In Vitro Action of *ent*-Manoyl Oxides against *Leishmania donovani*

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Several *ent*-manoyl oxides obtained from natural products through chemical and microbiological procedures were tested in vitro as inhibitors of the growth of the promastigote and amastigote forms of *Leishmania donovani*. Compounds **3**, **6**, **7**, and **15** showed significant antileishmanial activity. (3*S*)-*ent*-3*β*-Acetoxy-12-oxo-8α,13-epoxylabda-9,14-diene (**15**), whose semisynthesis is described, totally inhibited the promastigote forms of *L. donovani* at a concentration of 100 mg/mL after 24 h of incubation. Functionalization of this series of compounds plays an important role in the antileishmanial activity observed.

Species of the genus *Leishmania*, a protozoan member of the order Kinetoplastida, is the causative agent of human leishmaniasis, which is transmitted by mosquitoes of the genera *Phlebotomus* and *Lutzomia*. The term leishmaniasis comprises three clearly distinguishable clinical manifestations: generalized visceral infection (visceral leishmaniasis or "Kala-azar"), cutaneous leishmaniasis (Oriental button), and mucocutaneous leishmaniasis (ulceration of the skin and hyperdevelopment of the mucous membranes).[1,2]

Leishmaniasis poses considerable health care problems. This disease requires prolonged hospitalization and expensive treatments; side effects and resistance to medicinal treatment are often observed. It is necessary to combat the various forms of leishmaniasis by different methods. Visceral leishmaniasis, caused by *Leishmania donovani*, is considered by the World Health Organization as one of the most serious diseases caused by protozoa. Hepatomegaly and splenomegaly account for only 1.5 million of the 12 million new cases of leishmaniasis worldwide and produce the most serious illness, which, when untreated, can cause a 90% mortality rate.[3,4] The currently used medicines for visceral leishmaniasis (pentavalent antimonials and/or pentamidine salts) have some toxicity and numerous side effects.[5−9] One principal subject of current research in the field of leishmaniasis is the development of new effective molecules for chemotherapy. Some imidazole, oxazole, and thiazole derivatives are effective against leishmaniasis.[10,11] Carbazolylpyrimidine,[12] 9-anilinoacridin,[13] acridinobenzimidazole compounds,[14] and a naturally occurring labdane diterpenoid[15] are also promising antileishmanial agents. A series of organometalic complexes of Pt(II), Pt(IV), Rh(I), Rh(III), Rh(IV), and Sb(III) have also shown antileishmanical activity in vitro.[16,17] In the present study, we have compared the effects of different compounds in vitro on the promastigote and amastigote forms of *L. donovani*. These products possess an *ent*-manoyl oxide structure and were obtained by direct isolation from natural sources or through the chemical and microbiological transformation of natural products.

The compounds used in this study were obtained from natural products isolated initially from an Andalusian *Sideritis* species. The natural products ribenol (**1**) and varodiol (**2**) (Chart 1) were isolated from *Sideritis varoi*.[18,19] Compounds **3**−**14** were obtained as indicated in the Experimental Section. The ketone **15** was obtained from *ent*-3*β*-acetoxy-12-oxo-13-*epi*-manoyl oxide (**16**), previously obtained from varodiol **2**.[20] The treatment of ketone **16** with $CuBr_2$ in dioxane at reflux[21] gave rise to compound **17** (66%), not previously described in literature (Figure 1). The mass spectrum of **17** indicated that it was a monobrominated derivative (m/z 443/441 $[M + 1]^+$, CI). An AX system with doublets centered at 4.20 and 2.48 ppm (H-11 and H-9, $J = 8.9$ Hz) was observed in its ^{1}H-NMR spectrum. The magnitude of the coupling constant (8.9 Hz) indicated that the protons were in a *trans*-diaxial arrangement. The chemical shift of the proton at 4.2 ppm indicated that it was geminal to bromine. Comparison of the ^{13}C-NMR spectra of **17** and the ketone **16**[20] indicated a brominated carbon at 42.2 ppm. Thus, product **17** was assigned as *ent*-3*β*-acetoxy-11α-bromine-12-oxo-13-*epi*-manoyl oxide. Dehydrobromation[22] of **17** with LiBr and Li_2CO_3 gave rise to an olefin (**15**) with a molecular weight of 360 daltons (m/z 361 $[M + 1]^+$, CI), which indicated that a HBr unit had been removed. It was inferred from its NMR data that product **15** was an α,*β*-

