Synthesis and Antileishmanial Activity of C7- and C12-Functionalized Dehydroabietylamine Derivatives

M. Auxiliadora Dea-Ayuela\textsuperscript{a,b}, Pablo Bilbao-Ramos\textsuperscript{b}, Francisco Bolás-Fernández\textsuperscript{b} and Miguel A. González-Cardenete,\textsuperscript{c}

\textsuperscript{a} Departamento de Farmacia, Universidad CEU Cardenal Herrera, Avda. Seminario s/n, 46113 Moncada (Valencia), Spain
\textsuperscript{b} Departamento de Parasitología, Universidad Complutense de Madrid, Plaza Ramón y Cajal s/n, 28040 Madrid, Spain
\textsuperscript{c} Instituto de Tecnología Química, Universitat Politècnica de València-Consejo Superior de Investigaciones Científicas, Avda. de los Naranjos s/n, 46022 Valencia, Spain

Abstract— Abietane-type diterpenoids, either naturally occurring or synthetic, have shown a wide range of pharmacological actions, including antiprotozoal properties. In this study, we report on the antileishmanial evaluation of a series of (+)-dehydroabietylamine derivatives functionalized at C7 and/or C12. Thus, the activity \textit{in vitro} against \textit{Leishmania infantum}, \textit{Leishmania donovani}, \textit{Leishmania amazonensis} and \textit{Leishmania guyanensis}, was studied. Most of the benzamide derivatives showed activities at low micromolar concentration against cultured promastigotes of \textit{Leishmania} spp. (IC\textsubscript{50} = 2.2-46.8 µM), without cytotoxicity on J774 macrophage cells. Compound 15, an acetamide, was found to be the most active leishmanicidal agent, though it presented some cytotoxicity on J774 cells. Among the benzamide derivatives, compounds 8 and 10, were also active against \textit{L. infantum} intracellular amastigotes, being 18- and 23-fold more potent than the reference compound miltefosine, respectively. Some structure-activity relationships have been identified for the antileishmanial activity in these dehydroabietylamine derivatives. © 2017 Elsevier Science. All rights reserved

Keywords: Leishmanicidal, abietane, diterpene, dehydroabietylamine

Introduction

Leishmaniasis is a poverty-associated disease caused by more than 20 species of protozoan parasites of the genus \textit{Leishmania}. It is a wide spectrum of vector born diseases with great epidemiological and clinical diversity. Three major clinical forms of the disease are visceral, cutaneous, and mucocutaneous leishmaniasis, which differ in immunopathologies and degree of morbidity and mortality. Visceral leishmaniasis (VL, also known as Kala-azar) is the most severe form, fatal, if it is not treated. Moreover, VL has emerged as an important opportunistic infection associated with HIV and co-infection is becoming an increasing problem that requires urgent attention [1]. Administration of pentavalent antimonials remains as the first choice therapy, followed by amphotericin B and miltefosine. However, the growing incidence of parasitic resistance, the route of administration, long-term treatments, high toxicity and the drug cost makes the development of new drugs necessary [2].

Natural products are potential sources of new and selective agents for the treatment of a number of diseases, including those caused by parasites. To date, many compounds from plant sources have shown potential antileishmanial activity, though none of them has undergone clinical evaluation [3]. Among these, some naturally occurring abietane-type diterpenoids and semisynthetic derivatives have shown promising antiparasitic activities [4]. For example, ferruginol (1), isolated from \textit{Juniperus procera}, showed good antileishmanial activity (IC\textsubscript{50} = 12.2 µM) against \textit{Leishmania donovani} promastigotes and 12-methoxyarnosic acid (2) (Fig. 1), isolated from \textit{Salvia repens}, showed potent activity (IC\textsubscript{50} = 0.75 µM) against axenic \textit{L. donovani} amastigotes [5,6]. On the other hand, semisynthetic ferruginol analogues 3 (Fig. 1) displayed antileishmanial activity (IC\textsubscript{50}= 22.3-35.0 µM) on intracellular amastigotes (\textit{L. infantum} and \textit{L. braziliensis}), being more active and less toxic than the reference drug glucantime [7]. Recently, Moreira \textit{et al.} reported on the antileishmanial activity of some dehydroyabiatic acid derivatives (e.g. cyclohexyl-L alanine derivative 4, Fig. 1)
and some dehydroabietylamine amides (e.g. acrylic acid derivative 5, Fig. 1), which were able to kill intracellular *L. donovani* amastigotes in the range 0.4 to 9.0 µM [8].

