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Article

Activity of the Lupane Isolated from *Combretum leprosum* against *Leishmania amazonensis* Promastigotes

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O presente trabalho descreve a atividade do extrato etanólico (EE) dos frutos de *Combretum leprosum*, do triterpeno 3β, 6β, 16β-triidroxilup-20(29)-eno (**1**) e seus derivados sintéticos (**1a**-**1d**), sobre promastigotas de *Leishmania amazonensis*. O EE apresentou atividade leishmanicida e o valor de IC_{50} foi de 24,8 μg mL^{-1}. Já o triterpeno 3β, 6β, 16β-trihidroxilup-20(29)-eno (**1**), na concentração de 5,0 μg mL^{-1}, apresentou uma potente ação inibitória sobre a proliferação das promastigotas (IC_{50} = 3,3 μg mL^{-1}). Entre os derivados sintéticos, apenas **1b** e **1d** apresentaram atividade contra as promastigotas (IC_{50} = 3,48 μg mL^{-1} e 5,8 μg mL^{-1}, respectivamente). Por outro lado, o derivado sintético **1a** não apresentou atividade sobre as promastigotas de *L. amazonensis*. O EE, (**1**) e os derivados sintéticos **1a**-**1d** não apresentaram efeito citotóxico sobre macrófagos peritoneais de camundongos. Estes resultados fornecem evidencias de que o extrato etanólico e o lupano isolado de *C. leprosum* possui atividade contra promastigotas de *L. amazonensis*, podendo ser utilizados como ferramentas no estudo de novas drogas leishmanicidas.

This paper describes the activity of the ethanolic extract (EE), obtained from the fruits of *Combretum leprosum,* the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (**1**) and its synthetic derivatives **1a**-**1d** on *Leishmania amazonensis* promastigotes. The EE displayed leishmanicidal activity and the IC_{50} was 24.8 μg mL^{-1}. However, the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (**1**), at a concentration of 5.0 μg mL^{-1}, showed a potent inhibitory activity on promastigotes proliferation (IC_{50} = 3.3 μg mL^{-1}). Among the synthetic derivatives, only (**1b**) and (**1d**) were active against promastigotes (IC_{50} = 3.48 μg $mL^{-1}$and 5.8 μg mL^{-1}, respectively). Moreover, the synthetic derivative **1a** showed no activity on promastigotes of *L. amazonensis*. EE, (**1**) and the synthetic derivatives **1a**-**1d** showed no cytotoxic effect on mice peritoneal macrophages. These results provide evidence that the ethanolic extract and the lupane isolated from *C. leprosum* was active against promastigotes of *L. amazonensis*, and may be used as a tool in the studies of new antileishmanial drugs.

Keywords: promastigotes, *Leishmania amazonensis*, *Combretum leprosum*, triterpene, synthetic derivatives

Introduction

Leishmania, a protozoan parasite belonging to the family *Trypanosomatidae*, causes a broad spectrum of diseases, collectively known as leishmaniasis. Such conditions occur predominantly in tropical and subtropical regions. Approximately 350 million people live in areas of active *Leishmania* transmission, with 12 million people being directly affected by leishmaniasis in Africa, Asia, Europe and Americas.[1]

Leishmania parasites have a complex life cycle that involves *Phlebotomine* sandfly vectors and mammalian hosts, with the amastigotes being within the phagolysosome of macrophages and the promastigotes in the vector's midgut.[2]

The clinical manifestations of leishmaniasis are often divided in cutaneous, diffuse cutaneous, mucocutaneous and visceral leishmaniasis.[3,4] Cutaneous leishmaniasis can

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be spontaneously healed after a few months, or, depending on the *Leishmania* species, develop into diffuse cutaneous, relapsing cutaneous or mucocutaneous leishmaniasis. Visceral leishmaniasis, if untreated, leads to death in most patients.[5,6] This disease causes considerable morbidity and severe face-disfigurement lesions on the affected people.

Nowadays, chemotherapy for leishmaniasis is still based on pentavalent antimonials (Glucantime and Pentostam), diamines (Pentamidine) and antifungal polyene (Amphotericin B). These are only a few of the drugs available since 1940. Unfortunately, they are generally toxic, expensive, share a tendency to generate resistance and require long-term treatments, which would make the conclusion complicated.[7] Therefore, there is a great and urgent need to develop new, more effective and safer drugs for leishmaniasis control.

Plants provide a potential alternative source of therapeutic compounds in the search for new agents for leishmaniasis and others protozoan diseases treatment.[8-10] They are often used by traditional communities, and, based on documented history of folk usage, many compounds have been isolated. Chalcones, alkaloids, triterpenes, and acetogenins have promising activity against protozoan parasites.[11-14] Plants of the Combretaceae family are widely sold in the traditional-medicine markets in southern Africa.[15] Several authors have demonstrated that some extracts or purified compounds of the *Combretum* species have a broad spectrum of biological activities[16-19] including antiviral,[15,20] antibacterial,[20,21] antiprotozoal,[22,23] anticancer,[24-26] analgesic,[27] anti-inflamatory and hepatoprotective.[28] The usage of *Combretum* genera in folk medicine includes the treatment of a broad range of diseases, such as abdominal pain, back pain, cough, cold, conjunctivitis, diarrhea, dysmenorrhea, earache, fever, headache, fighting worms, infertility in women, leprosy, pneumonia, scorpion stings and snake bite.[16]

Combretum leprosum Mart., a member of the Combretaceae family, from northern Brazil, popularly known as "mufumbo" or "mofumbo" or "cipoaba" is used in folk medicine to treat haemorrhages and as a sedative.[27] In order to evaluated the antileishmanial activity of *Combretum leprosum*, ethanolic extract (EE) and 3β,6β,16β-trihydroxylup-20(29)-ene (**1**) isolated from the fruit, and four synthetic derivatives **1a**-**1d** were investigated in *L. amazonensis* promastigotes.

Experimental

Parasites

Promastigotes of *Leishmania (Leishmania) amazonensis* PH8 strain (IFLA/BR/67/PH8) were axenically cultured at 23 ºC in RPMI 1640, supplemented with 10% inactivated fetal bovine serum (FBS), 20 mmol L^{-1} Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and 50 μg mL^{-1} of gentamycin. On the 5th day of culture (stationary phase of growth), promastigotes were harvested for the *in vitro* assays.

Plant material

Botanical material was colleted in May 2001 in Viçosa, Ceará State, Brazil, and identified by Dr. Afrânio Fernandes (Federal University of Ceará, Fortaleza) as *Combretum leprosum* Mart. A voucher specimen was deposited (No. 12446) in the Herbarium Prisco Bezerra, Biology Department, Federal University of Ceará, Brazil.

Instrumentation and chromatography

Silica gel (Merck 70-230 mesh) was used for all column chromatographies and solvents were redistilled prior to use. ^{1}H and ^{13}C NMR spectra 1D and 2D dimensions, were recorded at 300 and 75 MHz, respectively, using $CDCl_3$ or pyridine-d_5, using TMS as internal standard; EIMS: Finnigan 3200 GC-MS instrument, electron impact mode, 70 eV. UV and IR spectra were obtained with a Perkin-Elmer PC FT-IR apparatus.