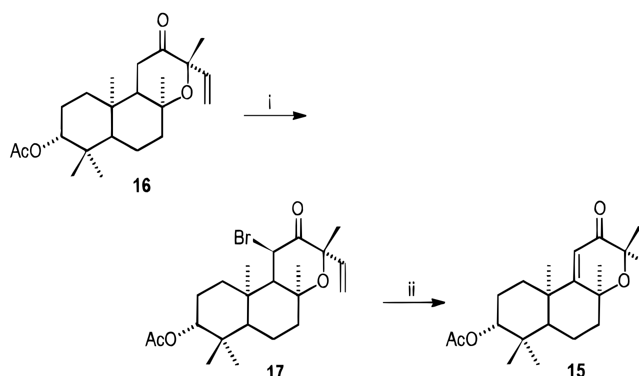

Figure 1. Conversion of ketone **16** to compound **15**: (i) $CuBr_2$, dioxane, reflux; (ii) Li_2CO_3, LiBr, DMF, reflux.

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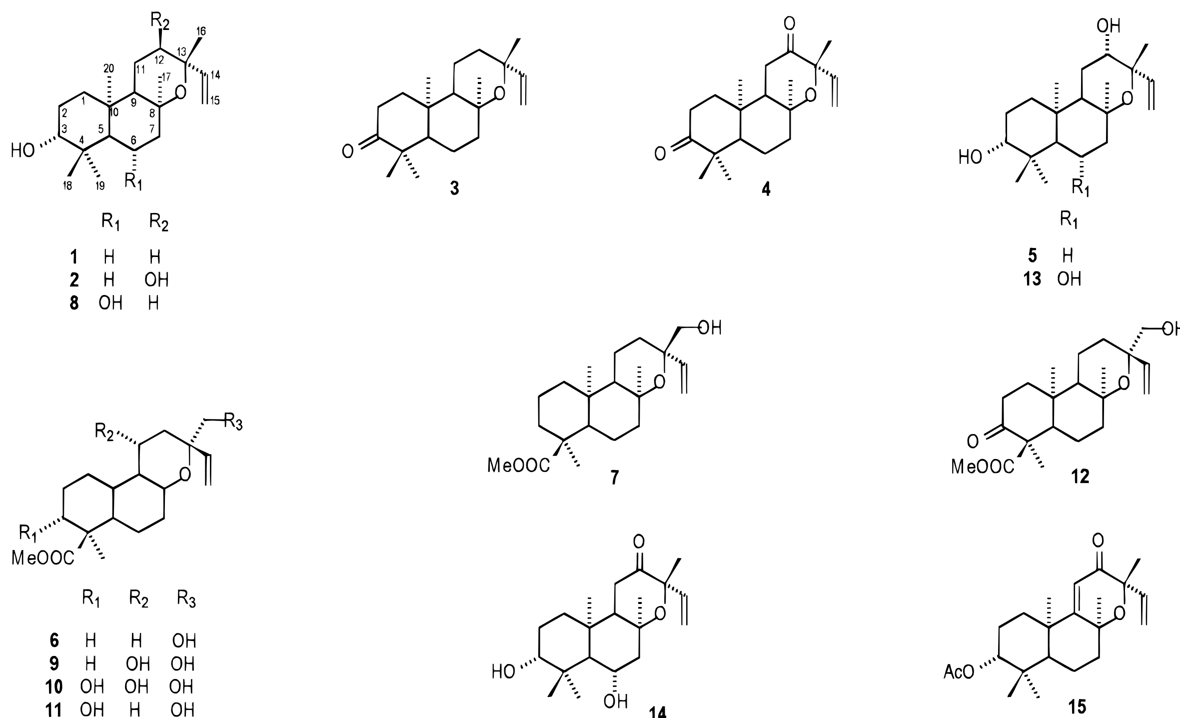
Chart 1



unsaturated ketone. Thus, the ^{1}H-NMR spectrum of **15** exhibited a singlet signal for H-11 (δ 5.80), and, in its ^{13}C-NMR spectrum, signals were observed at δ 198.30 (CO), 176.43 (C-9), and 117.22 (C-11). Therefore, product **15** was characterized as (13*S*)-*ent*-3β-acetoxy-12-oxo-8α,13-epoxylabda-9,14-diene.