The present study is a continuation of our research programs to synthesize and discover bioactive diterpenoids [9]. It includes the synthesis of several amide derivatives from commercially available (+)-dehydroabietylamine (6, Scheme 1) with potential antileishmanial activity. Among these derivatives, several nitroaromatic compounds were envisaged as antileishmanial prodrugs, as it is known that these compounds require activation before exerting their therapeutic effects [10]. Herein, we describe the antileishmanial activity of dehydroabietylamine derivatives (7-15) (Scheme 1) against *Leishmania* species (*L. infantum, L. donovani, L. amazonensis* and *L. guyanensis*), as well as for cytotoxicity against J774 macrophages, following established procedures (see Experimental section). The results on extracellular forms (promastigotes) are summarized in Table 1.

As can be seen in Table 1, compounds (benzamides) 7-10 showed relevant activity without significant cytotoxicity on J774 macrophage cells (CC50 > 200 µM), being *L. donovani* the most sensitive species. Compound 15, an acetamide, was found to be the most active leishmanicidal agent, though it presented some cytotoxicity on J774 cells. The remaining acetamides, compounds 11-14, also presented cytotoxicity and were not included in further studies.

Figure 1. Examples of antileishmanial abietane diterpenoids.

Scheme 1. Synthesis of the tested compounds 7-15.

**Results and Discussion**

**Chemistry**

The compounds 7-15 were synthesized from (+)-dehydroabietylamine (6), as shown in Scheme 1. Compounds 13 and 15 are novel derivatives.

Benzamides 7, 8 and 10 were obtained following similar conditions to those reported in the literature [11]. Thus, compound 7 (N-benzyloxydehydroabietylamine) was synthesized by acylation of dehydroabietylamine (6) with benzoyl chloride in high yield (87%). Then, benzamide 7 was subjected to nitration at C-12 with acetic anhydride-cupric nitrate to give nitroderivative 8 (66% yield), while benzylic oxidation with CrO₃ gave ketone 10 in 48% yield. Similar benzylic oxidation at C-7 of nitroderivative 8 gave benzamide 9 in 40% yield. A similar synthetic sequence using acetyl chloride as acylating agent of 6 instead of benzoyl chloride, afforded the corresponding acetamides 11-14 in reasonable yields. Finally, compound 15 was obtained by Fischer indole reaction of 14 with a fluorinated phenylhydrazine [12].

**Biological Evaluation**

Compounds 6-15 (Scheme 1) were evaluated for antileishmanial activity against four different *Leishmania* species (*L. infantum, L. donovani, L. amazonensis* and *L. guyanensis*), as well as for cytotoxicity against J774 macrophages, following established procedures (see Experimental section). The results on extracellular forms (promastigotes) are summarized in Table 1.
Table 1. IC₅₀ Leishmanicidal and cytotoxic effects (in µM) of C7- and C12-functionalized dehydroabietylamine derivatives on in vitro promastigote assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>L. infantum</th>
<th>L. donovani</th>
<th>L. amazonensis</th>
<th>L. guyanensis</th>
<th>Macrophages J774</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀±SD</td>
<td>SI</td>
<td>IC₅₀±SD</td>
<td>SI</td>
<td>CC₅₀±SD</td>
</tr>
<tr>
<td>7</td>
<td>3,1±0,2</td>
<td>&gt;64</td>
<td>2,2±0,2</td>
<td>&gt;90</td>
<td>3,7±0,2</td>
</tr>
<tr>
<td>8</td>
<td>11,1±0,8</td>
<td>&gt;18</td>
<td>3,2±0,1</td>
<td>&gt;62</td>
<td>20,7±0,5</td>
</tr>
<tr>
<td>9</td>
<td>23,9±1,2</td>
<td>&gt;8</td>
<td>5,4±0,4</td>
<td>&gt;37</td>
<td>12,2±2,2</td>
</tr>
<tr>
<td>10</td>
<td>8,7±0,8</td>
<td>&gt;23</td>
<td>4,0±0,3</td>
<td>&gt;50</td>
<td>4,9±0,5</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>1,5±0,1</td>
<td>19,2</td>
<td>2,8±0,4</td>
<td>10,3</td>
<td>4,0±0,2</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>3,4±0,6</td>
<td>40,1</td>
<td>0,15±0,02</td>
<td>909</td>
<td>47,7±5,0</td>
</tr>
</tbody>
</table>

It is interesting to note that benzamides 7-10 showed better activity against L. amazonensis parasites compared to the standard drug miltefosine, used as reference. They also showed better selectivity index (SI = CC₅₀/IC₅₀) in this Leishmania spp. In general, L. guyanensis was the least sensitive parasite strain to the tested compounds. Unfortunately, the tested acetamide derivatives displayed toxicity against the J774 cell line. On the contrary, the benzamide derivatives showed no cytotoxicity at 200 µM.

