aminosterols [23–26] since we had shown previously that 7α,β-aminocholesterol (77% α epimer and 23% β epimer) inhibits Δ^8-Δ^7-sterol isomerase and Δ^{14}-sterol reductase of fungi as morpholine inhibitors did. In addition, this epimeric mixture is fungicidal and active against *Saccharomyces cerevisiae* resistant strains. Then, 7α and 7β-aminocholesterol were then selectively synthesized. According to in vitro bioassay studies on resistant strains, these aminosterols were more active than AmB on *Candida tropicalis* (AmB-resistant). No significant variation in the cytotoxicity of the two epimers 7α or 7β was observed against the three resistant strains [26]. On the other hand, we have synthesized novel polyaminosterols as squalamine analogues. These molecules showed similar antifungal activities as squalamine [27,28].

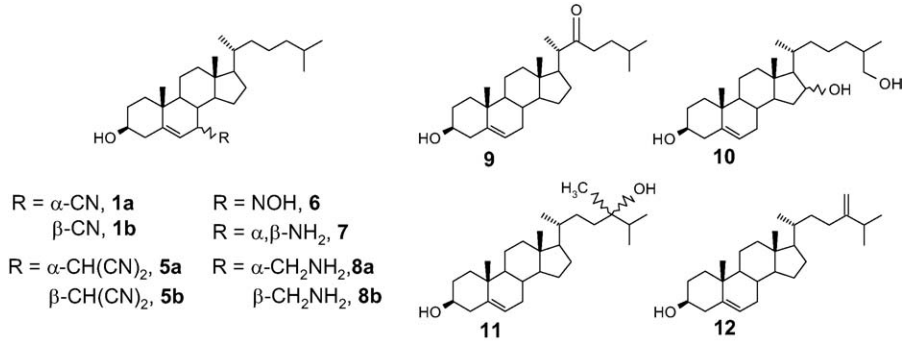
Considering the analogy in the sterol biosynthesis pathways between fungi and *Leishmania*, we decided therefore to synthesize oxysterols and nitrogenous sterols, especially designed to interact with the sterol metabolism. These molecules were then evaluated, in vitro, against *Leishmania donovani* and *T. brucei brucei*. The choice of *T. brucei* relies on the fact that this disease dramatically increases in Africa and new drugs are needed urgently whereas *T. cruzi* infections are in diminution in South America because of the improvement of habitat suppressing the contact between triatomine insect vector and humans (Scheme 1).

2. Results and discussion

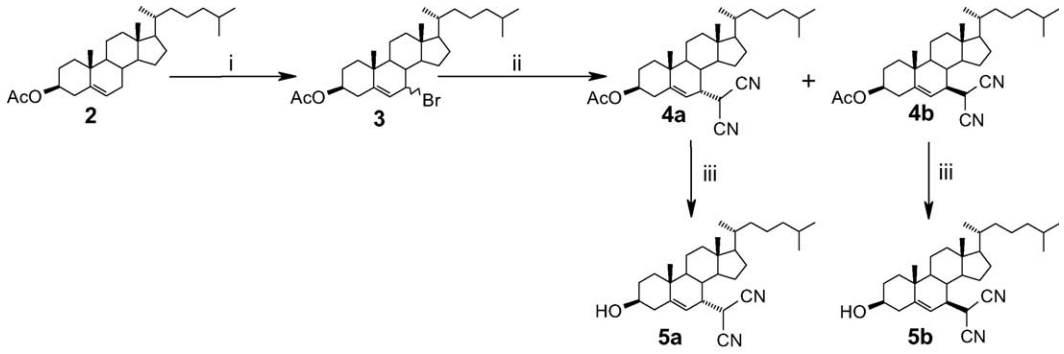
2.1. Chemistry

Compounds **1a**, **1b**, **7**, **8a** and **8b** were prepared according to our procedure described in literature [29].

Dicyanomethylcholesterols **5a** and **5b** were prepared as shown in Scheme 2: cholesteryl-3β-acetate **2** was brominated by *N*-bromosuccinimide, which gave an epimeric mixture of monobromide compounds **3** (7α/7β, 7:3 ratio). 7-Bromocholesteryl-3β-acetate was prepared by Confalone et al. as intermediate to prepare the 7-dehydrocholesterol using 5,5-dimethylhydantoin. Epimeric mixture **2** was not described by analysis spectra but 7α/7β bromides ratio was determined via 7-phenylsulfide after separation by chromatography on silica gel column [30]. In our case, epimers bromides were purified by flash chromatography. The structure and 7α/7β bromides ratio were established by ^{1}H NMR spectra. Due to the relative instability of the bromide product, the crude product was used to next step without further purification. The crude bromide mixture **3** was substituted by malononitrile in the presence of sodium hydride in THF. The nitrile mixture (**4a** and **4b**) was easily separated by chromatography. Each epimer **4a** and **4b**



Scheme 1. Structure of oxysterols and nitrogenous sterols.