*Ethanolic extract (EE) obtention, compound **1** isolation and purification*

Dried fruits (2.7 kg) were powdered and then extracted with ethanol (5 L), being stirred and macerated at room temperature for approximately 24 h. This procedure was repeated three times. The solvent was fully evaporated under reduced pressure and the EE (58.3 g) was concentrated and stored at –20 ºC prior to use. Part of the EE (32.0 g) was submitted to coarse chromatography over silica gel (600 g) using hexane, $CHCl_3$, EtOAc and MeOH as eluents. The fraction eluted with $CHCl_3$ was chromatographed on a silica gel column and was eluted with hexane-EtOAc, in increasing polarity. The fractions 27-30, eluted with hexane:EtOAc (30:70), were combined on the basis of thin layer chromatography (TLC) analysis and the presence of a white precipitate was observed, which after recrystallization from ethanol, was identified as **1** (2.37 g).[29]

*Preparation of the synthetic derivatives **1a**-**1d***

The derivatives **1a**-**1d** were prepared in accordance with Facundo *et al*.[29]

*Derivative **1a***

0.54 mmol of **1** was dissolved in acetic anhydride (5.0 cm^3) and pyridine (1.0 cm^3). After 24 h, the material was poured over ice and the resulting mixture extracted with ethyl ether. The ethereal solution was washed with 3% aqueous HCl to eliminate the presence of pyridine. After solvent distillation and silica gel column purification, it yielded 0.34 mmol of the acetylated derivative **1a**.

*Derivative **1b***

0.65 mmol of **1** was dissolved in dichloromethane (170 cm^3) and treated with pyridinium chlorochromate (1.53 mmol) for 2 h, under agitation, at room temperature. The mixture was filtered and extracted with ethyl ether solvent evaporation and silica chromatographic column purification, yielding 0.45 mmol of the oxidized product **1b**.

*Derivative **1c***

0.22 mmol of **1a** dissolved in dichloromethane (100 cm^3) was treated with pyridinium chlorochromate (0.65 mmol) for 2 h, under agitation. The mixture was filtered and extracted with diethyl ether. After solvent distillation and silica gel column purification, the crude product yielded **1c** (0.17mmol).

*Derivative **1d***

0.10 mmol of **1c** was dissolved in a saturated solution of methanol and hydroxide potassium. The mixture was kept under reflux for 3 h at 70 ºC. After organic solvent evaporation, 70 cm^3 of distilled water was added and the organic part was extracted with ethyl ether. After solvent evaporation and silica chromatographic column purification 0.08 mmol of **1d** was obtained.

The structure of compound **1** and its synthetic derivatives (**1a-d**) are shown in Figure 1. The spectroscopic data are presented in the Supplementary Information.

Antileishmanial activity

The antiparasitic activity of ethanolic extract (EE), isolated triterpene **1** and synthetic derivatives (**1a**, **1b**, **1d**) from *C. leprosum* were evaluated against *Leishmania amazonensis* promastigote forms. The EE, **1** and the synthetic derivatives were dissolved in ethanol (less 1%). Promastigotes (5×10^5 *per* well) in 24-well were incubated in RPMI-1640 culture medium in the absence or in the presence of 1, 2 and 5 μg *per* mL of triterpenes and 12.5, 25, 50 and 100 μg *per* mL of the EE during 5 days at 23 ºC. The number of living promastigotes was scored in the presence of erythrosin B. The results were expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a 5 days incubation period. Pentamidine was used as antileishmanial reference compound.[30]

Toxicity on macrophages

Cell viability was assessed using a modified MTT assay.[31] Briefly, elicited peritoneal mice macrophages (1×10^5 cells *per* well) were seeded in a 96-well plate and

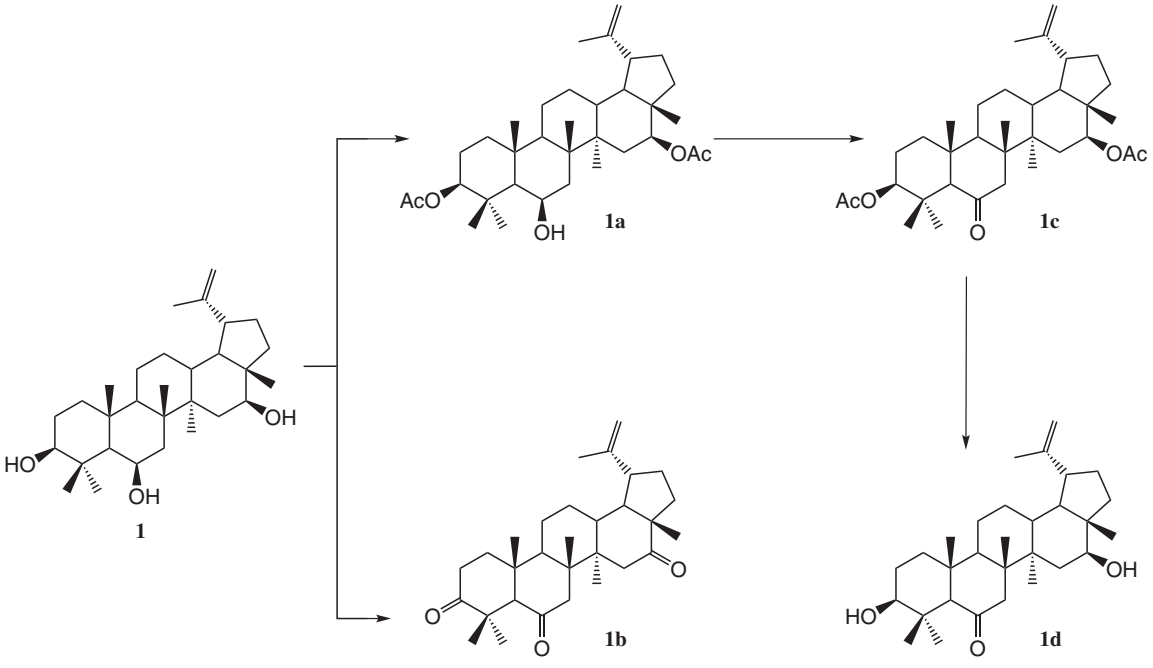

Figure 1. Natural triterpene **1** and synthetic derivatives **1a-1d**.

treated with 25 μg mL^{-1} of EE and 5 μg mL^{-1} of triterpenes. After 24 h, 10 μL of a MTT solution (5 mg mL^{-1} in phosphate buffered saline) was added to each well and furtherly incubated for 2 h at 37 °C. Subsequently, 100 μL of dimethylsulfoxide (DMSO) was added to each well to solubilize any deposited formazan and the optical density (OD) of each well was measured at 540 nm.

Statistical analysis

The inhibitory concentrations (ICs) were calculated by Probit Analysis using the program Minitab 14 (Minitab Inc). The statistical significance of group differences was evaluated using ANOVA and comparisons by Student-Newman-Keuls by the program SigmaStat (SPSS Inc, 1992-1997).

Results and Discussion

The pharmacological properties of *Combretum leprosum* have not been characterized up to this moment in details. Some studies had shown a promising potential for antinociceptive, anti-inflammatory, and antiulcerogenic activities.[32] The arjunolic acid, isolated from roots and flowers of *C. leprosum*, displayed anti-inflammatory, antinociceptive and anticholinesterasic activities.[33] Triterpene, 3β,6β,16β-trihydroxylup-20(29)-ene (**1**), isolated from flowers, showed antinociceptive activity.[34] In the present work we have shown an anti-leishmanicidal effect that had not been explored for this species yet.

