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Novel steroid derivatives: synthesis, antileishmanial activity, mechanism of action, and *in silico* physicochemical and pharmacokinetics studies

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ABSTRACT

The search for new drugs for the treatment of leishmaniasis is an important strategy for improving the current therapeutic arsenal for the disease. There are several limitations to the available drugs including high toxicity, low efficacy, prolonged parenteral administration, and high costs. Steroids are a diverse group of compounds with various applications in pharmacology. However, the antileishmanial activity of this class of molecules has not yet been explored. Therefore, in the present study, we investigated the antileishmanial activity and cytotoxicity of novel steroids against murine macrophages with a focus on the derivatives of cholesterol (**CD**), cholic acid (**CA**), and deoxycholic acid (**DA**). Furthermore, the mechanism of action of the best compound was assessed, and *in silico* studies to evaluate the physicochemical and pharmacokinetic properties were also conducted. Among the sixteen derivatives, **schiffbase2**, **CD2** and deoxycholic acid derivatives (**DOCADs**) were effective against promastigotes of *Leishmania* species. Despite their low toxicity to macrophages, the majority of **DOCADs** were active against intracellular amastigotes of *L. amazonensis*, and **DOCAD5** exhibited the best biological effect against these parasitic stages (IC_{50} = 15.34 μM). Neither the **CA** derivatives (**CAD**) nor **DA** alone inhibited the intracellular parasites. Thus, the absence of hydroxyl in the C-7 position of the steroid nucleus, as well as the modification of the acid group in **DOCADs** were considered important for antileishmanial activity. The treatment of *L. amazonensis* promastigote forms with **DOCAD5** induced biochemical changes such as depolarization of the mitochondrial membrane potential, increased ROS production and cell cycle arrest. No alterations in parasite plasma membrane integrity were observed. *In silico* physicochemical and pharmacokinetic studies suggest that **DOCAD5** could be a good candidate for an oral drug. The data demonstrate the potential antileishmanial effect of certain steroid derivatives and encourage new *in vivo* studies.

1. Introduction

Leishmaniasis is a complex of infectious diseases caused by more than 20 protozoan species of the genus *Leishmania*. These parasites are transmitted to humans through the bite of infected Diptera females of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) during their blood meal [1,2]. The disease can be asymptomatic or present a great variety of clinical manifestations, which are classically divided into cutaneous, mucocutaneous, and visceral forms [3]. The latter is the most severe form, which may lead to the death of patients who are immunosuppressed and those who are not treated in the initial phase of the disease [3].

Chemotherapy is used for the treatment of leishmaniasis, and a limited number of drugs is available. In some countries, the first choice drugs are pentavalent antimonials [4,5]; however, these medications cause several side effects such as myalgia, anorexia, fever, malaise, headache, metallic taste sensation, and lethargy [3–5]. Furthermore, these drugs present toxic effects such as high cardiotoxicity and nephrotoxicity. If the response to pentavalent antimonials is not satisfactory or there are any restrictions on their use, the option is to use

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second-choice drugs such as amphotericin B, paromomycin, and pentamidine. However, these drugs also present serious toxic effect. Miltefosine, originally developed as an anticancer drug, is only administered orally [6]. This drug was initially approved for treatment of leishmaniasis in India and Germany. Subsequently, Bangladesh, Latin America (Argentina, Bolivia, Colombia, Ecuador, Guatemala, Honduras, Mexico, Paraguay and Peru), Israel, Germany and USA also approved the use of this drug for treat leishmaniasis [7]. Although toxicity is not very common, miltefosine is associated with gastrointestinal problems and teratogenicity [3].

Steroids are a group of diverse compounds that include corticosteroids, progestins, estrogens, androgens, vitamin D, and cholesterol [8]. These compounds are of great interest to the pharmaceutical industry because of their varied pharmacological properties and strong ability to permeate the lipid bilayer of cells. In addition, small changes in the structure of these molecules are known to likely result in diverse biological effects [9]. Cholesterol is one of the most important steroids, as it is one of the constituents of the lipid bilayer of mammalian cells. In addition, it is a precursor of several other important compounds in organisms such as sex hormones, vitamin D, and bile acids [10]. There are two important classes of bile acids: primary bile acids, produced in hepatocytes through the modification of cholesterol and secondary bile acids, produced through modification by bacterial intestinal microbiota. Cholic (**CA**) and deoxycholic (**DA**) acids are themajor primary and secondarybile acids, respectively [11]. In addition to the biological importance of bile acids in organisms, they are expected to lead to great achievements in the field of pharmacology, such as the development of new drugs or improvement of the pharmacokinetics of existing drugs [12,13].

Recent studies have shown that the activity of compounds such as derivatives of aminoquinolines, natural cinchona alkaloids and 6-thipurine derivatives containing 1,2,3-triazole was effectively improved when these molecules were conjugated to bile acids such as **CA**, lithocholic acid, and chenodeoxycholic acid [14–16]. Considering the importance of this class of molecules and the undeniable need for alternative treatments for leishmaniasis, this study aimed to determine the activity of novel steroids against different species of *Leishmania*, with an emphasis on the derivatives of cholesterol, **CA**, and **DA**. The mechanism of action and the *in silico* physicochemical and pharmacokinetic properties of the most promising compound were evaluated.

2. Material and methods

2.1. Chemistry

2.1.1. General methods

Melting points (m.p.) were determined using a MQAPF-301-Microquimica digital apparatus and are uncorrected. Infrared spectra (wave numbers in cm^{-1}) were recorded on a Shimadzu 8400 series FTIR instrument. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-300 or a BRUKER AVANCE DRX300 HD 500. The chemical shifts (δ) are given in parts per million relative to tetramethylsilane (TMS). All MALDI spectra were obtained using a time-of-flight mass spectrometer. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry experiments were performed using a pulsed nitrogen laser with a wavelength of 337 nm of a Shimadzu Biotech Axima Performance MALDI-TOF. Elementary analysis data was performed in a Perkin-Elmer CHN analyzer model 2400. Column chromatography was performed on Merck silica gel (70–230 mesh). Reagents and materials were obtained from commercial suppliers and were used without purification. All reagents used were analytical reagent grade.

Compounds cholic acid derivative 1 (**CAD1**), deoxycholic acid derivative 1 (**DOCAD1**), cholic acid derivative 3 (**CAD3**), deoxycholic acid derivative 3 (**DOCAD3**), cholic acid derivative 4 (**CAD4**), deoxycholic acid derivative 4 (**DOCAD4**), schiffbase1 and schiffbase2 (Scheme 1) have been described in the literature by our research group [17–19].

2.1.2. Preparation of cholic (CAD) and deoxycholic acids derivatives (DOCAD)

2.1.2.1. General procedure for the synthesis of the amides CAD1, DOCAD1 and CAD2, DOCAD2. Bile amides syntheses were performed according to the procedure previous described by our research group [17]. For amides **CAD2** and **DOCAD2**, however, DMAP catalysis was used.