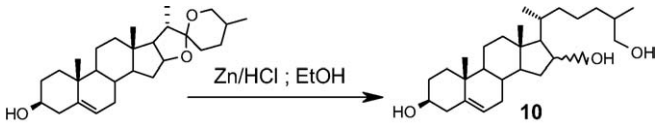
Scheme 2. Synthesis of **5a** and **5b**.

was saponified by potassium hydroxide in methanol to give **5a** and **5b**.

7*N*-hydroxyiminocholesterol **6** was obtained by saponification of 7*N*-hydroxyiminocholesteryl-3β-acetate which was synthesized according to previously described procedure [26]. 22-Ketocholesterol **9** was prepared from 3β-acetoxy-23,24-bisnor-5-cholenic acid according to the published procedure [31].

16(R,S),26-dihydroxycholesterol **10** was prepared from diosgenin as the starting material, allowing access to sterols containing substituents at the 16 and 26 positions using the established literature method (Scheme 3) [34].

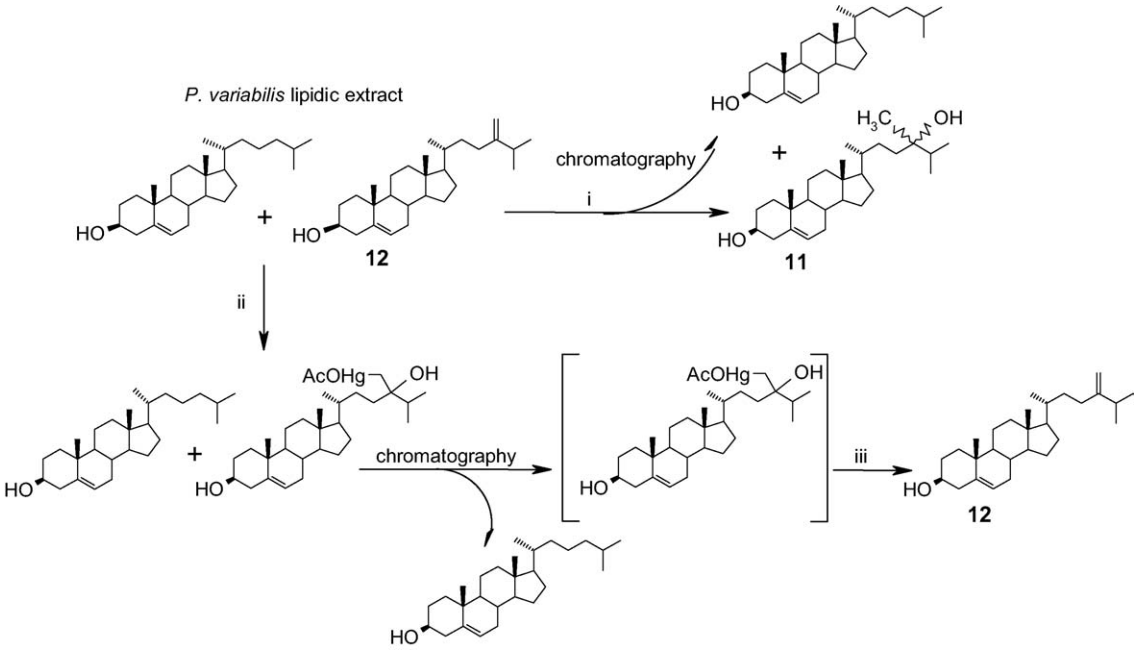
Compounds **11** and **12** were obtained from a marine invertebrate, *Palythoa variabilis* (genus *Cnidaria*). Lipidic extract of this *Cnidaria* was analyzed by Diop et al. [33] CPG analysis



Scheme 3. Synthesis of compound **10** from diosgenin.

showed a mixture of 24-methylenecholesterol, cholesterol and sitosterol (93:4:3 ratio). (24R,S)-24-hydroxy-24-methylcholesterol **11** was prepared from 24-methylenecholesterol (rare sterol) by hydroxymercuration. *P. variabilis* lipidic extract was used as the starting material which was treated with a mercuric acetate excess in Brown's conditions (THF/water, 5:5) followed by hydrodemercuration with $NaBH_4$/NaOH. After extraction, it was markedly separated by elution (cyclohexane/ethyl acetate, 5:5) on silica gel column. Unreacted sterol (cholesterol) was eluted first, followed by the hydroxysterol **11** (Scheme 4). Analysis was based on comparison by gas chromatography of the initial sterols mixture with unreacted sterol. Structures of the resulting hydroxylated compounds were established by NMR (^1H, ^{13}C and DEPT), mass spectrometry and IR.

The 24-methylenecholesterol **12** was isolated from *P. variabilis* lipidic extract by hydroxymercuration–desoxymercuration. The lipidic extract was treated with a mercuric acetate excess in THF/water (5:5). The organomercurial compound was easily separated by flash chromatography on silica gel col-



Scheme 4. Synthesis of (24R,S)-24-hydroxy-24-methylcholesterol **11** and isolation of 24-methylenecholesterol **12**.

umn and the reactive alkene was regenerated by treatment in a biphasic system (ethyl acetate/1 M HCl, 2:1) (Scheme 4).