The ethanolic extract (EE) of fruits of *C. leprosum* was tested *in vitro* against the promastigote forms of *L. amazonensis*. Quantifying *Leishmania* promastigote populational growth is involved in a number of investigations such as drug-efficacy testing and vaccine candidates.[35] In such studies, the promastigote stage is most oftenly used once the promastigote is the infective form of the parasite and the proliferation capacity of a strain plays a key role in its infectivity potential.[36]

The EE showed an inhibitory activity, interfering in the promastigotes growth. A significant leishmanicidal effect was evident after 5 days of culture (Table 1). Promastigotes treated with 100 μg mL^{-1} of the EE or pentamidine died after 24 h of treatment. In all concentrations of EE (12.5-100 μg mL^{-1}) studied, there was a significant difference in the growth of treated parasites compared to controls, not treated with the extract and treated with ethanol ($F=318.6$; $P < 0.001$). Promastigotes incubated with 1% (v/v) ethanol (the concentration necessary to dissolve the highest extract concentration used in the test) showed growth rates equivalent to the control cultures, indicating that the EE solvent was not toxic to the parasite.

The *L. amazonensis* promastigotes growth curve in the presence or absence of EE is shown in Figure 2. On the 5th day of culture the promastigotes treated with the EE (25 μg mL^{-1} and 50 μg mL^{-1}) showed a decrease in the parasite growth around 80% and 93%, respectively, compared with control or control treated with ethanol (Figure 2). The estimated IC_{50} of EE for *L. amazonensis* promastigotes was 24.8 μg mL^{-1}.

Table 1. Growth inhibition of *L. amazonensis* cultures by the ethanolic extract (EE) of *C. leprosum* Mart. *in vitro* after five days

Drug	Promastigotes × 10^5
Control (C)	162.1 + 3.1$^{(a)}$
EtOH	138.2 + 3.1$^{(b)}$
EE 12.5 μg mL^{-1}	113.2 + 3.4$^{(c)}$
EE 25 μg mL^{-1}	81.0 + 2.9$^{(d)}$
EE 50 μg mL^{-1}	22.6 + 1.9$^{(e)}$

One-way ANOVA, Student-Newman-Keuls (comparisons), n = 8 (mean ± SE). Different letters (a, b, c, d, e) indicate significant ($P < 0.05$) differences in a column. EE 100 μg mL^{-1} and Pentamidine killed all *L. amazonesis* promastigotes in culture after 5 days.

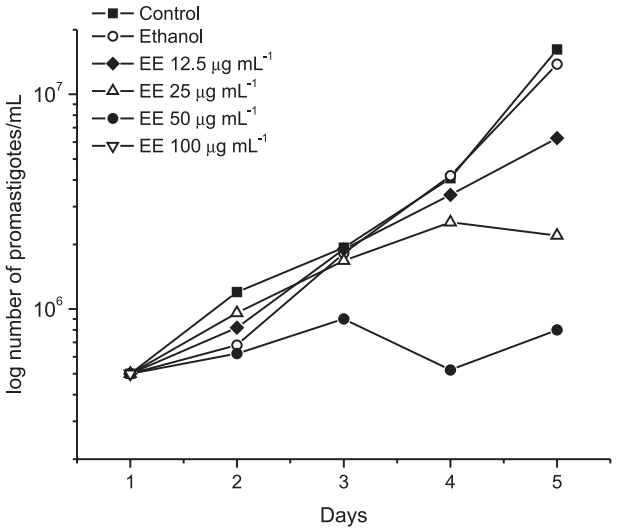

Figure 2. *In vitro* effect of ethanolic extract of *C. leprosum* fruits on parasite growth. Representative growth curve of *L. amazonensis* promastigotes. Promastigotes forms were incubated with a range of EE concentrations during 5 days at 24 ºC. The promastigote growth was scored by counting of the viable parasites daily. The data shown is representative of at least five independent experiments.

Phytochemical studies carried out with some species of the genus *Combretum* indicated the presence of many classes of constituents, including triterpenes, flavonoids, lignans, non-protein amino acids, among others.[29,37,38] McGaw *et al.*[16] using extracts of 20 species of *Combretum*, have shown that some of these, such as *C. apiculatum, C. imberbe* and

C. molle, present antiinflammatory activity; others, such as *C. hereroense* and *C. paniculatum* have antihelmint and antischistosoma activity, respectively. *C. molle* also has activity against *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum*.[39] Other *Combretum* species are capable of causing damages to the DNA of bacteria, such as *C. apiculatum, C. mossambicense* and *C. hereroense*. *C. fragrans*, *C. padoides* and *C. erythrophyllum* have antimicrobial activity against Gram-positive and Gram-negative bacteria[21,40,41] and *C. micranthum* have antiviral activity against herpes simplex virus types 1 and 2.[42]

In order to investigate the role of 3β,6β,16β-trihydroxylup-20(29)-ene (**1**), a lupane triterpene isolated from fruits of *C. leprosum*, we examined the effect of this compound in the *L. amazonensis* promastigotes growth. Various concentrations of this compound were used and the number of viable cells was scored every 24 h for up to 5 days. As shown in Figure 3A, after three days of culture, 5 μg mL^{-1} of compound **1** inhibited cell growth. The number of promastigotes reduced at least 80% at the fifth day of culture if compared to both controls; the one untreated and the other treated with ethanol. The estimated IC_{50} of **1** for *L. amazonensis* promastigotes was 3.3 μg mL^{-1} ($F = 1889.8$; $P < 0.001$). This compound has a potent effect of inhibiting the promastigote growth. The others concentrations (2 and 1 μg mL^{-1}) inhibited the parasite growth in 27% and 9% respectively, if compared to untreated control or ethanol-treated control (Figure 3A).

As another approach, in order to assess the biological activity and verify whether the role of structural modifications could increase or decrease biological activity, synthetic derivatives from this triterpene **1** (Figure 1) were prepared as described in Experimental. Derivative **1a**, which had the hydroxyl groups at 3β and 16β positions