In the mammalian hosts Leishmania parasites exist as intracellular amastigotes within phagolysosomes of macrophages. Therefore, benzamides 7-10, which are the most active compounds in the antipromastigote assay (SI from >2 to >90), could be good candidates for subsequent investigations against the clinically relevant Leishmania amastigote forms. Consequently, these benzamides were tested against L. infantum and L. amazonensis intracellular amastigotes (Table 2).

When the assay was carried out on L. infantum amastigotes, the antileishmanial activity of the tested derivatives was similar in comparison to extracellular forms. However, L. amazonensis amastigotes were more sensitive to these compounds. Compound 10 was the most potent for both parasite strains (IC₅₀ = 2.5 and 3.0 µM, respectively).

Different authors have claimed that a compound should have a selectivity index (SI) value > 20 to be considered to possess leishmanicidal properties [13]. This requirement is satisfied by compounds 7-10 against L. infantum and L. amazonensis, except compound 9 against L. infantum.

Table 2. IC₅₀ Leishmanicidal and cytotoxic effects (in µM) of C7- and C12-functionalized dehydroabietylamine derivatives on intracellular amastigote assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>L. infantum</th>
<th>L. amazonensis</th>
<th>Macrophages J774</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀±SD</td>
<td>SI</td>
<td>IC₅₀±SD</td>
</tr>
<tr>
<td>7</td>
<td>4,7±0,2</td>
<td>&gt;42</td>
<td>5,0±0,2</td>
</tr>
<tr>
<td>8</td>
<td>3,3±0,2</td>
<td>&gt;61</td>
<td>3,5±0,2</td>
</tr>
<tr>
<td>9</td>
<td>17,5±0,4</td>
<td>&gt;11</td>
<td>3,7±0,5</td>
</tr>
<tr>
<td>10</td>
<td>2,5±0,9</td>
<td>&gt;80</td>
<td>3,0±0,4</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>58,1±4,4</td>
<td>2,3</td>
<td>16,5±1,9</td>
</tr>
</tbody>
</table>

a IC₅₀, concentration of the compound that produced a 50% reduction in parasites; SD: standard deviation. b Selectivity index, SI = CC₅₀/IC₅₀. c CC₅₀, concentration of the compound that produced a 50% reduction of cell viability in treated culture cells with respect to untreated ones. d IC₅₀ values obtained from six data points instead of eight. The two points removed because of problems solubility corresponded to the more concentrated dilutions 100 and 50 µg/mL of test compound. e ND, not determined.
The resultant data indicates that the introduction of a nitro group at C-12 or a carbonyl group at C-7 increases the antileishmanial activity. In the case of L. infantum amastigotes both modifications on the parent molecule led to lower activity.

**Conclusions**

In summary, a study of antileishmanial activity of several dehydroabietylamine derivatives has been carried out in vitro. Particular modifications of the molecular scaffold abiet-8,11,13-triene functionalized at C-7, C-12, and C-18 have led to potent antileishmanial agents. Two benzamide derivatives, compounds 8 and 10, were found to consistently reduce parasitism at low micromolar concentrations. The presence of a benzamide at C-18 and a carbonyl group at C-7 in the molecule, compound 10, resulted in the highest antileishmanial activity and selectivity index against L. infantum amastigotes (IC50 = 2.5 µM, SI > 80). Moreover, the activity of compound 10 was remarkable, as it was more potent than the reference drug miltefosine, and showed good selectivity. Thus, the abietane-type diterpenoids are promising scaffolds for the development of treatments for Leishmaniasis.

**Experimental**

**General.**

See supporting information.

**Chemistry.**

**Materials.** Compounds 7-15 have been prepared following similar conditions of reported procedures [11, 12]. All compounds prepared in this work exhibit spectroscopic data in agreement with the proposed structures. Purity of all final compounds was 95% or higher.