The compounds selected for study were tested in vitro with promastigote and amastigote forms of *L. donovani.* Once infection had begun, the promastigote forms changed to the amastigote forms. The multiplication phase was entered 20 h later. These forms continued to divide until they occupied the entire cytoplasm of the host cells, after approximately 76 and 92 h of culture. The incubation periods examined with the different compounds were 24, 48, and 72 h, to determine the effect on multiplication of the parasite. Inhibition of cell multiplication is a viability criterion in the treatment of leishmaniasis. The compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1%, which does not influence growth of the parasites.[16] The concentrations tested were 100, 10, and 1 mg/mL, dissolved in TC-199 and 20% inactivated fetal bovine serum. Percentage growth inhibition was calculated for each experimental condition, and the results are shown in Tables 1 and 2.

Many of the test compounds inhibited growth of the promastigote forms of *L. donovani* at a concentration of 100 mg/mL; no inhibition was observed at lower concentrations (Tables 1 and 2). For some compounds inhibition was observed after 48 h, but such activity was not evident after 24 or 72 h. This apparently paradoxical behavior suggests that each compound produced a slight inhibition of parasite growth followed by the development of resistance to the action of the compound; similar results have been obtained with other substances.[16] The results of most interest were obtained with compounds **3**, **6**, **7**, and **15**, because inhibition increased with time of incubation. Compound **3** was effective at 72 h (80%) and showed greater inhibitory action than compound **1**. This indicated that structural modifications influence inhibition. Compound **1**, with a hydroxyl group at C-3, produced a slight inhibition at 24 h (27%). When this hydroxyl group was oxidized (compound **3),** inhibition was considerably increased (51%) for the same incubation period. However, a compound with hydroxyl groups at both C-3 and C-12 (compound **2**) failed to produce inhibition after any of the test periods of incubation. Compounds **6** and **7** also showed considerable inhibitory activity. These compounds are isomeric at C-13. Although neither showed activity at 24 h, inhibition was seen after 48 h (73% and 62%, respectively), and increased at 72 h (97% and 79%, respectively) (Table 1). Compounds with more than one hydroxyl group (**2**, **5**, **8**, **9**, **10**, **11**, **13**, and **14**) also showed no inhibition. The presence of a second hydroxyl

Table 1. Growth Inhibitory Effect (%) of Compounds **1**−**15** on Promastigote Forms of *Leishmania donovani* at 24, 48, and 72 h of Incubation with Concentrations of 100 mg/mL, 10 mg/mL and 1 mg/mL

	concentration (mg/mL)								
	(24 h incubation)			(48 h incubation)			(72 h incubation)		
compd	100	10	1	100	10	1	100	10	1
1	27	0	0	70	0	0	52	0	0
2	0	0	0	63	0	0	0	0	0
3	51	0	0	86	0	0	80	0	0
4	0	0	0	25	0	0	0	0	0
5	0	0	0	31	0	0	0	0	0
6	0	0	0	73	0	0	97	0	0
7	0	0	0	62	0	0	79	0	0
8	0	0	0	52	0	0	0	0	0
9	0	0	0	32	0	0	38	0	0
10	0	0	0	70	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	22	0	0	34	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	100	0	0	100	0	0	100	0	0
glucantime[a]	70	28	4	74	40	20	76	42	39

[a] Drug used as reference.

Table 2. Growth Inhibitory Efect (%) of Compounds **1**−**15** on Amastigote Forms of *Leishmania donovani* at 24, 48, and 72 h of Incubation with Concentrations of 100 μg/mL, 10 μg/mL, and 1 μg/mL

	concentration (mg/mL)								
	(24 h incubation)			(48 h incubation)			(72 h incubation)		
compd	100	10	1	100	10	1	100	10	1
1	17	0	0	25	13	7	39	18	0
2	12	0	0	29	7	0	33	12	0
3	26	7	0	48	17	10	56	25	10
4	22	0	0	31	7	0	36	11	0
5	15	0	0	31	6	0	35	11	0
6	39	13	0	48	20	0	57	30	0
7	47	17	0	58	26	12	67	35	13
8	34	10	0	51	24	0	67	33	14
9	17	0	0	32	7	0	41	15	0
10	27	0	0	36	6	0	40	15	0
11	20	0	0	34	0	0	38	6	0
12	9	0	0	14	0	0	19	0	0
13	35	7	0	46	11	0	50	16	0
14	14	0	0	15	0	0	19	0	0
15	32	12	0	45	21	0	50	34	12

group increased the polarity of the molecule, and this polarity may have influenced transport of the compound across the cellular membrane. Particularly interesting is the inhibition observed by compound **15**, which totally inhibited growth at all of the periods tested. We thus observe that the polarity of a compound seems to be related to its inhibitory activity inasmuch as compound **15** possesses an acetoxy group at C-3 and an α,β-unsaturated system in the C ring.