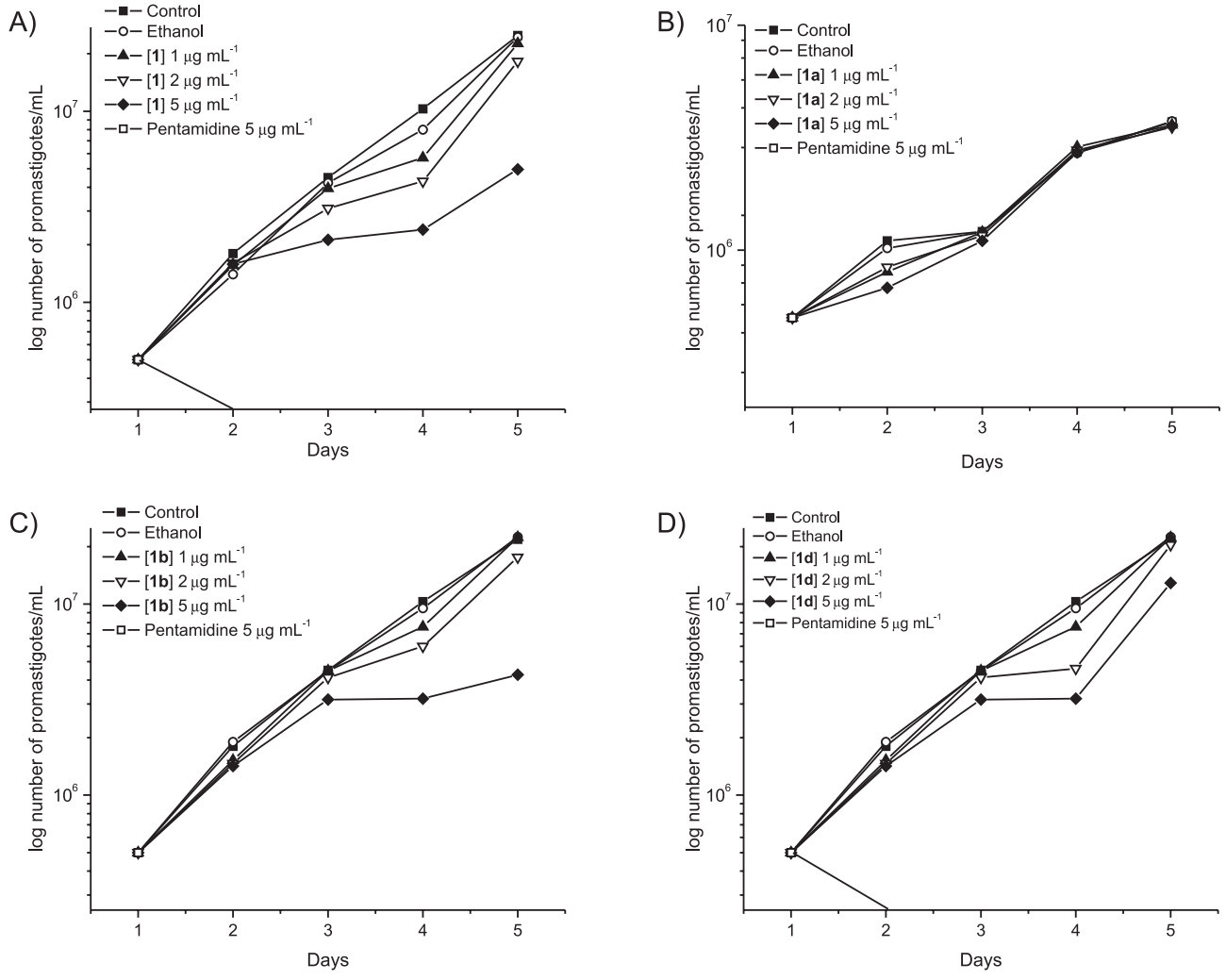

Figure 3. *In vitro* effect of triterpene **1** isolated from *C. leprosum* fruits and its synthetic derivatives **1a**-**1d** on parasite growth. Representative inhibition growth curves of *L. amazonensis* promastigotes incubated with a range of concentrations of natural triterpene **1** (A), derivative **1a** (B), derivative **1b** (C), and derivative **1d** (D) during 5 days at 24 ºC. The promastigote growth inhibition was scored by counting of the viable parasites daily. The data shown is representative of at least five independent experiments.

Table 2. Growth inhibition of *L. amazonensis* cultures by different concentrations of the triterpene **1** and its derivatives *in vitro* after five days

	Promastigotes × 10^5				
	1 μg mL^{-1}	2 μg mL^{-1}	5 μg mL^{-1}	C (±SE)	EtOH (±SE)
Triterpene **1**	226.8 (a1)	182.1 (a2)	49.6 (a3)	249.0 ± 7.0 (4)	244.2 ± 7.6 (4)
Derivative **1a**	197.1 (b1)	192.3 (a1)	202.3 (b1)	190.7 ± 3.9 (1)	190.7 ± 8.5 (1)
Derivative **1b**	223.5 (a1)	171.6 (b2)	42.6 (a3)	218.3 ± 6.8 (1)	224.3 ± 5.9 (1)
Derivative **1d**	222.6 (a1)	203.8 (a2)	129.0 (b3)	218.3 ± 6.9 (1)	224.3 ± 5.9 (1)

SE = Standard Error; C = control without treatment; EtOH = Control with ethanol; Concentration (μg mL^{-1}) ± 3.75 (SE) Two Way ANOVA, Student-Newman-Keuls (comparisons), n = 8. Different letters indicate significant ($P < 0.05$) differences in a column and different numbers indicate significant differences in the same row.

replaced by acetyl, became inactive (Figure 3B). Derivative **1b**, that was trioxydated at positions 3β, 6β and 16β, had a similar antileishmanial activity if compared to the natural compound (Figure 3C). This derivative **1b** at 5 μg mL^{-1} inhibited 80% of cell growth after 5 days of culture ($F = 3951.8$; $P < 0.001$). Incubation with 2 μg mL^{-1} of **1b** inhibited 21% of parasite growth and 1μg mL^{-1} had no effect (Figure 3C). The estimated IC_{50} of derivative **1b** for *L. amazonensis* promastigotes was 3.48 μg mL^{-1} similar of natural compound.

The derivative **1d** presents a carbonyl group at position C-6, differently from the natural triterpene **1**, which has a hydroxyl group at this position. This derivative **1d** also inhibited *L. amazonensis* growth *in vitro* at the concentration of 5 μg mL^{-1}, but the rate of inhibition was 41% ($F = 402.98$; $P < 0.001$). This compound interfered only slightly with parasite growth in culture. At the concentration of 2 μg mL^{-1} it inhibited only of 6.5% of promastigote growth (Figure 3D). The estimated IC_{50} of derivative **1d** was 5.9 μg mL^{-1}.

The derivative **1c** that was monooxydated at positions 6β and had the hydroxyl groups at 3β and 16β positions replaced by acetyl was insoluble in ethanol, and therefore was not tested. The promastigotes incubated with ethanol (the vehicle used to dissolve the compounds **1** and **1a**-**1d**) showed growth rates equivalent to ones of the control cultures, indicating that the solvent/vehicle was not toxic to the parasite. The promastigote culture treated with ethanol showed no significant difference compared to control. On the other hand there was a significant difference between the rate growth of natural triterpene-treated parasites and derivative **1a** and **1d** treated parasites at 5 μg mL^{-1} ($P < 0.05$). At this concentration the triterpene **1** and the synthetic derivative **1b** have the same leishmanicidal activity, inhibiting promastigote growth in a similar pattern (Table 2).

Structural modifications in natural compound **1** allowed us to conclude that the acetyl groups at positions 3β and 16β led to inactivation of the lupane. But, the replacement of hydroxyl groups by carbonyl groups did not significantly affect its activity, as shown in the results from derivatives **1b** and **1d**. Compound **1** has $IC_{50} = 3.3$ μg mL^{-1}, derivative **1b** has $IC_{50} = 3.48$ μg mL^{-1} and **1d**, $IC_{50} = 5.8$ μg mL^{-1}.

Screening compounds with known toxic effects against *Leishmania* and no effect against host cell is a useful approach in enhancing our knowledge of the biological events that regulate the processes of growth arrest and death in this parasite. By the way EE, the natural compound **1** and synthetic derivatives **1a**-**1d** were evaluated for their cytotoxicity against mouse peritoneal macrophages and none of them were cytotoxic to the mammalian cells by MTT assay (Table 3).

Table 3. Effect of EE, **1** and **1a**-**1d** on the cell viability

Drug	O.D. $_{540\ nm}$
Control	0.200 ± 0.013
EE 25 μg mL^{-1}	0.213 ± 0.020
(**1**) 5 μg mL^{-1}	0.223 ± 0.019
(**1a**) 5 μg mL^{-1}	0.205 ± 0.019
(**1b**) 5 μg mL^{-1}	0.217 ± 0.030
(**1d**) 5 μg mL^{-1}	0.206 ± 0.018

Values are the mean ± SE of the three independent experiments.