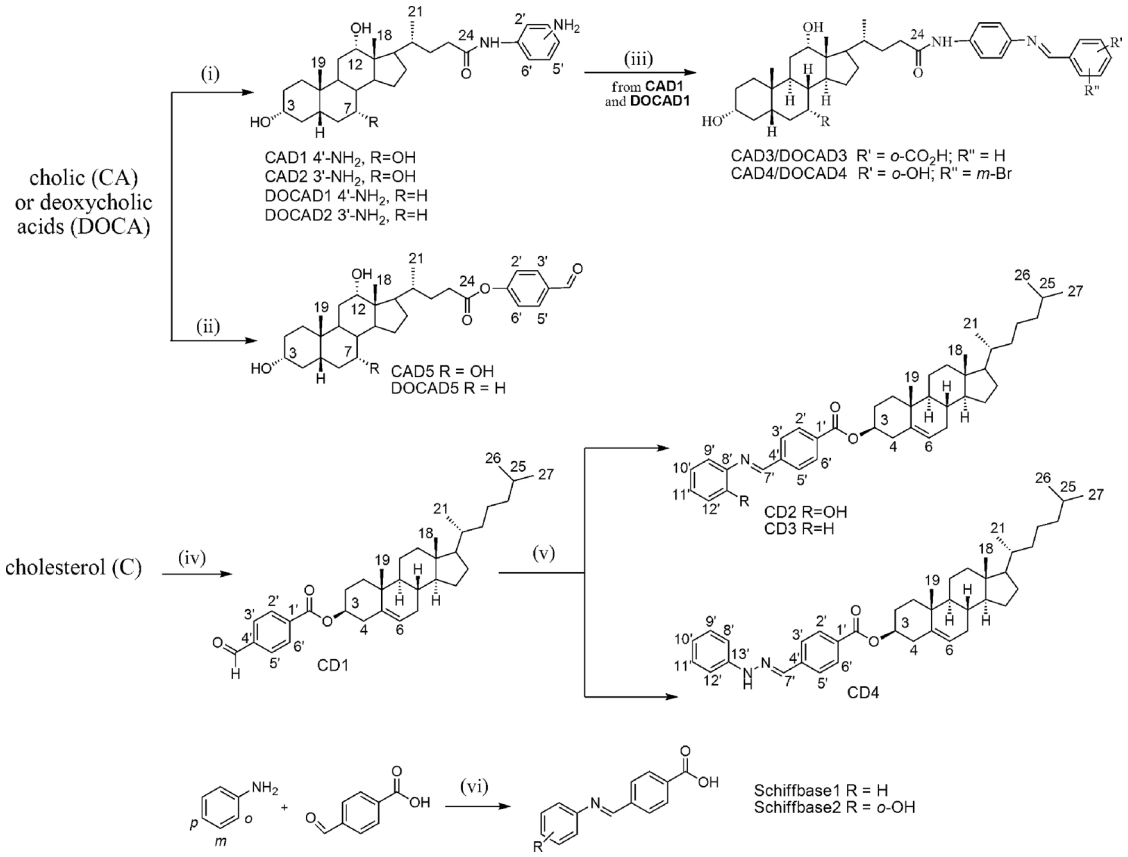
CAD2. Beige solid. Yield: 82%. M.p.: 153.2–154.0 °C. I.R. (KBr) *v*: 3390, 3135, 2936, 2868, 1664, 1552, 1400, 1386. ^{1}H-NMR (300 MHz, DMSO-d_6), δ (ppm), *J* (Hz): 0.59 (s, 3H, 18 − CH_3); 0.81 (s, 3H, 19 − CH_3); 0.97 (d, 2H, 21 − CH_3, *J* = 6.0); 1.26–2.29 (m, 24H, CH_2 and CH skeleton and 23 − CH_2); 3.17 (d, 1H, H-3β, *J* = 4.8); 3.62 (s, 1H, H-7β); 3.80 (s, 1H, H-12β); 4.04 (d, 1H, 7 − OH, *J* = 3.0); 4.14 (d, 1H, 12 − OH, *J* = 3.0); 4.36 (d, 1H, 3 − OH, *J* = 4.2); 5.02 (bs, 2H, -NH_2); 6.22 (d, 1H, H-4′, *J* = 7.8); 6.66 (d, 1H, H-6′, *J* = 7.8); 6.88 (t, 1H, H-5′, *J* = 7.9); 6.93 (s, 1H, H-2′); 9.55 (s, 1H, -NH). ^{13}C-NMR (75 MHz, DMSO-d_6), δ (ppm): 12.3 (C-18); 17.1 (C-21); 22.6 (C-19); 22.8-46.2 (CH_2 and CH esqueleto); 66.2 (C-7); 70.5 (C-3); 71.0 (C-12); 104.9 (C-2'); 107.2 (C-4'); 109.1 (C-6'); 128.7 (C-5'); 139.9 (C-1'); 148.8 (C-3'); 171.4 (C-24). MS (MALDI): m/z Calc. for $[C_{30}H_{47}N_2O_4]$ $[M+H]^+$ Calc. (499.3536) found 499.4271.

DOCAD2. Beige solid. Yield: 21%. M.p.: 136.5–138.0 °C. I.R. (KBr) *v*: 3335, 3149, 2935, 2862, 1666, 1551, 1450. ^{1}H-NMR (300 MHz, DMSO-d_6), δ (ppm), *J* (Hz): 0.59 (s, 3H, 18 − CH_3); 0.84 (s, 3H, 19 − CH_3); 0.95 (d, 2H, 21 − CH_3, *J* = 4.8); 1.20–1.78 (m, 23H, CH_2 and CH skeleton); 2.17–2.28 (m, 2H, 23 − CH_2); 3.80 (s, 1H, H-12β); 4.22 (s, 1H, 12 − OH); 4.51 (s, 1H, 3 − OH); 5.02 (bs, 2H, -NH_2); 6.22 (d, 1H, H-4′, *J* = 7.5); 6.66 (d, 1H, H-6′, *J* = 7.5); 6.87 (t, 1H, H-5′, *J* = 8.1); 6.93 (s, 1H, H-2′); 9.54 (s, 1H, -NH). ^{13}C-NMR (75 MHz, DMSO-d_6), δ (ppm): 12.5 (C-18); 17.1 (C-21); 23.1 (C-19); 23.5–46.3 (CH_2 and CH skeleton); 47.5 (C-7); 70.0 (C-3); 71.1 (C-12); 104.9 (C-2'); 107.2 (C-4'); 109.1 (C-6'); 128.8 (C-5'); 140.0 (C-1'); 148.8 (C-3'); 171.4 (C-24). MS (MALDI): m/z Calc. for $[C_{30}H_{47}N_2O_3]$ $[M]^+$ Calc. (482.3508) found 482.3510.

2.1.2.2. General procedure for the synthesis of the aldehydes CAD5 and DOCAD5. Bile acid (2.45 mmol) was partially dissolved in 50 mL of CH_2Cl_2 and to that reaction mixture were added 1.05 equimolar amount of 4-hydroxybenzaldehyde, 1.05 equimolar amount of DCC and catalytic amount of DMAP. The mixture was refluxed and stirred for 24 h, when the formations of the desired aldehydes were found by thin layer chromatography (TLC). A simple filtration was carried out to remove the byproduct (DCU) from the reaction medium and the residual organic phase was evaporated. The solid obtained was then subjected to purification on CCS using as eluent a mixture of solvents CH_2Cl_2 and MeOH.

CAD5. White solid. Yield: 30%. M.p.: 194.7–196.0 °C. I.R. (KBr) *v*: 3429, 3326, 2927, 2850, 1764, 1699, 1625, 1575. ^{1}H-NMR (500 MHz, DMSO-d_6), δ (ppm), *J* (Hz): 0.58 (s, 3H, 18 − CH_3); 0.61 (s, 3H, 19 − CH_3); 0.81 (d, 3H, 21 − CH_3, *J* = 4.5); 0.84–2.20 (m, 24H, CH_2 and CH skeleton and 23 − CH_2); 3.17 (d, 3H, *J* = 4.1); 3.20 (bs, 1H, H-3β); 3.60 (s, 1H, H-7β); 3.80 (s, 1H, H-12β); 4.02 (s, 1H, 7 − OH); 4.20 (s, 1H, 12 − OH); 4.40 (s, 1H, 3 − OH); 7.36 (d, 2H, H-2' and H-6', *J* = 8.0); 7.98 (d, 2H, H-3' and H-5', *J* = 8.0); 9.99 (s, 1H, HCO). ^{13}C-NMR (75 MHz, DMSO-d_6), δ (ppm): 12.3 (C-18); 16.8 (C-21); 22.6 (C-19); 22.7–45.9 (CH_2 and CH skeleton); 66.2 (C-7); 70.4 (C-3); 70.9 (C-12); 122.6 (C-2' and C-6'); 131.0 (C-3' and C-5'); 133.8 (C-4'); 155.1 (C-1'); 171.8 (C-24); 191.9 (HCO).