A high-yielding directed chemoselective hydroxymercuration was achieved on methylenic sterol. These conditions leave intact the ring double bonds of steroids. No isomerization of double bond was thus observed in these experimental conditions.

2.2. Biology

These series exhibited a strong activity against *Leishmania* whereas the activity against *Trypanosoma* was slight. The activities observed on the promastigote form of two *L. donovani* strains (LV9 and DD8) were similar. In addition, a similar activity was observed against a *L. donovani* DD8 AmB-R clone. This clone is resistant to AmB, a drug having sterols as the main membrane target [32]. The membranes of the *L. donovani* DD8 AmB-R clones lack of 24-methylated sterols such as ergosterol. This change in the membrane composition had no effect on the oxy- and nitrogenous sterols antileishmanial properties. Although *Leishmania* sp. cannot use the supply of cholesterol from the external medium for its own sterol synthesis, the activity observed against promastigotes suggests that oxysterols and nitrogenous sterols easily penetrate the parasite provoking the growth inhibition and cell death.

In our work, two sterol families have been studied: the first is nitrogenous sterols containing amino, *N*-hydroxyimino or cyano group and the second is oxysterols such as ketosterol and hydroxysterols (Tables 1 and 2).

The most active compounds against *Leishmania* promastigotes were aminosterols **7**, **8a** and **8b** with IC_{50} values in a range from 1.2 to 3.6 μM. 7*N*-hydroxyiminosterol **6** showed a comparable activity such as aminosterols (IC_{50} = 3.7 μM). Cyanosterols **1a**, **1b**, **5a** and **5b** showed a low activity (IC_{50} = 26.3–73.2 μM). Aminosterols **7**, **8a** and **8b** have been evaluated as antifungal agents and inhibit Δ^8-Δ^7-sterol isomerase and Δ^{14}-sterol reductase as morpholine inhibitors [23]. Morpholine derivatives contain a nitrogen protonated in biological medium, which mimics the carbocationic high energy intermediates (HEI) involved in the Δ^8-Δ^7-sterol isomerase and Δ^{14}-reductase. The Δ^8-Δ^7-isomerase reaction is conducted with

Table 1
In vitro antileishmanial and trypanocidal activities of oxysterols and nitrogenous sterols

Compounds	*L. donovani* LV9 promastigotes	*L. donovani* DD8 WT promastigotes	*L. donovani* DD8 AmB-R promastigotes	*T. brucei* MEC (μM)
	IC_{50} (μM ± S.D.)	IC_{50} (μM ± S.D.)	IC_{50} (μM ± S.D.)	
1a	73.2 ± 6.8	42.2 ± 5.6	35.8 ± 4.1	100
1b	76.3 ± 5.3	47.3 ± 5.9	40.7 ± 3.6	100
5a	62.6 ± 4.1	> 100	75.7 ± 8.2	> 100
5b	26.3 ± 2.8	36.8 ± 3.9	27.8 ± 3.2	100
6	3.7 ± 0.4	3.5 ± 0.4	2.9 ± 0.3	50
7	1.2 ± 0.2	3.2 ± 0.3	2.2 ± 0.3	100
8a	3.6 ± 0.5	3.6 ± 0.4	3.2 ± 0.4	100
8b	1.4 ± 0.2	1.8 ± 0.2	1.2 ± 0.1	100
9	4.4 ± 0.4	4.2 ± 0.3	1.6 ± 0.1	100
10	4.3 ± 0.5	4.7 ± 0.3	2.4 ± 0.2	25
11	4.2 ± 0.2	4.5 ± 0.5	3.0 ± 0.2	12.5
12	4.2 ± 0.3	4.2 ± 0.4	3.1 ± 0.3	50
AmB	0.2 ± 0.1	0.1 ± 0.1	2.2 ± 0.3	/
Pentamidine	2.8 ± 0.3	7.1 ± 0.8	8.6 ± 0.9	2.1 ± 0.3

Results are the mean of three independent experiments ± S.D. Results are expressed in IC_{50} after a 72 h incubation period for *L. donovani* promastigote lines (*L. donovani* LV9, *L. donovani* DD8 WT and *L. donovani* DD8 AmB-R). Results are expressed in MEC for *T. brucei brucei* GVR 35 trypomastigote forms.