In conclusion, the *C. leprosum* Mart. contains a bioactive triterpene, which has a significant activity against *L. amazonensis* promastigotes. The results presented in this paper furtherly support a new activity of triterpene **1** isolated from the fruits of *C. leprosum* and two of its synthetic derivatives **1b** and **1d**. The high pharmacological activity of 3β,6β,16β-trihydroxylup-20(29)-ene (**1**) from the *C. leprosum* fruits and the activity of the ethanolic extract on *L. amazonensis* promastigotes may be tools in further studies for the development of novel antileishmanial drugs.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as a PDF file.

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Supplementary Information

Activity of the Lupane Isolated from *Combretum leprosum* against *Leishmania amazonensis* Promastigotes

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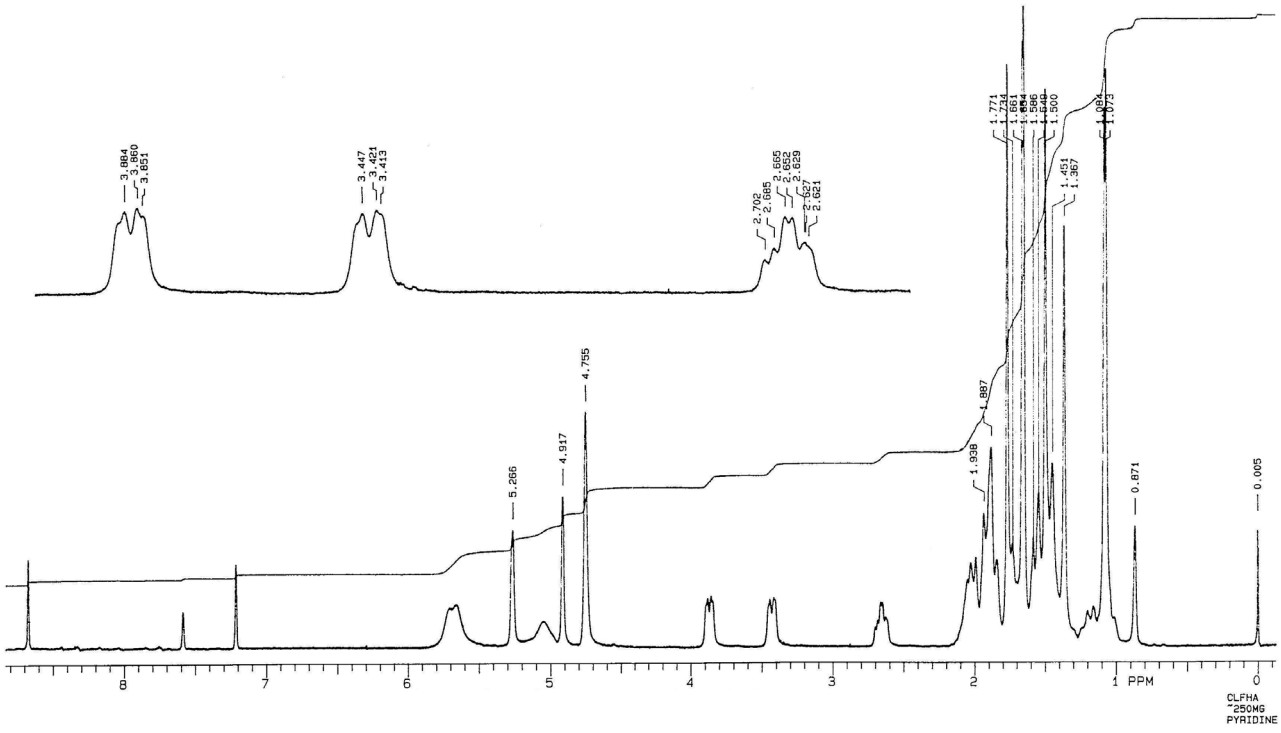

Figure S1. ^{1}H NMR spectrum of **1**.

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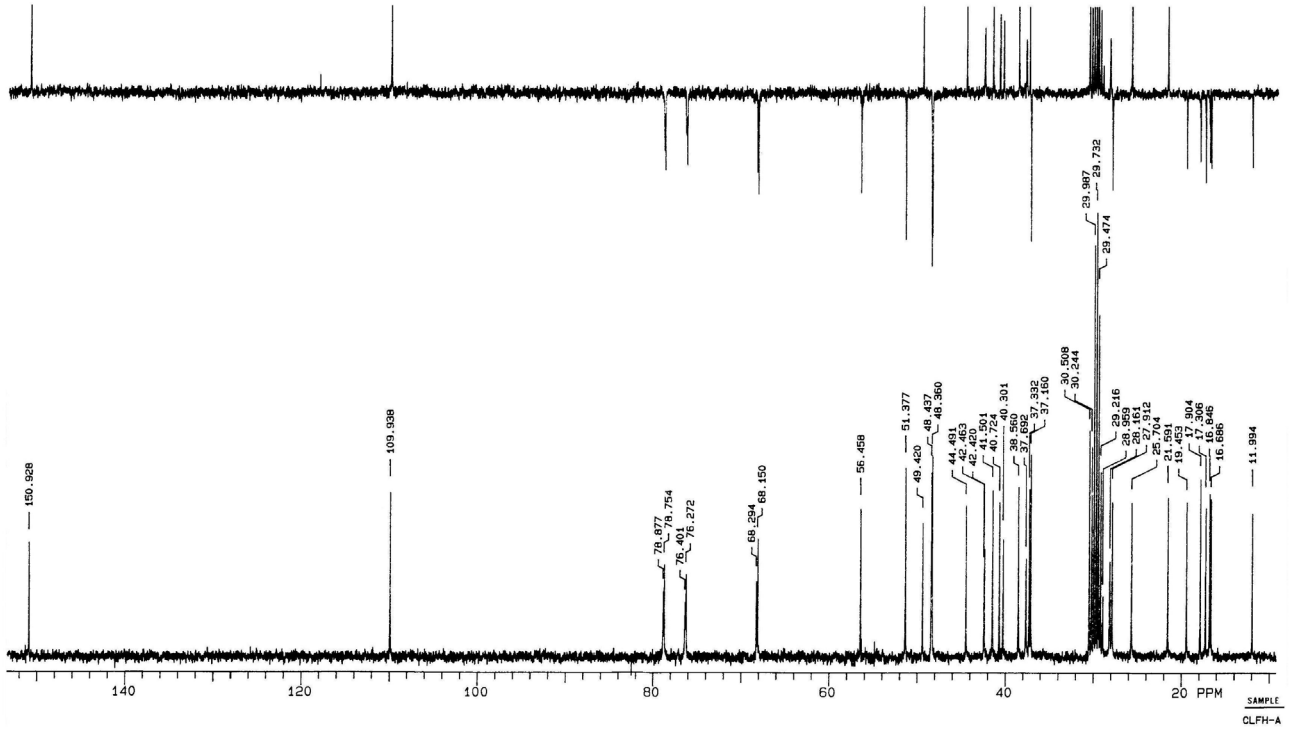

Figure S2. ^{13}C NMR spectrum of **1**.