More test compounds inhibited growth of the amastigote form of *L. donovani* than the promastigote form (Table 2), and these compounds were active at concentrations of 100 mg/mL, 10 mg/mL, and in some cases 1 mg/mL. The compounds active against promastigotes were also active against amastigotes, although inhibition was slightly weaker at 100 mg/mL. Compounds **6** and **7** showed potent inhibitory activity, even at 1 mg/mL; in this case compound **7** was the more active of the two. Compound **15** also inhibited the growth of amastigotes but did not produce total inhibition, as seen with the promastigote form.

In conclusion, these in vitro studies indicate that a series of *ent*-manoyl oxides inhibited the growth of promastigote and amastigote forms of *L. donovani*. The functionalizations of the compounds play an important role in the activities observed. Compounds **3**, **6**, **7**, and **15**, which showed the greatest activities, have been selected for subsequent in vivo studies designed to determine their possible usefulness as antileishmanial agents.

Experimental Section

General Experimental Procedures. The NMR spectra (300 MHz for ^{1}H and 75.4 MHz for ^{13}C) were determined in $CDCl_3$ solution (which also provided the lock signal) on a Bruker AM-300 spectrometer. Assignments of ^{13}C-NMR chemical shifts were made with the aid of distortionless enhancement by polarization transfer (DEPT) using a flip angle of 135°. IR spectra were run on a Perkin-Elmer 983 G spectrometer. CIMS (CH_4) were obtained on a Hewlett-Packard 5988 A spectrometer. Specific rotations were measured in $CHCl_3$ (1-dm tube) on a JASCO DIP-370 polarimeter at 20 °C. For column chromatography, Si gel 60 Merck (230−400 mesh ASTM) was used, with CH_2Cl_2 and increasing amounts of Me_2CO as eluents. Si gel (Merck G), with visualization by spraying with H_2SO_4−HOAc−H_2O followed by heating at 120 °C, was employed for TLC.

Sources of Test Compounds. The compounds used in this study were obtained from a *Sideritis* species. The compounds *ent*-3β-hydroxy-13-*epi*-manoyl oxide (ribenol, **1**) and *ent*-3β,12α-dihydroxy-13-*epi*-manoyl oxide (varodiol, **2**) were isolated directly from *S. varoi*.[18,19] Compounds **3**−**7** were obtained by us through chemical conversion from natural products.[19,20,23] Compounds **8**−**14** were obtained from biotransformations of *ent*-manoyl oxides with the microorganisms *Curvularia lunata*[20,24] or *Rhizopus nigricans*.[23] The ketone **15** was obtained from varodiol (**2**) via *ent*-3β-acetoxy-12-oxo-13-*epi*-manoyl oxide (**16**), which was previously obtained by acetylation of **2** and later oxidation of the *ent*-3β-acetoxy derivative.[20]

Bromation of Ketone 16. Compound **16** (500 mg) was dissolved in dioxane (50 mL), and $CuBr_2$ (1.2 g) was added.[21] The reaction was stirred and heated under reflux for 3 h. The CuBr was filtered off, and dioxane was removed under reduced pressure. The mixture was chromatographed over a Si gel column to give the starting product (**16**, 90 mg) and *ent*-3β-acetoxy-11α-bromo-12-oxo-13-*epi*-manoyl oxide (**17**, 380 mg, 66%): mp 124−126 °C; $[\alpha]_D$ −55° (*c* 1, $CHCl_3$); IR (KBr) ν max 3095 (C=C), 1725 (C=O, ester and ketone), 1640, 1247 (C−O−C, ester), 1126, 1087, 1032, 901 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.38 (1H, dd, J_1 = 17.3, J_2 = 10.8, H-14), 5.40 (1H, dd, J_1 = 17.3, J_2 = 1.5 Hz, H-15), 5.34 (1 H,dd, J_1 = 10.8, J_2 = 1.5 Hz, H-15), 4.51 (1H, dd, J_1 = 11.4, J_2 = 4.7 Hz, H-3), 4.20 (1H, d, J = 8.9 Hz, H-11), 2.48 (1H, d, J = 8.9 Hz, H-9), 2.05 (3H, s, AcO) and 1.71, 1.02, 0.90, 0.88, 0.85 (3H each, s, 5 × Me); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 207.18 (C-12), 170.90 (Me*CO*), 141.79 (C-14), 113.93 (C-15), 81.61 (C-13), 80.10 (C-3), 75.92 (C-8), 63.79 (C-9), 54.71 (C-5), 42.21 (C-11), 41.59 (C-7), 38.04 (C-1), 38.38 and 37.92 (C-4 and C-10), 32.46 (C-16), 28.11 (C-18), 23.48 (C-2), 22.24 (C-17), 21.34 (*Me*CO), 19.38 (C-6), 16.52 (C-19), 15.91 (C-20); CIMS (methane) m/z $[M + 1]^+$ 443/441 (31/35), ($[M + 1]^+$ − AcOH) 383/381 (100/100).