Table 2
In vitro antileishmanial activity of compounds **7**, **8a** and **8b** against intramacrophage amastigotes of *L. donovani* LV9

Compounds	Concentration		Mean number and S.D. of amastigotes/ infected macrophage	Mean number of amastigotes/100 macrophage nuclei	Macrophage infection (%)	Reduction in number of amastigotes/100 nuclei (%)	IC_{50} (μM)
	(μg ml^{-1})	(μM)					
7	1.56	3.88	/	/	/	/	1.31 (1.20–1.57)
	0.78	1.94	5.24 ± 3.16	208	40	59	(r = 0.982)
	0.39	0.97	8.82 ± 4.80	405	46	20	
	0.20	0.49	13.20 ± 7.40	515	52	0	
8a	2.50	6.00	/	/	/	/	ND
	1.25	3.00	12.50 ± 7.20	485	52	4	
8b	2.50	6.00	/	/	/	/	1.37 (1.17–1.61)
	1.25	3.00	1.25 ± 0.50	20	16	96	(r = 0.987)
	0.63	1.50	6.80 ± 2.61	184	27	64	
	0.31	0.75	9.51 ± 5.73	465	49	8	
Untreated control	/	/	12.50 ± 5.12	660	53	0	/

Compound **7**: toxic on macrophages at 3.88 μM. Compounds **8a** and **8b**: toxic on macrophages at 6 μM. IC_{50} of AmB, as reference compound was 1.05 (0.87–1.25) r = 0.986.

the initial addition of a proton of C-9 giving a stabilized carbonium ion at C-8 [35]. Amine function at C-7 protonated as ammonium in physiological media, is specially a better mimic. Moreover, the basic nitrogen is necessary of protonation and stronger interaction with the enzyme. However, Δ^8-Δ^7-sterol isomerase and Δ^{14}-sterol reductase have not yet been described in *Leishmania*. Oxysterols **9**, **10**, **11** and **12** have comparable activities (IC_{50} = 4.2–4.4 μM). No difference activity has been shown between these hydroxysterols.

Among the three compounds (**7**, **8a** and **8b**) tried on the intramacrophage amastigotes of *L. donovani* LV9, two of them (**7** and **8b**) exhibited an IC_{50} value of 1.3 μM. Compounds **7**, **8a** and **8b** were responsible for a change in macrophage shape at 3.88, 6 and 6 μM, respectively, suggesting a slight toxicity for macrophages at these concentrations. Despite this low therapeutic index (1.3/3.88 and 1.3/6) such an activity similar to those of AmB justifies further in vivo evaluation of these compounds on the *L. donovani*/Balb/c mouse model.

Against the bloodstream forms of *T. brucei*, the most active compound was compound **11** with a MEC value of 12.5 μM. Compounds **10**, **6** and **12** exhibited MEC values in a range from 25 to 50 μM. All other compounds, except inactive compound **5a**, were active at 100 μM. There is no positive correlation between antileishmanial and trypanocidal properties. Such results are not encouraging enough to perform further in vivo evaluation on the *T. brucei*/mouse model.

3. Conclusion

These series of 12 oxysterols and nitrogenous sterols were successfully prepared. The aim of this study was to design more active and selective compounds. Aminosterols exhibited a strong antileishmanial activity both against promastigote and intramacrophagic amastigotes of *L. donovani*. Such interesting in vitro results prompt us to carry out in vivo evaluation against *L. donovani*.

4. Experimental

4.1. Chemistry

All solvents were distilled and dried prior to use. Reagents and materials were obtained from commercial suppliers and were used without further purification. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick). The spots were detected by upon spraying with sulfuric acid/ethanol (2:8) and heating. Column chromatography was carried out using silica gel 60 (0.063–0.2 mm) (Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were recorded on a Perkin–Elmer 1600 FT-IR spectrometer. Specific rotation was measured in chloroform with a Perkin–Elmer 343 polarimeter. Mass spectra were recorded in positive mode on a Finnigan MAT 95 S spectrometer using electrospray ionization and EI mass spectra were recorded on a Jeol-GCmate (GC–MS system) spectrometer with ionization energy from 30 to 40 eV. ^{1}H NMR and ^{13}C NMR spectra were recorded using $CDCl_3$, respectively, at 400 MHz (Jeol Lambda 400 spectrometer) and at 100 MHz. Chemical shifts are reported relative to TMS; *J* values are given in Hz. ^{13}C NMR spectra are ^{1}H-decoupled.

Compounds **1a**, **1b**, **7**, **8a** and **8b** were prepared according to our literature procedure [29].

16(R,S),26-dihydroxycholesterol **10** was obtained according to the literature procedure starting from diosgenin [4].

4.1.1. 7α,β-Bromocholesteryl 3β-acetate *3*

Cholesteryl-3β-acetate **2** (6 g, 13.9 mmol) was dissolved in heptane (50 ml) at 50 °C. *N*-bromosuccinimide (3 g, 16.8 mmol) was added and the solution was irradiated with UV lamp for 15 min (yellow solution). The solution was cooled to 0 °C, filtered and evaporated. The sample crude product (flash chromatography on alumina (cyclohexane/ethyl acetate, 9:1)) was purified for characterization by ^{1}H NMR spectra. The crude product was used to next step without purification.