DOCAD5. White oil. Yield: 30%. I.R. (KBr) *v*: 3406, 3136, 2936, 2862, 1741, 1703. ^{1}H-NMR (300 MHz, DMSO-d_6), δ (ppm), *J* (Hz): 0.58 (s, 3H, 18-CH_3); 0.61 (s, 3H, 19-CH_3); 0.83 (d, 3H, 21-CH_3, *J* = 4.5); 0.87-2.30 (m, 25H, CH_2 and CH skeleton and 23-CH_2); 3.78 (bs, 1H, H-12β); 4.23 (m, 1H, 12-OH); 4.50 (s, 1H, 3-OH); 7.35 (d, 2H, H-2' and H-6', *J* = 8.4); 7.97 (d, 2H, H-3' and H-5', *J* = 8.4); 9.98 (s, 1H, HCO). ^{13}C-NMR (75 MHz, DMSO-d_6), δ (ppm): 12.0 (C-18); 16.8 (C-21); 23.1 (C-19); 23.5-45.9 (CH_2 and CH skeleton); 47.5 (C-7); 70.0 (C-3); 71.0



Scheme 1. Synthetic route to preparation of cholesterol and bile acids derivatives. Reagents and conditions: (i) 1,3- or 1,4-phenylenediamine, DCC, THF, r.t.; (ii) 4-hydroxybenzaldehyde, DCC, DMAP, CH_2Cl_2, reflux; (iii) aromatic aldehydes, MeOH, r.t; (iv) 4-formylbenzoic acid, DCC, DMAP, CH_2Cl_2, r.t.; (v) aromatic amine, EtOH, reflux; (vi) EtOH, r.t.

(C-12); 122.7 (C-2' and C-6'); 131.0 (C-3' and C-5'); 133.8 (C-4'); 155.1 (C-1'); 171.8 (C-24); 191.9 (HCO).

2.1.3. Preparation of cholesterol derivatives (CD)

CD1. Cholesterol (0.70 g; 1.8 mmol) was dissolved in CH_2Cl_2 (40 mL). After, 4-formylbenzoic acid (0.27 g; 1.8 mmol) and a catalytic amount of DMAP were added and the resulting mixture was stirred during few minutes, when a solution of dicyclohexylcarbodiimide (DCC) (0.41 g; 1.9 mmol) solved of CH_2Cl_2 was added. The mixture was stirred for 20 h at room temperature until no starting material was detected. The precipitated byproduct *N,N'*-dicyclohexylurea (DCU) was filtered off and the solution was concentrated to give a white solid was finally purified by column chromatography using CH_2Cl_2 as eluent. Yield: 92%. Compound **CD1** was characterized by ^{1}H and ^{13}C NMR spectroscopy, IR spectroscopy and melting point and were in accord with data in the literature [20].

2.1.3.1. General procedure for the synthesis of the compounds cholesterol derivative 2 (CD2), cholesterol derivative 3 (CD3) and cholesterol derivative 4 (CD4). Aldehyde **CD1** (0.10 g; 0.20 mmol) was dissolved under reflux in ethanol (2 mL). After, 0.20 mmol (equimolar amount) an aromatic amine (2-hydroxyaniline to give **CD2** or aniline to give **CD3**) or phenylhydrazine (to give **CD4**) was added and the resulting mixture was refluxed for 6 h. Stirring and heating were then stopped and the mixture was allowed to stand for about 12 h. After this time, the precipitate was filtered, washed with ethanol and dried in an oven.

CD2. Yellow solid. Yield: 98%. M.p.: 174.1–176.0 °C. I.R. (KBr) *v*: 3420, 2938, 2865, 1715, 1626, 1589, 1486, 1276, 1118 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$), δ (ppm), *J* (Hz): 0.71 (s, 3H, 18-CH_3); 0.89 (d, 6H, 26-CH_3 and 27-CH_3, *J* = 6.3); 0.94 (d, 3H, 21 − CH_3, *J* = 6.3); 1.10–2.03 (m, 32H, 19 − CH_3 and CH_2 and CH skeleton); 2.51 (d, 2H, 4 − CH_2, *J* = 7.2); 4.90 (bs, 1H, 3-H); 5.46 (bs, 1H, 6-H); 6.78 (bs, 1H, − OH); 6.94 (t, 1H, H-10', *J* = 7.2); 7.05 (d, 1H, H-12', *J* = 7.5); 7.25 (t, 1H, H-11', *J* = 8.2); 7.35 (d, 1H, H-9', *J* = 7.8); 7.98 (d, 2H, H-3'and H-5', *J* = 8.1); 8.16 (d, 2H, H-2'and H-6', *J* = 8.1); 8.75 (s, 1H, H-7'). ^{13}C-NMR (75 MHz, $CDCl_3$), δ (ppm): 12.1 (C-18); 18.9 (C-21); 19.6 (C-19); 21.3(C-11); 22.8 and 23.0 (C-26 and C-27); 24.1–56.9 (CH_2 and CH skeleton); 75.3 (C-3); 115.5 (C-9'); 116.0 (C-12'); 120.4 (C-6); 123.1 (C-10'); 128.5 (C-3' and C-5'); 129.8 (C-11'); 130.2 (C-2' and C-6'); 133.5 (C-1'); 135.2 (C-1'); 139.6 (C-5); 152.8 (C-13'); 155.9 (C-7'); 165.6 (COO). Elemental analysis for $C_{41}H_{55}NO_3$: calcd. C 80.74, H 9.09, N 2.30; Found: C 80.46, H 9.19, N 2.40. MS (MALDI): *m/z* Calc. for $[C_{41}H_{56}NO_3]$ $[M+H]^+$ Calc. (610.4260) found 610.4174.

CD3. Yellow solid. Yield: 76%. M.p.: 163.0–164.0 °C. I.R. (KBr) *v*: 2947, 2864, 1712, 1626, 1466, 1382, 1277. ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm), *J* (Hz): 0.72 (s, 3H, 18 − CH_3); 0.89 (2xd, 6H, 26 − CH_3 and 27 − CH_3, *J* = 2.0); 0.96 (d, 3H, 21 − CH_3, *J* = 6.5); 1.02–2.07 (m, 32H, 19 − CH_3 and CH_2 and CH skeleton); 2.52 (d, 2H, 4 − CH_2, *J* = 8.0); 4.91 (bs, 1H, 3-H); 5.45 (d, 1H, 6-H, *J* = 3.9); 7.26–7.28 (m, 3H, H-10', H-11' and H-12'); 7.42–7.45 (m, 2H, H-9' and H-13'); 7.99 (d, 2H, H-3'and H-5', *J* = 8.5); 8.16 (d, 2H, H-2'and H-6', *J* = 8.0); 8.54 (s, 1H, H-7'). ^{13}C-NMR (75 MHz, $CDCl_3$), δ (ppm): 12.1 (C-18); 18.9 (C-21); 19.6 (C-19); 21.3(C-11); 22.8 and 23.0 (C-26 and C-27); 24.1–56.9 (CH_2 and CH skeleton); 75.2 (C-3); 121.1 (C-9' and C-13'); 123.0 (C-6); 126.6 (C-11'); 128.8 (C-3' and C-5'); 129.4 (C-2' and C-6'); 130.1 (C-10' and C-12'); 133.3 (C-1'); 139.8 and 140.0 (C-5'and C-4'); 151.8 (C-8'); 159.3 (C-7'); 165.6 (COO).