**N-benzoyl-dehydroabietylamine (7).** To a solution of dehydroabietylamine (6) (2.0 g, 7 mmol) and triethylamine (2.0 mL, 14 mmol) in THF (35 mL) at 0 °C, benzoyl chloride (0.9 mL, 7.7 mmol) was added dropwise. Then, it was allowed to warm to rt and stirred overnight (16 h). The resulting reaction mixture was treated with 1 M HCl (20 mL), phases separated, and the aqueous phase was extracted twice with ethyl acetate. The combined organic extracts were washed with brine, dried over Na2SO4 and concentrated. The resulting crude was purified by flash chromatography, using hexane-ethyl acetate (6:4) as eluent, to give ketone 7 (2.44 g, 87%) as a white solid: [α]20D +2.7 (c 3.0, CHCl3); 1H NMR (300 MHz) δ 7.75-7.70 (2H, m), 7.52-7.35 (3H, m), 6.19 (1H, m), 3.35 (1H, m), 3.25 (1H, m), 2.32 (1H, br, J = 6.5, 8.08 (1H, s), 7.75-7.70 (2H, m), 5.91 (1H, s), 5.22-5.25 (3H, m), 3.10 (1H, t, J = 6.5), 2.05 (3H, s), 1.98 (6H, d, J = 6.5), 0.24 (3H, s), 11C NMR (75 MHz) δ 167.8 (s), 157.0 (s), 145.6 (s), 135.4 (s), 131.3 (s), 128.6 x 2 (d), 126.9 (d), 126.8 x 2 (d), 124.2 (d), 123.8 (d), 50.3 (t), 45.8 (d), 38.5 (t), 37.5 (s), 36.4 (t), 33.4 (d), 30.4 (t), 25.4 (q), 23.9 (q), 23.9 (q), 19.1 (t), 18.8 (q), 18.6 (t); HRMS (ESI) m/z 390.2825 [M+H]+, calcd for C23H21NO3: 390.2797. The NMR and MS data agree with those reported in the literature [8b, 11].

**N-benzoyl-12-nitrodehydroabietylamine (8).** Powdered cupric nitrate trihydrate (2.20 g, 9.0 mmol) was added in portions to a stirred solution of benzamide 7 (1.74 g, 4.47 mmol) in acetic anhydride (70 mL) and the blue solution was stirred at 5 °C for 48 h. Then, the mixture was poured into water with stirring and the resulting precipitate was filtered off, washed with water and crystallized from methanol to give the nitro derivative 8 (1.28 g, 66%) as a pale yellow solid: [α]20D +24.2 (c 3.0, CHCl3); 1H NMR (300 MHz) δ 7.75-7.70 (2H, m), 7.62 (1H, s), 7.52-7.39 (3H, m), 7.06 (1H, s), 6.18 (1H, m), 3.56 (1H, dd, J = 15.0, 6.0), 3.20 (1H, dd, J = 15.0, 6.0), 2.29 (1H, br d, J = 12.0), 1.27-1.19 (9H, m), 1.01 (3H, m); 13C NMR (75 MHz) δ: 167.7 (s), 149.0 (s), 148.3 (s), 141.0 (s), 139.5 (s), 134.8 (s), 131.5 (d), 128.6 x 2 (d), 127.9 (d), 126.2 x 2 (d), 120.3 (d), 50.2 (t), 45.1 (d), 38.3 (t), 37.7 (s), 37.6 (s), 36.3 (t), 30.2 (t), 28.7 (d), 25.3 (q), 2 x 23.5 (q), 18.7 (t), 18.6 (q), 18.4 (t); HRMS (ESI) m/z 435.2739 [M+H]+, calcd for C23H21N2O3: 435.2648. The NMR and MS data agree with those reported in the literature [11].

**N-benzoyl-12-nitrodehydroabietylamine-7-one (9).** A solution of CrO3 (0.36 g, 5.0 mmol) in 25 mL of acetic acid was added dropwise to a solution of nitro-benzamide 8 (1.1 g, 2.50 mmol) in 10 mL of acetic acid. The mixture was stirred at room temperature for one hour and heated at 60 °C for 17 h. Then, the mixture was poured into water and ice, and extracted with chloroform. The combined organic extracts were washed with brine, dried over Na2SO4 and concentrated. The resulting crude was purified by flash chromatography, using hexane-ethyl acetate (6:4) as eluent, to give ketone 9 (455 mg, 40%) as a yellowish foam: [α]20D +16.9 (c 1.3, CHCl3); 1H NMR (300 MHz) δ 8.08 (1H, s), 7.75-7.70 (2H, m), 7.59 (1H, s), 7.52-7.35 (3H, m), 6.19 (1H, m), 3.35 (1H, m), 3.25 (1H, m), 2.32 (1H, br, J = 12.0), 1.30-1.19 (9H, m), 1.10 (3H, m), 13C NMR (75 MHz) δ: 197.2 (s), 167.9 (s), 154.0 (s), 153.0 (s), 140.0 (s), 134.8 (s), 133.0 (s), 131.6 (d), 128.6 x 2 (d), 127.4 (d), 126.8 x 2 (d), 119.1 (d), 49.7 (t), 44.4 (d), 37.9 (s), 37.9 (s), 37.2 (t), 36.0 (t), 35.9 (t), 28.4 (d), 23.9 (q), 2 x 23.4 (q), 18.4 (q), 17.9 (t); HRMS (ESI) m/z 449.2463 [M+H]+, calcd for C23H23N3O5: 449.2440.