IR (KBr) υ (cm^{-1}): 2946–2868 (C–H alkane), 1732 (C=O ester), 738 (C–Br). ^{1}H NMR ($CDCl_3$, 400 MHz, 25 °C), δ: 0.70 (s, 3 H, 18-Me), 0.87 (d, J = 6.5 Hz, 6 H, 26-Me and 27-Me), 0.93 (d, J = 6.5 Hz, 3 H,21-Me), 1.06 (s, 3 H, 19-Me), 2.03 (s, 3 H, CH_3COO–), 4.60 (m, 1 H, 3-H), 4.66 (dd, $J_{7\alpha\text{-}8}$ = 8.0 Hz, $J_{7\alpha\text{-}6}$ = 2.1 Hz, 0.3 H, 7α-H of β epimer), 4.69 (dd, $J_{7\beta\text{-}8}$ = 5.0 Hz, $J_{7\beta\text{-}6}$ = 5.0 Hz, 0.7 H, 7β-H of α epimer), 5.62 (d, $J_{6\text{-}7\alpha}$ = 2.1 Hz, 0.3 H, 6-H of β epimer), 5.80 (d, $J_{6\text{-}7\beta}$ = 5.2 Hz, 0.7 H, 6-H of α epimer).

4.1.2. 7α-Dicyanomethylcholesteryl 3β-acetate *4a* and 7β-dicyanomethylcholesteryl 3β-acetate *4b*

A solution of malononitrile (1.018 g, 13.9 mmol) in dry THF (5 ml) was added to a solution of sodium hydride (0.6 g, 15.4 mmol) in dry THF (40 ml). The solution was stirred for 30 min at room temperature under argon atmosphere. The mixture was then refluxed for 1 h, cooled to 0 °C and **3** (6.5 g as crude product) dissolved in THF (7 ml) was added dropwise. The mixture was stirred for 48 h at room temperature. The solution was diluted with water (30 ml) and extracted with dichloromethane (3 × 60 ml). The organic layer was washed with 1 M HCl (10 ml), 5% $NaHCO_3$ (10 ml), water (2 × 10 ml), and dried over anhydrous sodium sulfate. The solution was evaporated under reduced pressure and the crude product was purified by chromatography on silica gel column (cyclohexane/dichloromethane, 3:7) to afford compounds **4a** (α epimer, 2.24 g, 34%) and **4b** (β epimer, 0.96 g, 14%) as yellow solids.

4.1.3. 7α-Dicyanomethylcholesteryl-3β-acetate *4a*

$[\alpha]_D$ = –30° (C = 1 in $CHCl_3$). IR (KBr) υ (cm^{-1}): 2949–2870 (C–H alkane), 2215 (C≡N), 1736 (C=O ester). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ: 0.71 (s, 3 H, 18-Me), 0.86 (dd, J = 6.5 Hz, J = 2.0 Hz, 6 H, 26-Me and 27-Me), 0.92 (d, J = 6.5 Hz, 3 H, 21-Me), 1.08 (s, 3 H, 19-Me), 2.05 (s, 3 H, CH_3COO–), 2.46 (m, 1H, 7-H), 3.92 (d, $J_{\text{Hmalon.-}7\beta}$ = 2.7 Hz, 1 H, –$CH(CN)_2$), 4.72 (m, 1 H, 3-H), 5.65 (dd, $J_{6\text{-}7\beta}$ = 4.8 Hz,

$J_{6\text{-}4}$ = 2 Hz, 1 H, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C), δ: 12.0, 18.7, 19.4, 21.2, 21.3, 22.5, 22.7, 23.8, 26.4, 26.9, 27.5, 28.0, 28.3, 35.4, 35.6, 36.0, 36.1, 36.4, 37.4, 39.4, 39.4, 43.6, 43.6, 49.9, 55.1, 56.3, 72.8, 111.8, 112.6, 118.5, 147.8, 170.4. ESIMS: m/z = 515.4 $[M + Na]^+$.

4.1.4. 7β-Dicyanomethylcholesteryl-3β-acetate **4b**

$[\alpha]_D$ = +30° (C = 1 in $CHCl_3$)IR (KBr) υ (cm^{-1}): 2942–2868 (C–H alkane), 2254 (C≡N), 1728 (C=O ester). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ: 0.72 (s, 3 H, 18-Me), 0.86 (dd, J = 6.5 Hz, J = 2.0 Hz, 6 H, 26-Me and 27-Me), 0.92 (d, J = 6.5 Hz, 3 H, 21-Me), 1.09 (s, 3 H, 19-Me), 2.05 (s, 3 H, CH_3COO–), 2.43 (m, 1H, 7-H), 4.08 (d, $J_{\text{Hmalon.-7}\alpha}$ = 3.2 Hz, 1 H, –$CH(CN)_2$), 4.62 (m, 1 H, 3-H), 5.43 (dd, $J_{6\text{-}7\alpha}$ = 2.2 Hz, $J_{6\text{-}4}$ = 2.0 Hz, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C), δ: 12.2, 18.7, 19.5, 21.3, 21.3, 22.5, 22.8, 23.8, 26.5, 26.9, 27.6, 28.0, 28.3, 35.5, 35.6, 36.1, 36.2, 36.5, 37.9, 39.4, 39.5, 43.6, 43.7, 49.9, 55.3, 56.4, 72.9, 111.9, 112.7, 118.6, 148.0, 170.5. MS (30 eV, EI): m/z (%) = 432 (13) [M^+–AcOH], 367 (100) [M^+– (AcOH + $CH(CN)_2$)], 145 (66), 109 (28), 81 (80).