These spectroscopic data are in agreement with the literature [21]

CD4. Yellow solid. Yield: 70%. M.p.: 214.3–215.0 °C. I.R. (KBr) *v*: 3464, 3291, 2936, 2867, 1697, 1605, 1582, 1535, 1266. ^{1}H-NMR (300 MHz, $CDCl_3$), δ (ppm), *J* (Hz): 0.67 (s, 3H, 18 − CH_3); 0.89 (d, 6H, 26 − CH_3 and 27 − CH_3, *J* = 7.0); 0.95 (d, 3H, 18 − CH_3, *J* = 6.5); 1.01–2.03 (m,

32H, 19 − CH_3 and CH_2 and CH skeleton); 2.46 (d, 2H, 4 − CH_2, J = 7.8); 4.85 (m, 1H, 3-H); 5.40 (d, 1H, 6-H, J = 4.2); 6.89 (t, 1H, H-11', J = 7.3); 7.12 (m, 2H, H-10' and H-12'); 7.25–7.30 (m, 2H, H-9' and H-13'); 7.65 (s, 2H, H-3'and H-5'); 7.68 (s, 1H, H-7'); 8.01 (d, 2H, H-2' and H-6'). ^{13}C-NMR (75 MHz, $CDCl_3$), δ (ppm): 12.1 (C-18); 18.9 (C-21); 19.6 (C-19); 21.3(C-11); 22.8 and 23,0 (C-26 and C-27); 24.1–56.9 (CH_2 and CH skeleton); 74.9 (C-3); 113.2 (C-9' and C-13'); 120.9 (C-6); 123.0 (C-11'); 125.9 (C-3' and C-5'); 129.6 (C-10' and C-12'); 130.1 (C-2' and C-6'); 130.4 (C-1'); 135.9 (C-4'); 139.8 and 139.9 (C-5'and C-7'); 144.4 (C-8'); 166.0 (COO).

2.2. Biological assays

2.2.1. Parasites

Promastigotes of *L. amazonensis* (IFLA/Br/67/PH8) were cultured in Brain heart infusion (BHI - Himedia Mumbai, India) medium supplemented with hemin (0.01 mg/mL) and folic acid (0.01 mg/mL). *Leishmania braziliensis* MHOM/BR/75/M2903) and *L. major* (MRHO/SU/59/P) were cultured in BHI medium supplemented with L-glutamine. In this work, *L. amazonensis* (IFLA/Br/67/PH8) transfected with plasmid red fluorescent protein (RFP) B5947 piR1SAT-LUC(a) DsRed2(b) integrated into the genome was used [22]. *Leishmania amazonensis*-RFP promastigotes were kindly provided by Dr. Rodrigo P. Soares (Centro de Pesquisas René Rachou- FIOCRUZ/MG- Brazil). *Leishmania infantum* (MCAN/BR/2008/1112) and *L. amazonensis*-RFP were cultured in 199 medium. Under all cultivation conditions were added 10% of Fetal Bovine Serum (FBS) and the parasites were maintained at 25 °C in BOD incubator.

2.2.2. Antileishmanial activity on promastigote forms of Leishmania species

The antipromastigote assay was performed by using the MTT (Sigma-Aldrich, St. Louis, MO, USA) colorimetric method [23]. Therefore, promastigote forms of *L. amazonensis* (2×10^6 cells/mL), *L. braziliensis, L. infantum* and *L. major* (3×10^6 cells/mL) in stationary phase were placed in 96-well culture plates in the presence of steroids derivatives (3.12–100.00 μM). After 72 h, the viability of cells was measured using spectrophotometer microplate reader (Multiscan MS, LabSystem Oy, Helsink, Finland), at 540 nm. The results were expressed as concentration needed to inhibit 50% the cellular growth (IC_{50}). The IC_{50} values were obtained of three independent experiments conducted in duplicates. Amphotericin B and miltefosine were used as the reference drugs.

2.2.3. Cytotoxicity on macrophages

The cytotoxicity on murine macrophages was performed in accordance with Carmo and colleagues [24], with some modifications. Murine peritoneal macrophages were obtained from BALB/c mice previously stimulated with sodium thioglycollate (3%). In brief: the macrophages (2×10^6 cells/mL) were placed in 96-well culture plates in RPMI-1640 medium supplemented with 10% FBS. After 24 h, the non-adherent cells were removed and the adherent cells were unexposed (negative control) or exposed to the presence of steroidal derivatives (4.68–150.00 μM) for 72 h at 33 °C and 5% CO_2. Cell viability was determined by the MTT method, as previously described. Cytotoxicity concentration to reduce 50% of viable cells (CC_{50}) was obtained of three independent experiments conducted in duplicates. The procedures involving the animals were previously approved by the Ethical Committee for animal handling of UFJF (# 013/2015-CEUA).

2.2.4. Antileishmanial activity on amastigote forms of *L. amazonensis*

Murine peritoneal macrophages were obtained as described above. Adherent macrophages were infected with promastigote forms of *L. amazonensis*-RFP in stationary phase as previously reported [25]. Then, the infected macrophages were untreated (negative control) or treated with different concentrations of steroidal derivatives (3.12–100.00 μM), at 33 °C and 5% CO_2 for 72 h. Uninfected macrophages were used as a blank, and the fluorescence intensity was obtained after using spectrofluorimeter (FLx 800, Biotek Instruments, Winooski, USA) at 540 nm excitation/600 nm emission. The activity of the compounds was expressed as IC_{50}, i.e., the concentration that inhibit 50% intracellular parasites compared to untreated infected macrophages. Results were obtained of three independents experiments conducted in duplicates. All procedures involving the animals were previously approved by the Ethical Committee for animal handling of UFJF (# 012/2015-CEUA). Amphotericin B and miltefosine were used as the reference drugs.

2.2.5. Evaluation of plasma membrane integrity

To assess whether treatment with compound **DOCAD5** alters the integrity of the cell membrane, promastigotes of *L. amazonensis* (1×10^7) were incubated in the presence or absence of this compound (54.4 and 108.8 μM) for 24 h at 25 °C. Therefore, the cells (5×10^6) were washed with PBS and seeded in 96-well clear bottom black microplate. Then, the parasites were incubated with propidium iodide (PI - Sigma-Aldrich, St. Louis, MO, USA) (1 μg/mL) during 15 min at the room temperature in the dark. The intensity of fluorescence was measured by spectrofluorimeter (FLx800) at 540/600 nm of excitation and emission, respectively. Data were obtained from at the three independent experiments performed in duplicates. Parasites warmed at 65 °C for 10 min were used as positive control.

2.2.6. Evaluation of the mitochondrial potential membrane ($\Delta\psi_m$)

To evaluate the $\Delta\psi_m$, promastigote forms of *L. amazonensis* (1×10^7) treated with the compound **DOCAD5** (54.4 and 108.8 μM) were incubated with 500 nM of Mitotracker® Red CM-H2XROS (MtR) for 40 min at 26 °C protect from the light. Untreated parasites were used as negative control. Then, the cells (5×10^6) were washed in PBS, and placed in 96-well clear bottom black microplate. Fluorescent intensity was measured by spectrofluorimeter (FLx800), wavelengths of 485/528 nm of excitation and emission, respectively. FCCP (Sigma-Aldrich, St. Louis, MO, USA) (20.0 μM) was used as a positive control.