**N-benzoyl-dehydroabietylamine-7-one (10).** A solution of CrO3 (0.36 g, 5.0 mmol) in 20 mL of acetic acid was added dropwise to a solution of benzamide 7 (700 mg, 1.8 mmol) in 10 mL of acetic acid. The mixture was stirred at room temperature for one hour and heated at 60 °C for 17 h. Then, the mixture was poured into water and ice, and extracted with chloroform. The combined organic extracts were washed with brine, dried over Na2SO4 and concentrated. The resulting yellowish crude oil was purified by flash chromatography, using petroleum ether-ethyl acetone (3:1) as eluent, to give ketone 10 (350 mg, 48%) as a yellowish foam: [α]20D -2.1 (c 2.8, CHCl3); 1H NMR (300 MHz) δ 7.82-7.83 (1H, m), 7.75-7.70 (2H, m), 7.49-
N-acetyl-dehydroabietylamine (11). To a solution of dehydroabietylamine (6) (1.45 g, 5 mmol) and triethylamine (1.4 mL, 10 mmol) in THF (25 mL) at 0 ºC, acetyl chloride (0.4 mL, 5.5 mmol) was added dropwise. Then, it was allowed to warm to rt and stirred overnight (16 h). The resulting reaction mixture was treated with 1 M NaOH solution of CrO3 (600 mg, 6.0 mmol) in 28 mL of acetic acid was added dropwise to a solution of nitro-acetamide 12 (1.1 g, 3.0 mmol) in 12 mL of acetic acid. The mixture was stirred at room temperature for one hour and heated at 60 ºC for 17 h. The mixture was poured into water and ice, and extracted with chloroform. The combined organic extracts were washed with brine, dried over Na2SO4 and concentrated. The resulting yellowish crude oil was purified by flash chromatography, using hexane-ethyl acetate (4:6) as eluent, to give ketone 13 (235 mg, 30%) as a light orange foam: [α]D20 +27.4 (c 5.0, CHCl3); 1H NMR (300 MHz) δ 8.05 (1H, s), 7.63 (1H, s), 6.33 (1H, m), 3.26 (1H, m), 3.14 (2H, m), 2.34 (1H, br d, J = 12.0), 1.94 (3H, s), 1.32-1.25 (9H, m), 1.05 (3H, s); 13C NMR (75 MHz) δ 197.3 (s), 170.6 (s), 154.1 (s), 152.8 (s), 139.6 (s), 132.9 (s), 127.0 (d), 119.0 (d), 48.9 (t), 43.5 (d), 37.5 (s), 37.4 (t), 35.7 (t), 35.4 (t), 28.2 (d), 23.6 (q), 23.2 (q), 23.2 (q), 23.1 (q), 18.3 (q), 17.8 (t); HRMS (EI) m/z 387.2245 [M+H]+, calecd for C22H21N2O3: 387.2284.

N-acetyl-dehydroabietylamine-7-one (14). A solution of CrO3 (0.45 g, 4.5 mmol) in 20 mL of acetic acid was added dropwise to a solution of acetamide 11 (740 mg, 2.2 mmol) in 10 mL of acetic acid. The mixture was stirred at room temperature for one hour and heated at 60 ºC for 17 h. Then, the mixture was poured into water and ice, and extracted with chloroform. The combined organic extracts were washed with brine, dried over Na2SO4 and concentrated. The resulting yellowish crude oil was purified by flash chromatography, using hexane-ethyl acetate (4:6 to 3:7) as eluent, to give ketone 14 (565 mg, 73%) as a yellowish oil: [α]D20 -26.7 (c 0.6, CHCl3); 1H NMR (300 MHz) δ 7.70 (1H, d, J = 8.5), 7.38 (1H, d, J = 8.5, 2.0), 7.31 (1H, d, J = 8.5, 6.4), 6.64 (1H, t, J = 6.0, 3.33 (1H, dd, J = 15.0, 6.0), 3.00 (1H, dd, J = 15.0, 6.0), 2.36 (1H, br d, J = 12.0), 1.95 (3H, s), 1.27 (3H, s), 1.19 (6H, d, J = 6.0), 1.04 (3H, s); 13C NMR (75 MHz) δ 199.3 (s), 170.7 (s), 153.5 (s), 146.4 (d), 132.6 (d), 130.2 (d), 124.5 (d), 123.6 (d), 48.7 (t), 43.7 (d), 2 x 37.5 (q), 37.4 (t), 35.8 (t), 29.8 (t), 25.0 (q), 23.6 (q), 23.6 (q), 22.7 (q), 18.6 (t), 18.4 (q), 18.3 (t); HRMS (ESI) m/z 328.2621 [M+H]+, calecd for C22H21N2O3HNO2: 328.2640. The NMR and MS data agree with those reported in the literature [14].