4.1.5. 7α-Dicyanomethylcholesterol **5a**

Compound **4a** (1.1 g, 2.23 mmol) was added to a solution of potassium hydroxide (0.38 g, 6.69 mmol) in methanol (20 ml) and the mixture was stirred for 24 h at room temperature. The solution was acidified (pH 6), diluted with water and extracted with dichloromethane (3 × 20 ml). The organic layer was washed with 5% $NaHCO_3$, water and dried over anhydrous sodium sulfate. The solution was evaporated and the crude product was purified by chromatography (cyclohexane/ethyl acetate, 8:2) to afford 0.5 g (50%) of nitrile **5a** as a white solid. M.p. 163 °C. $[\alpha]_D$ = –50° (C = 0.1 in $CHCl_3$). IR (KBr) υ (cm^{-1}): 3292 (O–H alcohol), 2932–2869 (C–H alkane), 2253 (C≡N). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ: 0.70 (s, 3 H, 18-Me), 0.86 (dd, J = 6.5 Hz, J = 2.0 Hz, 6 H, 26-Me and 27-Me), 0.93 (d, J = 6.5 Hz, 3 H, 21-Me), 1.04 (s, 3 H, 19-Me), 2.39 (m, 1 H, 7β-H), 3.69 (m, 1 H, 3-H), 3.91 (d, $J_{\text{Hmalon.-7}\beta}$ = 2.7 Hz, 1 H, –$CH(CN)_2$), 5.63 (dd, $J_{6\text{-}7\beta}$ = 5.0 Hz, $J_{6\text{-}4}$ = 1.7 Hz, 1 H, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C), δ: 11.7, 18.7, 19.1, 19.2, 20.8, 21.3, 22.5, 23.6, 24.3, 24.6, 26.8, 27.9, 31.1, 34.6, 35.5, 36.8, 37.2, 38.8, 39.7, 42.3, 42.4, 42.9, 49.9, 55.5, 56.4, 71.3, 111.8, 113.0, 117.6, 149.0. ESIMS: m/z = 473.4 $[M + Na]^+$.

4.1.6. 7β-Dicyanomethylcholesterol **5b**

Nitrile **4b** (0.5 g, 1.02 mmol) was saponified in the same manner as **4a** to afford **5b** (0.35 g, 80%). M.p. 174 °C. $[\alpha]_D$ = +50° (C = 0.1 in $CHCl_3$). IR (KBr) υ (cm^{-1}): 3420 (O–H alcohol), 2950–2868 (C–H alkane), 2254 (C≡N). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ: 0.72 (s, 3 H, 18-Me), 0.86 (dd, J = 6.5 Hz, J = 2.0 Hz, 6 H, 26-Me and 27-Me), 0.92 (d, J = 6.3 Hz, 3 H, 21-Me), 1.08 (s, 3 H, 19-Me), 2.35 (m, 1H, 7-H), 3.56 (m, 1 H, 3-H), 4.09 (d, $J_{\text{Hmalon.-7}\alpha}$ = 2.9 Hz, 1 H, –$CH(CN)_2$), 5.40 (dd, $J_{6\text{-}7\alpha}$ = 2.2 Hz, $J_{6\text{-}4}$ = 2.2 Hz, 1 H, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C), δ: 12.0, 18.7, 19.5, 21.3, 22.5, 22.8, 23.8, 26.5, 26.8, 28.0, 28.3, 31.3, 35.5, 35.6, 36.0, 36.1, 36.7, 39.4, 39.5, 42.0, 43.6, 43.7, 49.9, 55.1, 56.4, 70.9, 111.8, 112.7, 117.7, 148.9. FABMS: m/z (%) = ESIMS: m/z = 473.4 $[M + Na]^+$.

4.1.7. N-Hydroxyiminocholesterol **6**

7*N*-(Hydroxy)iminocholesteryl 3β-acetate was obtained to according procedure [26].

This oxime (1.2 g, 2.63 mmol) and potassium hydroxide (0.44 g, 7.89 mmol) were stirred in ethanol (20 ml) for 10 h. The solution was neutralized (1 M HCl), concentrated and diluted with water. The mixture was extracted with dichloromethane (3 × 20 ml). The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated. The residue **6** was crystallized from acetone (0.582 g, 80%). IR (KBr) υ (cm^{-1}): 3452 and 3282 (O–H oxime and alcohol), 2944–2870 (C–H alkane), 1646 (C=N oxime). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ: 0.70 (s, 3 H, 18-Me), 0.86 (dd, J = 6.6 Hz, J = 1.5 Hz, 6 H, 26-Me and 27-Me), 0.92 (d, J = 6.5 Hz, 3 H, 21-Me), 1.13 (s, 3 H, 19-Me), 3.64 (m, 1 H, 3-H), 6.54 (d, $J_{6\text{-}4}$ = 2.0 Hz, 1 H, 6-H), 6.77 (br s, 1 H, N–OH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C), δ: 12.1, 18.0, 18.9, 20.8, 22.6, 22.8, 23.8, 27.2, 28.0, 28.3, 31.3, 35.6, 36.2, 36.6, 38.0, 38.4, 38.6, 39.5, 42.2, 42.8, 49.7, 50.2, 54.7, 71.1, 112.7, 153.5, 158.2. MS (30 eV, EI): m/z (%) = 415 (36) [M^+], 400 (100) [M^+ – Me], 398 (53) [M^+ – OH], 381 (14), 186 (19). ESIMS: m/z = 438.4 $[M + Na]^+$.