2.2.7. Evaluation of ROS levels

Intracellular ROS levels in *L. amazonensis* promastigotes was evaluated using H_2DCFDA, as previously reported [26]. The parasites were untreated or treated with the compound **DOCAD5** at 54.4 and 108.8 μM, at 25 °C. After 24 h of incubation, the parasites (20×10^6) were harvested, washed in PBS and incubated with 2',7'-dichlorodihydrofluorescein diacetate - H_2DCFDA (1 mM) for 30 min in dark at the room temperature (22 ~ 24 °C). The ROS levels were evaluated fluorimetrically (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) at 485/528 nm of excitation and emission, respectively. Miltefosine (44.0 μM) was used as a control.

2.2.8. Cell cycle analysis

Leishmania amazonensis promastigotes (1×10^7) were untreated (negative control) or treated with the compound **DOCAD5** at 54.4 and 108.8 μM for 24 h. Then, the parasites were fixed in 70% ethanol, ressuspended in RNAse (Sigma-Aldrich, St. Louis, MO, USA) (200 μg/mL) for 60 min, and incubated with PI (1 μg/mL) for 15 min in dark at the room temperature (22 ~ 24 °C). Data acquisition were performed using a Cytoflexflow cytometer (Beckman Coulter, Indianapolis, IN 46268 United States) equipped with CytExpert 2.0 software (Beckman Coulter, Indianapolis, IN 46268 United States). A total of 10,000 events were acquired using PI610ND-A channel. Miltefosine (40.0 μM) was used as a positive control.

2.3. In silico analysis

To evaluate the possible oral effectiveness **DOCAD5**, physicochemical and pharmacokinetic proprieties of molecule were analyzed as determined Lipinski's rules (RO5) [27], including octanol/water partition coefficient (LogP), molecular weight (MW), number of hydrogen

bond donor (HBD), and number of hydrogen bond acceptor (HBA), using Molinspiration software. In addition, the ADMET (absorption, distribution, metabolism, excretion and toxicity) properties were evaluated using the admetSAR software.

2.4. Statistical analysis

All data were obtained from three independent experiments performed in duplicates. The 50% inhibitory concentration (IC_{50}) were calculated by *Grafit*® (Erithacus Software Ltd) software. The data were statically analyzed using one or two-way-ANOVA (analyzes of variance) followed by the Dunnett or Bonferroni post-test. All statistical analyses were performed using GraphPad Prism version 5 (GraphPad software, San Diego, CA, USA). Values were considered as significant when $P \leq 0.05$(*); $P \leq 0.001$ (**) and $P \leq 0.0001$ (***).

3. Results

3.1. Chemistry

Bile acids and cholesterol derivatives (**CD**) were obtained from commercially available **CA**, **DA**, and cholesterol (Scheme 1).

CA derivatives (**CAD**) and **DA** derivatives (**DOCAD**) were subjected to an amide-forming reaction with aromatic amines (1,3- and 1,4-phenylenediamine), producing the biliar amides **CAD1**, **DOCAD1**, **CAD2,** and **DOCAD2**.Furthermore, from the biliar amides **CAD1** and **DOCAD1**, the conjugates **CAD3/DOCAD3** and **CAD4/DOCAD4** were obtained through the condensation of aromatic aldehydes in ethanol, with good yields. Bile aldehydes **CAD5** and **DOCAD5** were obtained directly from **CA** and **DA** moiety, respectively, using a Steglich esterification between 4-formylbenzoic acid and a hydroxyl group of the C-3 steroidal nucleus of **CA** or **DA**. Conjugates **CD2**, **CD3,** and **CD4**, derived from cholesterol, were synthesized via C-3 condensation between the intermediate aldehyde **CD1** and aromatic amines with good yields. **Schiffbase1** and **schiffbase2** were prepared by condensation of equimolar amounts of aromatic aldehydes and amines in ethanol as shown in Scheme 1.

3.2. Antileishmanial activity of steroid derivatives on Leishmania sp

All compounds were assayed against promastigote and amastigote forms of *Leishmania* species and their toxicity on macrophages was evaluated. Evaluation of the antipromastigote activity of the **CDs** revealed that only **CD2** was active against at least two of the *Leishmania* species examined in this study (Table 1). The precursor of compound **CD2**, **schiffbase2**, exhibited better antileishmanial activity against promastigotes of *L. amazonensis*, *L. braziliensis*, and *L. major* (IC_{50} ~2 μM) and amastigotes of *L. amazonensis* (IC_{50} = 35.45 μM). Cholesterol only inhibited the growth of promastigotes of *L. braziliensis* (IC_{50}, 27.9 μM). None of the compounds tested were toxic to murine macrophages at concentrations up to the maximum tested (150 μM), and the compounds **CD1**, **ShiffBase2** and **CD2** exhibited a selectivity index (SI) above 1 (Table 1).

The antileishmanial effect of **CAD** and **DOCADs** is shown in Table 2, and only **DOCADs** exhibited relevant biological activity. Most **DOCADs** inhibited the growth of all promastigotes of *Leishmania* species, and their activities were comparable among the species. The majority of **DOCADs** were active on intracellular amastigotes, with IC_{50} values between 15.34 and 40.43 μM, being **DOCAD5** the best compound. The tested **CADs** were not toxic to mammalian cells (CC_{50} > 150 μM), but only three **DOCAD** compounds (**DOCAD2**, **DOCAD3**, and **DOCAD4**) showed low toxicity to these cells. The compound **DOCAD5** showed the best SI, being ~10 times more destructive to intracellular parasites than to the macrophages (Table 2).

3.3. *DOCAD5 induced biochemical alterations in promastigotes of L. amazonensis, but plasma membrane integrity of parasites remained intact*

The **DOCADs** exhibited promising biological activity, and the most active compound (**DOCAD5**) was selected to further study its mechanism of action. First, we sought to determine if the plasma membrane of the parasite was intact after treatment. Incubation of **DOCAD5**-treated parasites with PI did not significantly increased the fluorescence intensity, which indicated that the plasma membrane of the parasites remained intact after treatment with this compound, like the untreated group (Fig. 1). As expected, parasites heated at 65 °C (positive control) showed an increase in fluorescence intensity (83.21%).

Leishmania mitochondria, a single organelle in trypanosomatids, is considered a good drug target; therefore, the effect of the compound **DOCAD5** on this organelle was evaluated. First, the mitochondrial function in promastigotes was evaluated using the MtR dye. A decrease in fluorescence intensity, as shown in Fig. 2, indicated the depolarization of the mitochondrial membrane potential ($\Delta\psi_m$) in *L. amazonensis* treated with **DOCAD5** at 54.4 μM (48.83%) and 108.8 μM (50.71%). Treatment with FCCP, used as the positive control, decreased the fluorescence intensity by 61.58% relative to the negative control. To

Table 1
Effect of cholesterol derivatives and Schiff bases on promastigotes and amastigotes of *Leishmania sp* and murine macrophages.