N-acetyl-12-nitrodehydroabietylamine (12). Powdered cupric nitrate trihydrate (1.10 g, 4.55 mmol) was added in portions to a stirred solution of acetamide 11 (745 mg, 2.27 mmol) in acetic anhydride (30 mL) and the blue solution was stirred at 5 ºC for 17 h. Then, the mixture was poured into water and extracted with ethyl acetate. The organic extracts were washed with brine, dried over Na2SO4 and concentrated. The resulting residue was purified by chromatography eluting with hexane-ethyl acetate (4:6) to give 0.64 g (76%) of pure nitro derivative 12 as a slightly orange foam: [α]D20 +45.3 (c 0.55, CHCl3); 1H NMR (300 MHz) δ 7.29 (1H, d, J = 8.5), 7.12 (1H, dd, J = 8.5, 1.5), 7.02 (1H, br s), 6.89 (1H, t, J = 6.0), 3.40 (1H, dd, J = 15.0, 6.0), 3.16 (1H, dd, J = 15.0, 6.0), 2.41 (1H, br d, J = 12.0), 2.08 (3H, s), 1.36 (6H, d, J = 6.0), 1.35 (3H, s), 1.07 (3H, s); 13C NMR (75 MHz) δ 170.2 (s), 146.8 (s), 145.0 (s), 134.4 (s), 126.5 (d), 123.7 (d), 123.4 (d), 53.1 (s), 49.6 (t), 44.8 (d), 37.9 (t), 37.0 (s), 35.8 (t), 33.0 (d), 29.8 (t), 25.0 (q), 23.6 (q), 23.6 (q), 22.7 (q), 18.6 (t), 18.4 (q), 18.3 (t); HRMS (ESI) m/z 328.2621 [M+H]+, calecd for C22H21N2O3: 328.2640. The NMR and MS data agree with those reported in the literature [14].

N-acetyl-12-nitrodehydroabietylamine-7-one (13). A solution of CrO3 (600 mg, 6.0 mmol) in 28 mL of acetic acid was added dropwise to a solution of nitro-acetamide 12 (1.1 g, 3.0 mmol) in 12 mL of acetic acid. The mixture was stirred at room temperature for one hour and heated at 60 ºC for 17 h. Then, the mixture was poured into water and ice, and extracted with chloroform. The combined organic extracts were washed with brine, dried over Na2SO4 and concentrated. The resulting crude oil was purified by flash chromatography, using hexane-ethyl acetate (15:85) as eluent, to give ketone 13 (330 mg, 30%) as a yellowish foam: [α]D20 -26.7 (c 0.6, CHCl3); 1H NMR (300 MHz) δ 8.05 (1H, s), 7.63 (1H, s), 6.33 (1H, m), 3.26 (1H, m), 3.14 (2H, m), 2.34 (1H, br d, J = 12.0), 1.94 (3H, s), 1.32-1.25 (9H, m), 1.05 (3H, s); 13C NMR (75 MHz) δ 197.3 (s), 170.6 (s), 154.1 (s), 152.8 (s), 139.6 (s), 132.9 (s), 127.0 (d), 119.0 (d), 48.9 (t), 43.5 (d), 37.5 (s), 37.4 (t), 35.7 (t), 35.4 (t), 28.2 (d), 23.6 (q), 23.2 (q), 23.2 (q), 23.1 (q), 18.3 (q), 17.8 (t); HRMS (ESI) m/z 328.2245 [M+H]+, calecd for C22H21N2O3: 328.2243.