Dehydrobromation of Compound 17. Compound **17** (350 mg) was dissolved in dry *N,N*-dimethylformamide (15 mL), and dry LiBr (755 mg) and Li_2CO_3 (1.4 g) were added.[22] The suspension was stirred at 120 °C under an argon atmosphere for 2 h. The reaction mixture was cooled, poured into diluted AcOH, and extracted with $CHCl_3$. The organic layer was washed with H_2O and saturated NaCl, dried over $MgSO_4$, and concentrated *in vacuo*. Chromatographic separation yielded (13*S*)-*ent*-3β-acetoxy-12-oxo-8α,13-epoxylabda-9,14 diene (**15**, 270 mg, 94%): mp 72−74 °C; $[\alpha]_D$ −133° (*c* 1, $CHCl_3$); IR (KBr) ν max 3093, 1734, 1675, 1244, 1109, 1032, 927 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.04 (1H, dd, J_1 = 17.7, J_2 = 10.9 Hz, H-14), 5.80 (1H, s, H-11), 5.11 (1H, d, J = 17.7 Hz, H-15), 5.04 (1H, d, J = 10.9 Hz, H-15), 4.46 (1H, dd, J_1 = 10.9, J_2 = 4.4 Hz, H-3), 2.04 (3H, s, AcO), 1.55, 1.37, 1.18, 0.90, 0.98 (3H each, s, 5 × Me); ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 198.30 (C-12), 176.43 (C-9), 170.79 (Me*CO*), 142.50 (C-14), 117.22 (C-11), 113.83 (C-15), 80.24 (C-13), 79.68 (C-3), 75.03 (C-8), 52.46 (C-5), 42.56 (C-7), 39.98 (C-10), 38.54

(C-4), 35.52 (C-1), 28.08 (C-16), 28.24 (C-18), 23.75 (C-2), 30.40 (C-17), 21.28 (*Me*CO), 18.89 (C-6), 16.73 (C-19), 21.67 (C-20); CIMS (methane) m/z $[M + 1]^+$ 361 (100), ($[M + 1]^+ - $ AcOH) 301 (67).

Bioassays. Parasites. *L. donovani* strain (LCR-L133) (Leishmania Reference Center, Jerusalem, Israel) was isolated in 1967, from a human clinical case of "Kala-azar" in the vicinity of Begemder, Ethiopia. The parasites were maintained in our laboratory by culture in Nicole Novi and MacNeal (NNN) medium supplemented with Minimal Essential Medium (MEM) plus 20% Inactivated Fetal Calf Serum (Md-NNN).

Mass Culture of Promastigote and Amastigote Forms. The promastigote forms of *L. donovani*, from Md-NNN medium, were cultivated in TC 199 medium supplemented with 30% of Inactivated Fetal Calf Serum (IBFS) as indicated previously.[25] The promastigote forms in the exponential growth phase were cultured at 28 °C in TC199 medium, with a density of 3×10^6 cell/mL being transfered at 38 °C to obtain amastigote-like forms, which were separated to obtain a mass culture of amastigote forms of *L. donovani*.[25]

Treatment of Promastigote and Amastigote Forms with Test Compounds. The test compounds assayed were dissolved in small quantities of DMSO so as not to inhibit the growth of the parasites in any way. Screening in vitro was carried out in order to determine the leishmanicidal effects of the various compounds. The concentrations assayed were 100, 10, and 1 mg/mL, and the parasites were counted in the chamber hemocytometer after 24, 48, and 72 h. The results were referred to control experiments, and thus it was possible to calculate the growth-inhibition percentages for each compound at each concentration and time. The results are shown in Tables 1 and 2.

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