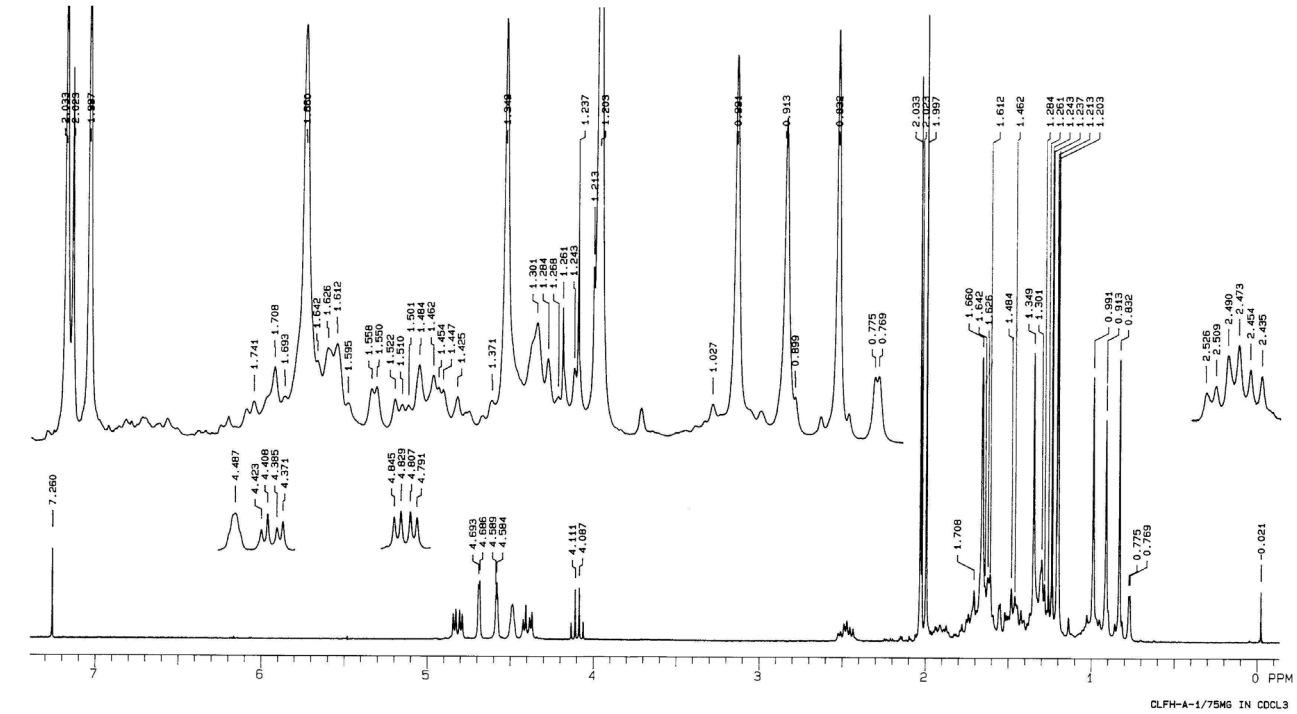

Figure S3. ^{1}H NMR spectrum of **1a**.

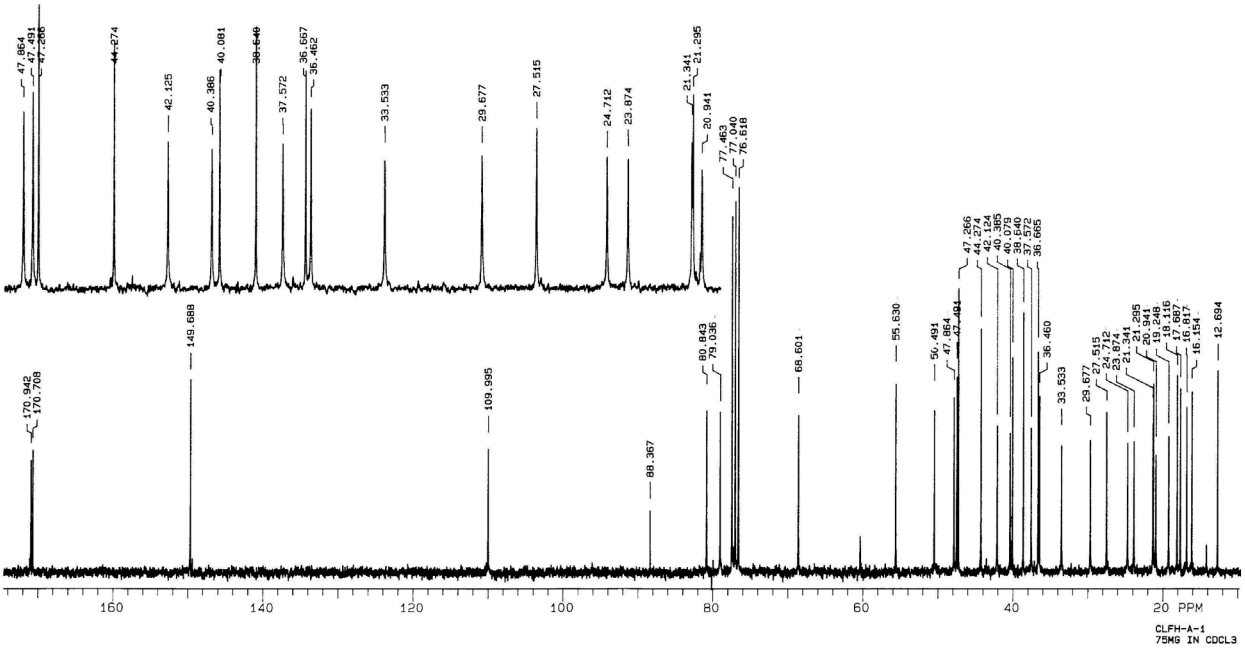

Figure S4. [13]C NMR spectrum of **1a**.

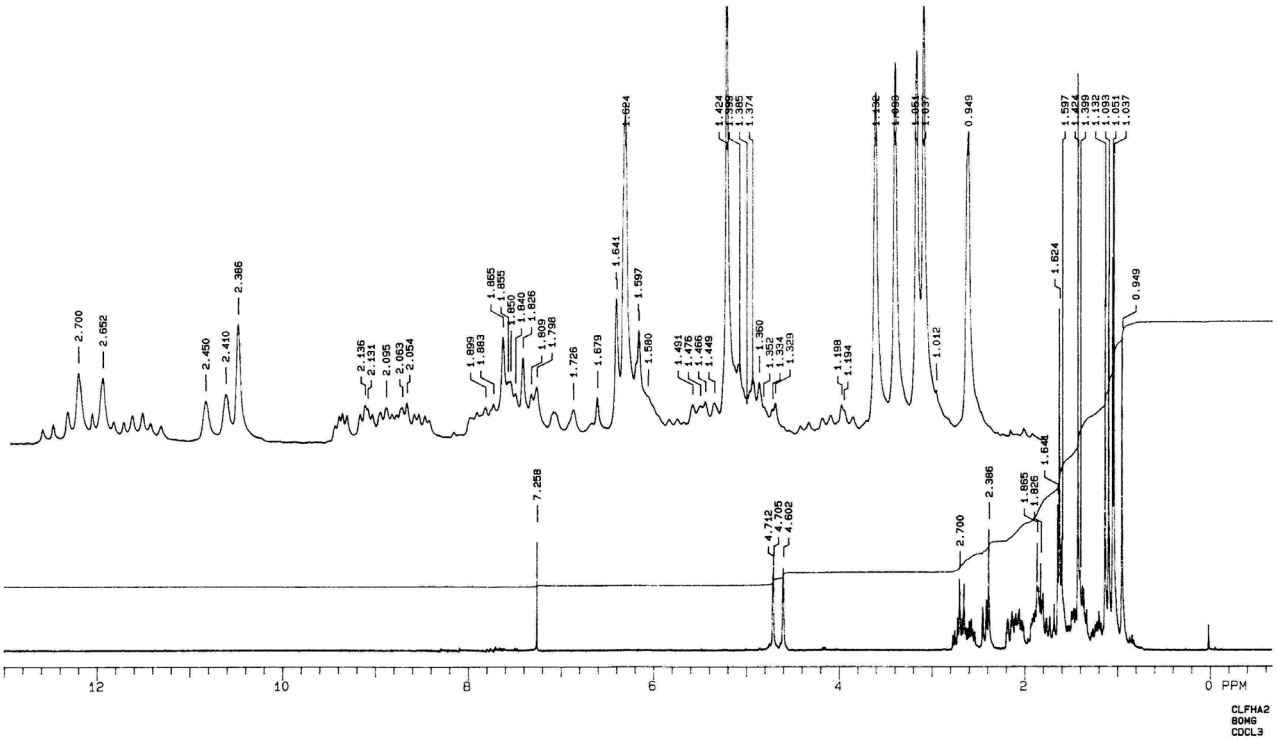

Figure S5. [1]H NMR spectrum of **1b**.