N-(1R,4aS,13cR)-12-fluoro-7-isopropyl-1,4a-dimethyl-2,3,4,4a,9,13c-hexahydro-1H-dibenzo[a,c]carbazol-1-yl)methyl)acetamide (15). It was followed a method reported in the literature [12]. To a solution of 4-fluoroaniline (50 mmol) in 50 ml of 20% HCl was added dropwise the solution of NaNO2 (3.63 g, 52.5 mmol) in 8 mL of water with cooling with an ice-water bath. The reaction mixture was stirred at 0 ºC for 1 h to give a clear solution. Then to the solution was added dropwise a solution of SnCl2 (19 g, 0.1 mol) in 30 ml of 35% HCl at 0 ºC. Then, the mixture was stirred at room temperature for 1-2 h. The solid product was filtered, washed with 35% HCl for 3 times and dried in a vacuum desiccator containing phosphorous pentoxide. The product (7 g) could be used in subsequent reactions without further purification.
To a solution of ketone-acetamide (14) (190 mg, 0.56 mmol) in EtOH (2 mL) was added the above prepared phenylhydrazine hydrochloride (200 mg, 1.21 mmol) and 36% HCl (0.2 mL). The mixture was refluxed for 3 h, water/ice added and extracted with DCM. The combined organic extracts were washed with saturated NaHCO₃, brine, dried over Na₂SO₄ and concentrated. The resulting brown crude oil was purified by flash chromatography, using DCM-MeOH (95:5) as eluent, to give carbazole 15 (77 mg, 32%) as a dark orange oil: [α]D 0 (c 3.5, CHCl₃); 1H NMR (300 MHz) δ 9.38 (1H, s, NH), 7.73 (1H, dd, J = 11.0, 2.0), 7.39 (1H, d, J = 1.5), 7.34 (1H, dd, J = 8.5, 5.0), 7.27 (1H, d, J = 8.5), 7.16 (1H, dd, J = 8.5, 1.5), 6.90 (1H, dt, J = 8.5, 2.0), 5.76 (1H, t, J = 6.0), 4.15 (1H, dd, J = 15.0, 6.0), 3.16 (1H, dd, J = 15.0, 6.0), 2.28 (1H, br d, J = 12.0), 1.99 (3H, s). C 170.7 (s), 157.0 (d, J C,F = 255), 146.7 (s), 146.7 (s), 139.3 (s), 126.7 (s), 126.2 (d), 123.9 (d, J C,F = 20), 123.5 (d), 119.3 (d), 111.6 (d, J C,F = 110), 111.0 (d, J C,F = 5), 109.0 (d, J C,F = 25), 107.7 (d, J C,F = 25), 53.4 (s), 51.3 (t), 48.8 (d), 39.9 (s), 37.2 (t), 36.4 (t), 33.7 (d), 23.9 (q), 23.9 (q), 21.3 (q), 19.6 (q), 17.6 (t); HRMS (ESI) m/z 433.2676 [M+H]+, calcd for C16H13F2N2O: 433.2655.

**Intracellular amastigote assay**

We performed on *L. amazonensis* and *L. infantum* according to the novel fluorometric method reported by us [17]. To carry out the *in vitro* macrophage infections, 5×10⁴ cells were cultured in RPMI-1640 medium and then were infected with 5×10⁵ promastigotes. In *L. infantum* assays, the plates were incubated during 48 h at 37 °C, while in *L. amazonensis* assays, were incubated at 33 °C the first 24 h and then the temperature was increased to 37 °C for the next 24 h. After, the culture medium was removed and the cells were washed with buffered RPMI-HEPES at pH 7.4 for several times in order to eliminate the non-internalized promastigotes. One hundred microliters of fresh medium tempered at 37 °C was added to the infected cells, which then were exposed to 100 µL of test compounds in RPMI-1640 at different concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/mL in a final volume 200 µL) for 48 h at 37 °C. The culture medium was removed by centrifugation at 3500 rpm for 5 min (Centrifuge 5403 Eppendorf) and a lysis solution (0.02 % sodium dodecyl sulphate in RPMI-HEPES) was added. After 20 min, the treated cells were harvested (centrifugation at 3,500 rpm for 5 min at 4° C) and the supernatants were replaced by 200 µL of fresh Schneider’s culture medium. The plates were then incubated at 26 °C for other 3 days to allow the transformation of viable amastigotes into promastigotes and proliferation. Afterwards, 20 µL/well of 2.5 mM resazurin was added and incubated for another 3 h. Finally, URF was measured and IC₅₀ was estimated as described above. All tests were carried out in triplicate. Miltefosine was used as reference drug.

**Cytotoxic activity**

**Cell culture**

J774 murine macrophages were grown in RPMI-1640 medium (Sigma) supplemented with 10 % heat-inactivated FBS (30 min at 56 °C), penicillin G (100 U/mL) and streptomycin (100 µg/mL). Cell cultures were maintained in a humidified 5% CO₂/95 % air atmosphere at 37 °C. Cells in the pre-confluence phase were harvested mechanically to accomplish the assays.