Compounds	Antileishmanial activity IC_{50} (μM)[a]					Macrophages CC_{50} (μM)[b]	Selectivity Index (SI)***
	Promastigotes				Amastigotes		
	L.ama[1*]	L. braz[2]	L. maj[3]	L. inf[4]	L. ama[5**]		
CD1	> 100	> 100	> 100	> 100	69.18 ± 13.76	> 150	> 2.16
ShiffBase1	> 100	> 100	> 100	> 100	> 100	> 150	–
ShiffBase2	1.81 ± 0.29	1.86 ± 0.17	1.93 ± 1.93	> 100	35.45 ± 0.74	> 150	> 4.23
CD2	> 100	7.00 ± 0.61	23.89 ± 2.9	> 100	97.97 ± 20.87	> 150	> 1.53
CD3	> 100	> 100	> 100	> 100	> 100	> 150	–
CD4	> 100	> 100	> 100	> 100	> 100	> 150	–
C[c]	> 100	27.9 ± 3.9	> 100	> 100	> 100	> 150	–
AmB[d]	0.15 ± 0.02	1.24 ± 0.06	1.31 ± 0.07	1.08 ± 0.004	0.71 ± 0.22	85.8± 30.4	120.8
Miltefosine[d]	22.0 ± 0.5	29.66 ± 0.02	20.0 ± 0.52	2.64 ± 0.29	12.52 ± 0.83	131.9± 3.9	10.53

[a] Values of inhibitory concentration of 50% of parasites (IC_{50}).
[b] Values of citotoxicity concentration of 50% of macrophages (CC_{50}).
[c] C (cholesterol) and [d]AmB (amphotericin B). [1]*L. ama* (*L. amazonensis*), [2]*L. braz* (*L. braziliensis*), [3]*L. maj* (*L. major*), [4]*L. inf* (*L. infantum*). **Leishmania amazonensis* wild-type (IFLA/BR/1967/PH8). ***L. amazonensis*-RFP (IFLA/BR/1967/PH8). *** SI = CC_{50}/ IC_{50} (amastigotes).
[d] Miltefosine and AmB (amphotericin B) were used as reference drugs.

Table 2
Effect of colic and deoxycholic acid derivatives on promastigotes and amastigotes of *Leishmania sp* and murine macrophages.

Compounds	Antileishmanial activity IC_{50} (μM)[a]					Macrophages CC_{50} (μM)[b]	Selectivity Index (SI)***
	Promastigotes				Amastigotes		
	L.ama[1*]	L. braz[2]	L. maj[3]	L. inf[4]	L. ama[5**]		
CAD1	> 100	> 100	> 100	> 100	> 100	> 150	–
DOCAD1	32.81 ± 0.76	19.33 ± 1.36	31.67 ± 0.61	29.33 ± 3.07	23.89 ± 1.99	> 150	> 6.27
CAD2	> 100	> 100	> 100	> 100	> 100	> 150	–
DOCAD2	33.31 ± 0.24	42.28 ± 2.26	33.54 ± 0.90	21.88 ± 4.08	23.33 ± 4.05	80.81± 0.32	3.46
CAD3	> 100	> 100	> 100	> 100	> 100	> 150	–
DOCAD3	29.06 ± 0.53	15.83 ± 0.29	31.80 ± 0.46	28.88 ± 2.33	40.43 ± 2.97	53.13± 3.36	1.31
CAD4	> 100	> 100	> 100	> 100	> 100	> 150	–
DOCAD4	> 100	56. 10 ± 8.89	56.17 ± 0.15	52.33 ± 0.14	> 100	89.85± 7.97	< 0.89
CAD5	> 100	> 100	> 100	> 100	> 100	> 150	–
DOCAD5	54.40 ± 2.17	78.67 ± 11.99	41.19 ± 0.58	48.49 ± 0.52	15.34 ± 1.16	> 150	> 9.77
CA[c]	> 100	> 100	> 100	> 100	> 100	> 150	–
DOCA[d]	> 100	> 100	> 100	> 100	> 100	> 150	–
AmB[e]	0.15 ± 0.02	1.24 ± 0.06	1.31 ± 0.07	1.08 ± 0.004	0.71 ± 0.22	85.8± 30.4	120.8
Miltefosine[e]	22.0 ± 0.5	29.66 ± 0.02	20.0 ± 0.52	2.64 ± 0.29	12.52 ± 0.83	131.9± 3.9	10.53

[a] Values of inhibitory concentration of 50% of parasites (IC_{50}).
[b] Values of citotoxicity concentration of 50% of macrophages (CC_{50}).
[c] CA (cholic acid), [d]DOCA (deoxycholic acid) and.
[d] AmB (amphotericin B). [1]*L. ama* (*L. amazonensis*), [2]*L. braz* (*L. braziliensis*), [3]*L. maj* (*L. major*), [4]*L. inf* (*L. infantum*). **Leishmania amazonensis* wild-type (IFLA/BR/1967/PH8). ***L. amazonensis*-RFP (IFLA/BR/1967/PH8). *** SI = CC_{50}/ IC_{50} (amastigotes).
[e] Miltefosine and AmB (amphotericin B) were used as reference drugs.

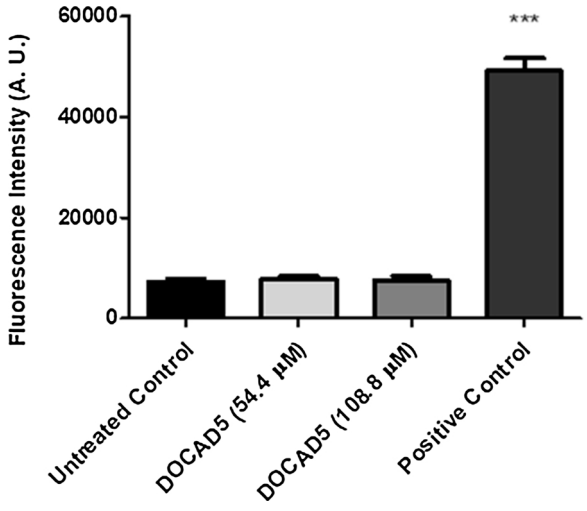


Fig. 1. DOCAD5 does not induce loss of integrity of the plasma membrane of *L. amazonensis* promastigotes. To evaluate the integrity of the plasma membrane of *L. amazonensis* treated with **DOCAD5**. Promastigotes were incubated with 54.4 and 108.8 μM of the compound for 24 h. then the cells were washed and stained with PI. The parasites were analyzed by spectrofluorimetry. Heated promastigotes (65 °C) were used as positive control. Statistical analyzes were performed by GraphPad Prism 5.0 software by analysis of variance (One-Way ANOVA) and statistical differences were analyzed by the Dunnett's test: $p < 0.001$ *(***)*. Data were expressed as the mean of three independent experiments.

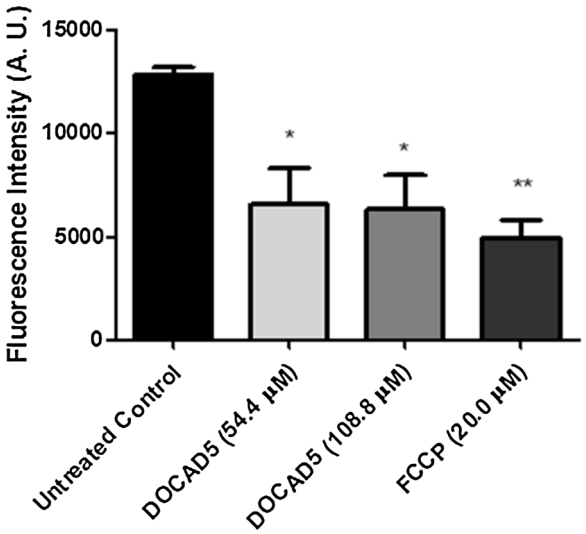


Fig. 2. Treatment with **DOCAD5** decreases the mitochondrial membrane potential ($\Delta\psi m$) of *L. amazonensis* promastigotes. Parasites were treated with 54.4 and 108.8 μM of the compound. The cells were then washed and incubated with Mitotracker Red CM-H2XROS® (500 nM). Fluorescence intensity was determined by fluorimetry using the wavelengths of 540 nm and 600 nm of excitation and emission, respectively. FCCP (20.0 μM) was used as a positive control. Statistical analyzes were performed using GraphPad Prism 5.0 software using analysis of variance (One-way ANOVA), followed by Dunnett's post-test: *$p < 0.05$ (*); $p < 0.01$ (**)*. Data were expressed as the mean of three independent experiments.

verify the ROS production in *L. amazonensis* promastigotes treated with **DOCAD5**, the parasites were incubated with H_2DCFDA. The data showed that the treatment with **DOCAD5** (54.4 and 108.8 μM) increased ROS production by 50.5% and 60.9%, respectively, relative to the untreated control (Fig. 3). In parasites treated with miltefosine (44.0 μM), the ROS production was increased by 98.25%.