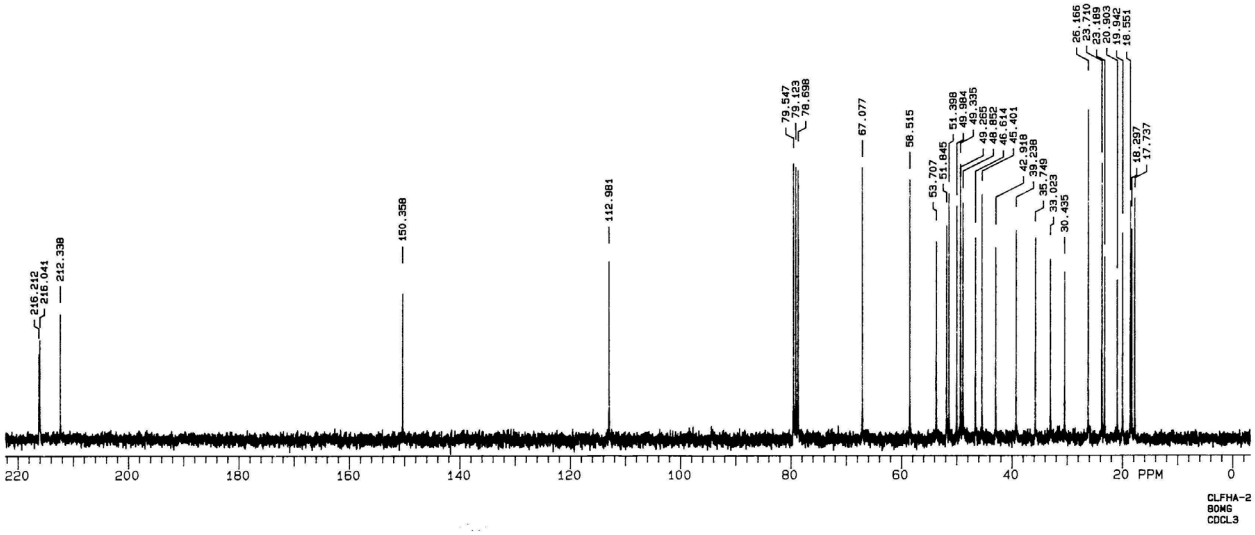

Figure S6. ^{13}C NMR spectrum of **1b**.

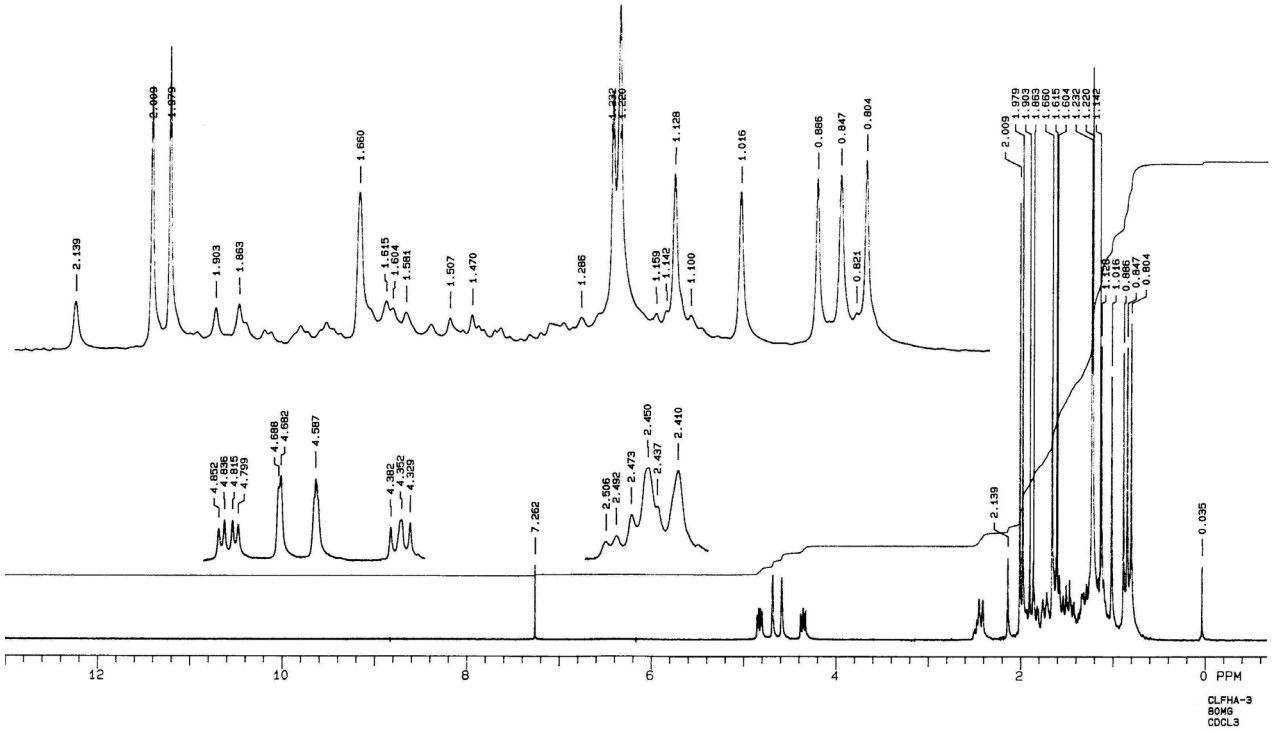

Figure S7. ^{1}H NMR spectrum of **1c**.