**Cytotoxicity Assay**

The assay was carried out according our methods previously described by us [18]. J774 macrophages cell lines were seeded (5×10⁴ cells/well) in 96-well flat-bottom plates with 100 µL of RPMI 1640 medium. The cells were allowed to attach for 2 h at 37 °C, 5% CO₂ and then was added 100 µL of RPMI-1640 medium with test compound at different concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µg/mL to the final volume) and exposed for several times in order to eliminate the non-internalized promastigotes. One hundred microliters of fresh medium tempered at 37 °C was added to the infected cells, which then were exposed to 100 µL of test compounds in RPMI-1640 at different concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/mL in a final volume 200 µL) for 48 h at 37 °C. The culture medium was removed by centrifugation at 3500 rpm for 5 min (Centrifuge 5403 Eppendorf) and a lysis solution (0.02 % sodium dodecyl sulphate in RPMI-HEPES) was added. After 20 min, the treated cells were harvested (centrifugation at 3,500 rpm for 5 min at 4° C) and the supernatants were replaced by 200 µL of fresh Schneider’s culture medium. The plates were then incubated at 26 °C for other 3 days to allow the transformation of viable amastigotes into promastigotes and proliferation. Afterwards, 20 µL/well of 2.5 mM resazurin was added and incubated for another 3 h. Finally, URF was measured and IC₅₀ was estimated as described above. All tests were carried out in triplicate. Miltefosine was used as reference drug.

**Promastigote assay**

The activity of functionalized dehydroabietylamide amide derivatives on *Leishmania* promastigotes was performed according to the method previously reported by us [16]. Parasites (2.5×10⁶ promastigotes/well) were cultured in 96-well flat-bottom plates. Stock solutions of the tested compounds were solubilized at 100 mg/mL in DMSO. Serial dilutions 1:2 of test compounds in fresh culture medium were performed (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µg/mL) in a final volume of 200 µL; the solutions then were added to the parasites suspension. After an incubation period of 48 h at 26 °C, a volume of 20 µL of 2.5 mM resazurin solution in PBS was added and the plates were incubated 3 h under the same conditions. Finally, the Relative Fluorescence Units (RFU) ([35S]ex – 590em nm wavelength) was determined in a fluorimeter (Infinite 200 Tecan i-Control). Growth inhibition (%) was calculated by 100 - [RFU treated wells – RFU signal-to-noise]/(RFU untreated – RFU signal-to-noise) x 100. All tests were carried out in triplicate and the IC₅₀ was calculated by probit analysis using SPSS 17.0 Statistics Software. Miltefosine (Sigma) was used as reference drug and all tests were carried out in triplicate.

**Antileishmanial activity**

**Parasites and culture procedure**

The following species of *Leishmania* were used: *L. donovani* (MHOM/IN/80/DD8) was purchased (ATCC, USA), an autochthonous isolate of *L. infantum* (MCAN/ES/96/BCN150) obtained from an asymptomatic dog from the Priorat region (Catalunya, Spain), kindly given by Prof. Montserrat Portús (Universidad de Salud Carlos III, Madrid). Promastigotes were cultured in Schneider's Insect Medium supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS) and 1000 U/L of penicillin plus 100 mg/L of streptomycin in 25 mL culture flasks at 26 °C.

**Biology**

**Promastigote assay**

The activity of functionalized dehydroabietylamide amide derivatives on *Leishmania* promastigotes was performed according to the method previously reported by us [16]. Parasites (2.5×10⁶ promastigotes/well) were cultured in 96-well flat-bottom plates. Stock solutions of the tested compounds were solubilized at 100 mg/mL in DMSO. Serial dilutions 1:2 of test compounds in fresh culture medium were performed (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µg/mL) in a final volume of 200 µL; the solutions then were added to the parasites suspension. After an incubation period of 48 h at 26 °C, a volume of 20 µL of 2.5 mM resazurin solution in PBS was added and the plates were incubated 3 h under the same conditions. Finally, the Relative Fluorescence Units (RFU) ([35S]ex – 590em nm wavelength) was determined in a fluorimeter (Infinite 200 Tecan i-Control). Growth inhibition (%) was calculated by 100 - [RFU treated wells – RFU signal-to-noise]/(RFU untreated – RFU signal-to-noise) x 100. All tests were carried out in triplicate and the IC₅₀ was calculated by probit analysis using SPSS 17.0 Statistics Software. Miltefosine (Sigma) was used as reference drug and all tests were carried out in triplicate.
another 48 h. Growth controls and signal-to-noise were also included. Afterwards, a volume of 20 μL of 2.5 mM resazurin solution in PBS was added, and plates were returned to the incubator for another 3 h to evaluate cell viability. The reduction of resazurin was determined by fluorometry as in the promastigote assay. Each concentration was assayed by triplicate. Cytotoxicity effect of compounds was defined as the 50% reduction of cell viability of treated culture cells with respect to untreated culture (CC50).

Acknowledgments

Financial support by the Spanish Government MINECO is gratefully acknowledged.

References and Notes