To evaluate changes in the promastigote cell cycle induced by treatment with **DOCAD5**, the parasites were stained with PI and analyzed using flow cytometry. Fig. 4 shows that the proportion of parasites in the sub-G0/G1 phase increased to 15.12% and 18.55%, respectively, compared with the untreated control (9.27%). Miltefosine, used as a positive control, increase the proportion to 48.88% of cells in the sub-G0/G1 phase. These results show that treatment of promastigotes with the compound significantly changed the parasite cell cycle.

3.4. In silico physicochemical and pharmacokinetic analyses indicate compound DOCAD5 is a good oral drug

The data of the *in silico* analysis using the molinspiration and admetSAR software packages are shown in Table 3. Thus, **DOCAD5** violated only one of Lipinski's "rule of five" (LogP ≤ 5, molecular mass

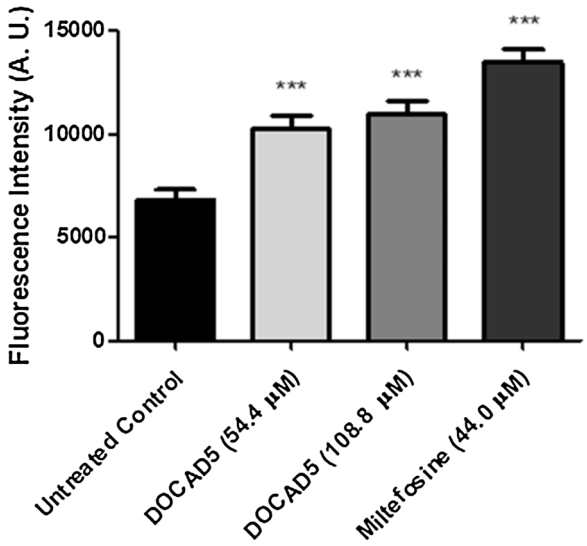


Fig. 3. Treatment with **DOCAD5** significantly increases ROS production in *L. amazonensis* promastigotes. The parasites were treated with 54.4 and 108.8 Mm of **DOCAD5**. Then, the cells were washed and incubated with H2DCFDA (1 Mm). Fluorescence intensity was determined by fluorimetry, using 485 nm excitation wavelengths and 528 nm emission. Miltefosine (44.0 μM) was used as a positive control. Statistical analyzes were performed using GraphPad Prism 5.0 software using analysis of variance (One-way ANOVA), followed by Dunnett's post-test: $p < 0.001$ (***). Data were expressed as the mean of three independent experiments.

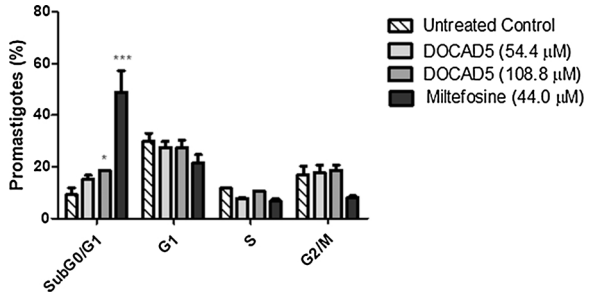


Fig. 4. Treatment with **DOCAD5** at 108.8 μM induces DNA fragmentation in *L. amazonensis* promastigotes. Cells were incubated with compound **DOCAD5** (54.4 and 108.8 μM) for 24 h. After this period, the parasites were permeabilized with 70% ethanol, and incubated with PI (μg/mL). Evaluation of the cells cicle was performed using flow cytometry. Graphical representation of the cells present at each stage of the cell cycle. Statistical analyzes were performed using GraphPad Prism 5.0 software using two-way ANOVA, followed by the Bonferroni post-test: $p < 0.05$ (*) and $p < 0.001$ (***).Data were expressed as the mean of three independent experiments.

Table 3
Physicochemical properties of **DOCAD5**.

Compound	logP	MW	nHBA	nHBD
DOCAD5	5.76	496.69	5	2

LogP (Partition coefficient between n-octanol and water; MW (Molecular weight); nHBA (number of hydrogen bond acceptor); nHBD (number of hydrogen bond donor).

≤ 500, number of hydrogen bond acceptor groups ≤ 5, and number of hydrogen bond donor groups ≤ 10), with a logP of 5.76, which is not considered to limit the bioavailability of a compound [27]. In addition, the prediction of ADMET properties suggested that **DOCAD5** could cross biological barriers such as the blood-brain barrier and human intestinal epithelium. Furthermore, metabolically, the compound was not classified as a cytochrome P450 (CYP) inhibitor. In addition, this compound did not show carcinogenic effect or AMES test toxicity and was classified as risk class III for acute oral toxicity (Table 4).

Table 4
Pharmacokinetic properties of **DOCAD5**.

Model	DOCAD5
Absorption	
Blood-brain barrier	+
Human intestinal absorption	+
Caco-2 Permeability	–
Metabolism	
CYP450 2C9 Substrate	NS
CYP450 2D6 Substrate	NS
CYP450 3A4 Substrate	S
CYP450 1A2 Inhibitor	NI
CYP450 2C9 Inhibitor	NI
CYP450 2D6 Inhibitor	NI
CYP450 2C19 Inhibitor	NI
CYP450 3A4 Inhibitor	NI
CYP Inhibitory promiscuity	low
Excretion	
Renal Organic Cation Transporter	NI
Toxicity	
AMES toxicity	NT
Carcinogens	NC
Acute Oral Toxicity	III

+ (positive absorption); - (negative absorption); NS (non-substrate); S (substrate); NI (non-inhibitor); NT (non-toxic); NC (non-carcinogenic).

4. Discussion

Steroids are an important and diverse class of compounds that perform a variety of functions in eukaryotic organisms and some prokaryotes. The ability to easily penetrate the plasma membrane and bind to cellular receptors makes this group of compounds an interesting target for the development of new drugs [9]. The results presented in this work provide information on the antileishmanial activity of steroid derivatives.

At least 20 *Leishmania* species are considered to be infective to humans, and the chemotherapies used to treat leishmaniasis have varied responses to *Leishmania* species. Therefore, an initial drug screen was performed against promastigotes of four *Leishmania* species: *L. amazonensis*, *L. braziliensis*, *L. major*, and *L. infantum*. The first three are related to cutaneous leishmaniasis, whereas the fourth is associated with visceral leishmaniasis. The promastigotes of *L. infantum* were less sensitive to **CD**, including the **Schiffbase2**, which showed antipromastigote activity against the cutaneous leishmaniasis-inducing species. In addition, no **CADs** affected promastigotes of *Leishmania* species, but, serendipitously, most **DOCADs** exhibited biological activity against these parasitic stages. The differences in sensitivity to the compounds among *Leishmania* species may reflect the biochemical and molecular peculiarities of each species, which would affect the drug effectiveness with important implications in disease treatment [28].