4.1.8. (24R,S)-24-hydroxy-24-methylcholesterol **11** and 24-methylenecholesterol **12**

Lipidic extract: *P. variabilis* was crushed (800 g) and refluxed in a methanol/dichloromethane mixture (ratio 1:2) for 48 h. The mixture was filtered and the solution was washed with water, dried under anhydrous sodium sulfate and evaporated to give 5.54 g of extract. The crude product (5.54 g) was then treated with potassium hydroxide (3 g) in ethanol (50 ml) and refluxed for 2 h. The solution was acidified (1 M HCl) and extracted with dichloromethane. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated to give 1.6 g of saponified lipidic mixture. GC analytical (operating conditions: column temperature: 240 °C; detector temperature: 320 °C; injector temperature: 300 °C; capillary column (BPX-35)) showed the mixture of 24-methylenecholesterol and cholesterol (94.5:4.5 ratio). Relative retention time/cholesterol (RRT): cholesterol: 1 and 24-methylenecholesterol: 1.42. No sitosterol was detected.

4.1.9. (24R,S)-24-hydroxy-24-methylcholesterol **11**

Mercuric acetate (2.99 g) in water (15 ml) was added to a stirred solution of lipidic crude extract (0.6 g) in tetrahydrofuran (15 ml) at room temperature and stirred for 12 h. The solution was treated with 3 M aqueous sodium hydroxide (10 ml) and with 0.5 M aqueous sodium borohydride (10 ml) and stirred for 30 min at room temperature. The solution was filtered and the solvent was concentrated and extracted with

chloroform. The two compounds were separated by column chromatography (eluent: cyclohexane/ethyl acetate, 9:1) to give 0.55 g of (24R,S)-24-hydroxy-24-methylcholesterol **11** as epimeric mixture and 0.019 g of cholesterol. IR (KBr) υ (cm^{-1}): 3367 (O–H alcohol), 2964–2866 (C–H alkane). ^1H NMR (400 MHz, $CDCl_3$, 25 °C), δ: 0.68 and 0.69 (2 × s, 2 × 3 H, 18-Me), 0.89 and 0.90 (2 × d, $J = 6.9$ Hz and $J = 7.0$ Hz, 2 × 3 H, 21-Me), 0.92 and 0.93 (2 × d, $J = 6.9$ Hz and $J = 6.5$ Hz, 2 × 3 H, 26-Me and 27-Me), 1.00 and 1.02 (2 × s, 2 × 3 H, 19-Me), 1.07 and 1.09 (2 × s, 2 × 3 H, 28-Me), 3.52 (m, 2 H, 3-H), 5.36 (s, 2 H, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C), δ : 11.8, 16.9, 16.9, 17.5, 17.5, 18.8, 19.4, 21.1, 23.3, 24.3, 28.2, 29.2, 29.2, 31.6, 31.9, 36.1, 36.5, 36.6, 37.2, 39.7, 42.3, 50.1, 55.9, 56.7, 71.8, 74.8, 121.6, 140.7. MS (30 eV, EI): m/z (%) = 416 (12) [M^+], 398 (28) [$M^+ - H_2O$], 373 (31), 314 (100), 271 (39). ESIMS: $m/z = 439.5$ $[M + Na]^+$.

4.1.10. 2.4. Methylenecholesterol **12**

Mercuric acetate (1.5 g) in water (10 ml) was added to a stirred solution of lipidic crude extract (0.5 g) in tetrahydrofuran (10 ml) at room temperature and stirred for 12 h. The solution was filtered, diluted with water (30 ml) and extracted with dichloromethane (3 × 15 ml). The crude product was purified by flash chromatography (cyclohexane/ethyl acetate, 8:2 and ethyl acetate/acetic acid, 9.4:0.6). The organomercurial in ethyl acetate (20 ml) was treated by 1 M HCl (10 ml) and stirred for 2 h at room temperature. The solution was diluted with water and extracted with dichloromethane. The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated. The residue was purified by chromatography on silica gel column (cyclohexane/ethyl acetate, 8:2) afforded product **12** (0.43 g, 91%) as a white solid. M.p. 141 °C. IR (KBr) υ (cm^{-1}): 3435 (O–H alcohol), 2928–2854 (C–H alkane). ^1H NMR (400 MHz, $CDCl_3$, 25 °C), δ: 0.68 (s, 3 H, 18-Me), 0.94 (d, $J = 6.6$ Hz, 3 H, 21-Me), 1.01 (s, 3 H, 19-Me), 1.02 (d, $J = 6.5$ Hz, 6 H, 26-Me and 27-Me), 3.52 (m, 1 H, 3-H), 4.65 and 4.71 (d, 2H, $J = 1.28$, $=CH_2$), 5.34 (d, $J_{6\text{-}7\beta} = 5.1$ Hz, 1 H, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C), δ: 11.8, 18.7, 19.4, 21.8, 22.0, 22.7, 24.3, 28.2, 29.7, 31.0, 31.6, 31.9, 33.8, 34.7, 35.7, 36.5, 37.2, 39.8, 42.2, 42.3, 50.1, 56.0, 56.7, 71.8, 105.9, 121.7, 140.7, 156.8. MS (30 eV, EI): m/z (%) = 398.5 (3) [M^+], 314.4 (28) [M^+–$CHC(CH_2)CH(CH_3)_2$], 256.3 (48), 129.1 (71), 97.1 (79), 73.9 (100). Anal. Calcd for $C_{28}H_{46}O$: C 84.36; H 11.63. Found: C 84.39; H 11.67.