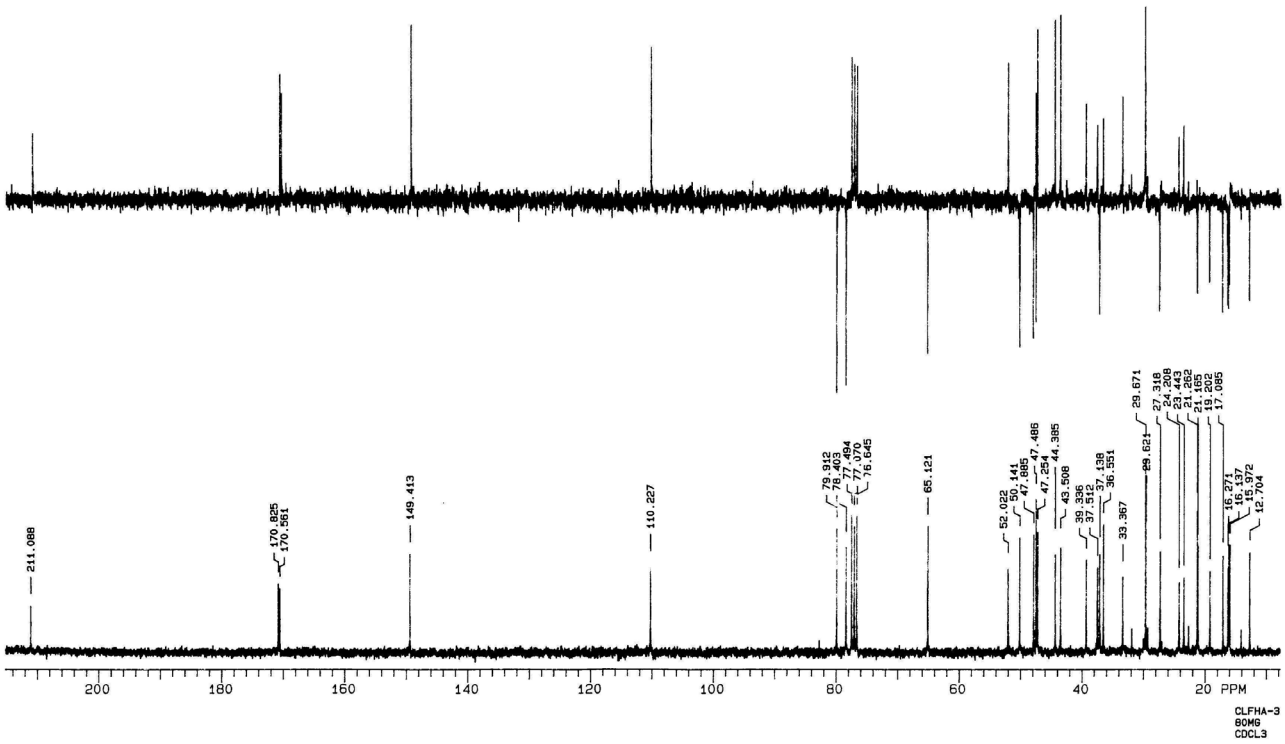

Figure S8. ^{13}C NMR spectrum of **1c**.

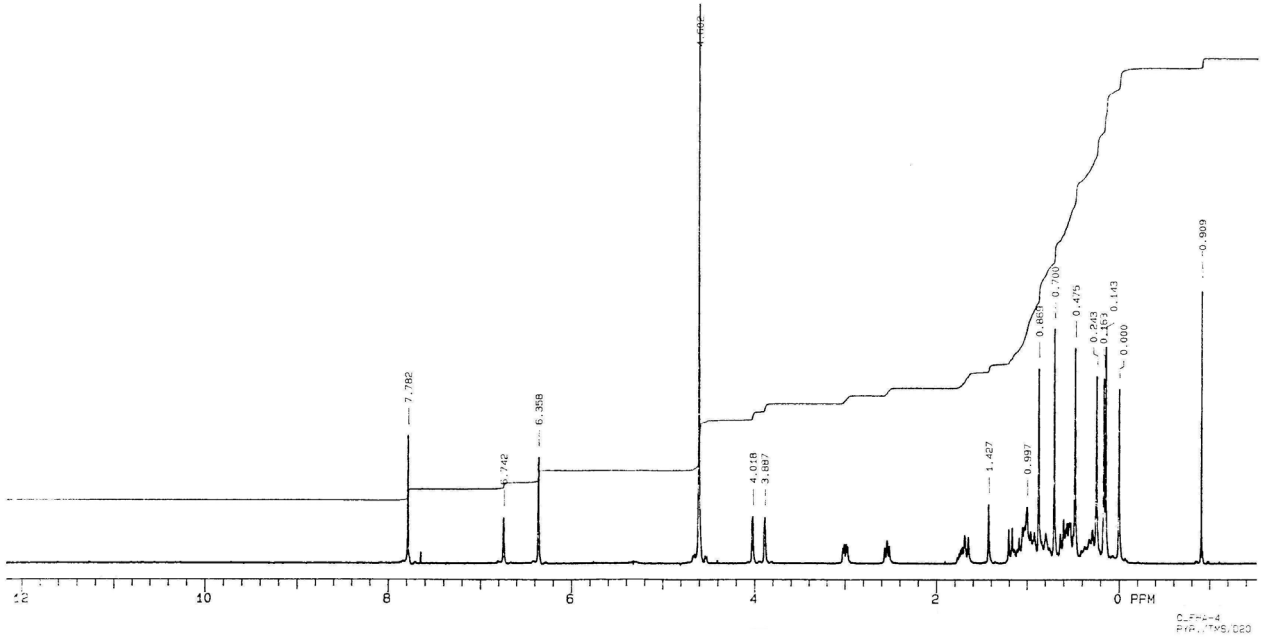

Figure S9. ^{1}H NMR spectrum of **1d**.

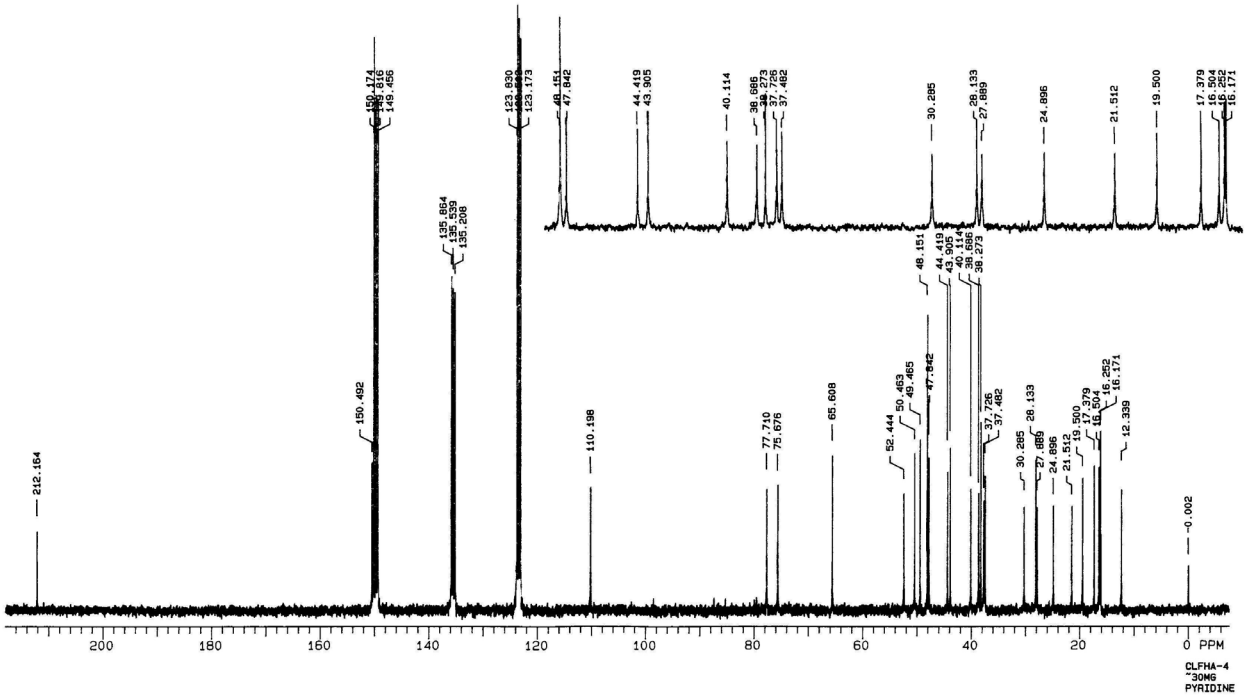

Figure S10. ^{13}C NMR spectrum of **1d**.