Leishmania amazonensis was chosen as a model for the anti-amastigote assays due to the variety of clinical forms of leishmaniasis that this species can cause, ranging from localized cutaneous leishmaniasis (LCL) to anergic cutaneous diffuse leishmaniasis [29]. Recently, this species was isolated from dogs that presented classic clinical symptoms of visceral leishmaniasis, but this is still under observation [30]. The data indicate that (i) **DOCA** alone did not show an inhibitory effect against intracellular parasites, (ii) **DOCADs** were effective against these parasitic stages; and (iii) none of the **CADs** were active against amastigotes. These results showed that the absence of hydroxyl in the C-7 position of the steroid nucleus, as well as the modification of the acid group, is important to antileishmanial activity. **DOCAD** compounds are more lipophilic than **CADs** are, which facilitates their

permeation into the cellular lipid bilayer [31]. However, this physicochemical characteristic may also have contributed, although only slightly, to the cytotoxicity of some **DOCADs**. Studies of aminoquinoline conjugates of **CA** revealed the increased antileishmanial activity of compounds, confirming the importance of this bile acid as a drug carrier [14].The amphiphilic molecular structure of bile acids encourages its wide use as a drug carrier owing to the ease of permeating cell membranes [31].

Regarding structure-activity relationship analysis based on biological activity, the organization of the steroidal derivatives was into two groups: those with **CD** and those bile acid derivatives. Among the **CDs** (**CD1–CD4**), only the **CD2** conjugate showed leishmanicidal activity, specifically against promastigote forms of *L. braziliensis* and *L. major*. Notably, the IC_{50} value was 7.00 μM in *L. braziliensis*, which was approximately four times more potent than miltefosine was (IC_{50}, 28.07 μM in *L. braziliensis*). In contrast, the comparison of the biological activity of the active conjugate **CD2** with that of the free Schiff base (**schiffbase2**) suggests that the conjugation decrease the activity, and the unrelated imine had a lower IC_{50} value than the conjugate did. The effect of the **CDs** on amastigotes of *L. amazonensis*-RFP was similar to that observed in promastigote forms. The relationship between the chemical structures of bile acid derivatives, including amides, aldehydes, and conjugates of steroid-Schiff bases, and their antipromastigote activity indicates a specific trend in the antipromastigote profile of synthetic derivatives. Specifically, only the **DOCADs** showed antileishmanial activity. Therefore, the absence of a hydroxyl group at the C-7 position of the steroidal skeleton contributed positively to the antileishmanial activity of this class of molecules. Among the steroid-Schiff bases conjugates (**CAD3**, **DOCAD3**, **CAD4**, and **DOCAD4**) the **DOCAD3** conjugate was the most active synthetic compound in the series against *L. braziliensis* promastigotes (**DOCAD3** IC_{50} = 15.83 μM). Finally, of all compounds in the series, the biliar aldehyde **DOCAD5** showed the most relevant activity against the intracellular form of the parasite.

The best compound, **DOCAD5**, has shown itself ~10 times more destructive to amastigotes than to the host cell. A good or remarkable SI value for new compounds (natural or synthetic origin) is controversial, ranging from 1 to 20 [32–35]. Although most of the compounds did not show remarkable antileishmanial activity and SI values, the fact that they are not toxic to macrophages makes them selective and, therefore, interesting.

Numerous studies of new compounds with antileishmanial effects have been conducted; however, the mechanisms of action of these compounds have not been well investigated, leading to gaps in the literature. In this study, the *L. amazonensis* death-inducing mechanism of the most promising compound investigated, **DOCAD5**, was evaluated. Mitochondria is an important drug target in trypanosomatids owing to the unique nature of the organelle in these protozoa. In addition, this organelle is the main site of the production of ROS, the byproducts of oxidative phosphorylation generated in the electron transport chain complex (ETC) during mitochondrial respiration. High concentrations of ROS are damaging to cells, and the maintenance of the $\Delta\Psi_m$ is critical to the proper function of the ETC to generate ATP and avoid high levels of ROS [36–38]. Thus, ROS production and changes in $\Delta\Psi_m$ are related events. Our results showed that treatment with **DOCAD5** depolarized the $\Delta\Psi_m$ and significantly increased ROS production. These results suggest that treatment of the parasites with **DOCAD5** affected mitochondrial function, culminating in oxidative stress. The functional impairment of the mitochondria in the treated parasites is sufficient to generate irreversible damage, leading to cell death. However, in association with this event, we also confirmed that the cycle cell of the parasites treated with **DOCAD5** was altered; the population of cells in the sub-G0/G1 phase was higher than that in the untreated cells was. As the total DNA of the parasite was analyzed, it was not possible to determine if the affected DNA was nuclear or mitochondrial. These data presented here demonstrate that **DOCAD5** exhibited promising antileishmanial activity by altering important organelle functions that are essential for complete cellular development. Interesting, the treatment did not alter membrane integrity of promastigotes, excluding necrosis.

In a recent review, the authors showed that many lipids, including sterols, play important roles in numerous cellular pathways such as cell signaling and transcriptional regulation [39]. These authors related the importance of steroids in responses to mitochondrial stress and the maintenance of mitochondrial homeostasis. In addition, they associated the effects of **DA** on the membrane with the activation of the signal cascades that trigger apoptotic cell death. Some authors also provided evidence that **DA** had major effects on the plasma membrane, such as increased membrane cholesterol and changes in the membrane fluidity of HTC116 cells [40]. More studies would be necessary to clarify the effect of **DOCAD5** on the plasma membrane of *L. amazonensis*, including potential interactions with lipids and cholesterol. However, we did not exclude the possibility that **DOCAD5** acts as an exogenous toxic lipid that alters the organization or composition of the plasma and organelle membranes, thereby interfering with signaling pathways and mitochondrial homeostasis, triggering changes that lead to cell death.

In silico studies of physicochemical and pharmacokinetic properties can be used to predict the behavior of chemicals substances in humans. Some structural parameters of the molecule provide information that can be used for the theoretical prediction of whether a drug is a good candidate for oral administration [27,41]. Thus, we evaluated the physicochemical properties of **DOCAD5,** and the results suggest that it is suitable for oral administration, since it only violated one of Lipinski's rules, which does not represent a limitation for oral administration [27]. The pharmacokinetic characteristics of **DOCAD5** indicate that this compound showed favorable permeation properties through the human blood-brain barrier and intestinal epithelium. The blood-brain barrier absorption may be advantageous for the treatment of visceral leishmaniasis because amastigotes of *Leishmania* species are sometimes found in tissues of the central nervous system [42]. The metabolic characteristics exhibited by the compound indicate that it was metabolized by CYP3A4, the enzyme responsible for metabolizing most currently available drugs [43]. Furthermore, based on the AMES test, the compound has been classified as non-toxic and non-carcinogenic, being assigned to the risk class III for acute oral toxicity, which, according to the U.S. Environmental Protection Agency (EPA), are substances that present median lethal dose (LD_{50}) above 500 mg/kg [44,45]. Interestingly, *in silico* physicochemical and pharmacokinetic studies with the compounds **Schiffbase 2**, **CD2**, **DOCAD1** and **DOCAD2** presented results very similar to that of the compound with the best antileishmanial activity **DOCAD5** (data not shown). Based on these data, the compounds exhibited satisfactory characteristics that allow their classification as good candidates for orally administered drugs.

In summary, the **DOCADs** showed better antileishmanial effect than those of cholesterol and **CADs** did, and the most effective compound was **DOCAD5**. Treatment with this compound strongly targeted several important cellular organelles. In addition, studies of the physicochemical and pharmacokinetic properties showed that this compound is suitable for oral administration.

Conflict of interest

The authors declare that there is no conflict of interest regarding this work.

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