4.2. Antileishmanial evaluation

4.2.1. Parasites

Three strains of *L. donovani* were used in this study: *L. donovani* (MHOM/ET/67/HU3) called LV9 and two strains of *L. donovani* (MHOM/IN/80/DD8) promastigotes called wild-type (WT) and AmB-resistant line (AmB-R). Promastigotes were cultured in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% heat-inactivated fetal calf serum (hi-FCS) and 50 μg ml^{-1} gentamycin at 27 °C in a dark environment.

4.2.2. In vitro evaluation on L. donovani promastigote forms

The antileishmanial screening was performed in flat-bottomed 96-well plastic tissue-culture trays maintained at 27 °C in an atmosphere of 95% air/5% CO_2. Promastigote forms from a logarithmic phase culture were suspended to yield 10^6 cells per ml after hemocytometer counting. Each well was filled with 100 μl of the parasite suspension, and plates were incubated at 27 °C for 1 h before drug addition. The compounds to be tested were dissolved in DMSO and then added to each well to obtain the final concentration of 100 μM and further concentrations were twice diluted. At up to 2% (v/v), DMSO had no effect on parasite growth. Each concentration was screened in triplicate. The viability of promastigotes was checked using the tetrazolium-dye (MTT) colorimetric method. The MTT cell proliferation assay is a colorimetric assay system, which measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. After incubation of the cells with the MTT reagent, a detergent solution was added to lyse cells and solubilize the colored crystals. The samples were read using an ELISA plate reader at a wavelength of 570 nm. The amount of color produced was directly proportional to the number of viable cells. The results are expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a 3-day incubation period. AmB and pentamidine were used as antileishmanial reference compounds.

4.2.3. In vitro evaluation on intramacrophage amastigotes

Concerning the amastigote in vitro model, peritoneal macrophages were harvested from female CD1 mice (Charles River, Cléon, France) 3 days after an intraperitoneal injection of 1.5 ml of sodium thioglycolate (Biomérieux) and were dispensed into eight-well chamber slides (LabTek Ltd.) at a concentration of 5×10^4 per well (400 μl per well) in RPMI 1640 medium supplemented with 10% hi-FCS, 25 mM HEPES, and 2 mM L-glutamine (Life Technologies, Cergy-Pontoise, France). Four hours after the macrophages were plated, they were washed in order to eliminate fibroblasts. After a 24 h-incubation period, the macrophages were infected with promastigote forms of *L. donovani* LV9 in a stationary phase at a ratio of 10 parasites per macrophage, to obtain 87% of infected macrophages and 10 ± 3 amastigotes per macrophage. At 18 h after the promastigotes had entered macrophages, the free promastigotes were eliminated and intramacrophagic amastigotes were treated at various concentrations of the compounds. Pentamidine was used as reference compound. The culture medium was renewed 48 h later and a new culture medium containing the drug was added. The experiment was stopped at day 5, and the percentage of infected macrophages was evaluated microscopically after Giemsa staining. The 50% inhibitory concentrations (IC_{50}) were determined by linear

regression analysis, and expressed in μM ± S.D. Each experiment was performed in triplicate.

4.3. Antitrypanosomal evaluation

The method used was previously described by Loiseau et al. [36]. Briefly, the bloodstream forms of *T. brucei brucei* were maintained in vitro for 48 h in the dark at 37 °C in a 5% CO_2 atmosphere, in minimum essential medium (Gibco BRL) including 25 mM HEPES and Earle's salts and supplemented with 2 mM L-glutamine, 1 g of additional glucose per l, 10 ml of minimum essential medium non-essential amino acids (100×; Gibco BRL) per l, 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine, 15% heat-inactivated horse serum (Gibco BRL), and 50 μg of gentamycin per ml. The 96-well plates were filled up like in the antileishmanial assay on promastigotes, except that the culture medium was complemented with 2×10^5 trypomastigotes from the blood of a mouse collected aseptically from the retro-orbital sinus. The minimum effective concentration (MEC) was defined as the minimum concentration at which no viable parasite was observed microscopically.

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