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SYNTHESIS OF *ENANTIO*-MANOYL OXIDES: MODIFIERS OF THE ACTIVITY OF ADENYLATECYCLASE ENZYME

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Key Word Index—*Rhizopus nigricans*; fungus; *ent*-manoyl oxides; adenylatecyclase inhibitors; biotransformation.

Abstract—Cyclization of methyl *ent*-8α-hydroxylabd-13(16),14-dien-18-oate with *m*-chloroperbenzoic acid gave methyl (13*S*)-*ent*-16-hydroxy-8α,13-epoxylabd-14-en-18-oate and its epimer at C-13. Biotransformation of the former (which exhibits antileishmania activity) with *Rhizopus nigricans* cultures produced the methyl (13*S*)-*ent*-11β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate (carbomanoyl, which inhibits the activity of the adenylatecyclase enzyme), methyl (13*S*)-*ent*-3β,16-dihydroxy-8α,13-epoxilabd-14-en-18-oate, methyl (13*S*)-*ent*-3β,11β,16-trihydroxy-8α,13-epoxilabd-14-en-18-oate and the (14*S*)-*ent*-3β-hydroxy-14,15-epoxy derivative that cyclized spontaneously to a spiran compound. Biotransformation of methyl (13*S*)-*ent*-16-hydroxy-3-oxo-8α,13-epoxilabd-14-en-18-oate with *R. nigricans* produced *ent*-11β-hydroxylation, reduction of the keto group at C-3 (to give 3*S*-alcohol) and 14(*S*),15-epoxidation, which also rearranged to a spiro compound.

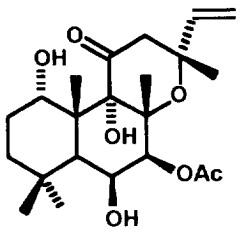
INTRODUCTION

The biotransformation of natural products is a useful tool for the hemisynthesis of biologically active products. On the other hand, chirality is frequently a decisive factor in the biological activity of a chemical compound. Forskolin **1** [1] is an 8,13-epoxy derivative of a labdane skeleton of the normal series, which also has normal configuration at C-13. Because of the importance of forskolin **1** and related compounds [2–4], an extensive series of manoyl oxides of the *enantio* series is being tested in order to determine their biological activity.

In previous papers [5, 6] we described the preparation of *ent*-manoyl oxides, epimers at C-13 and hydroxylated at C-16, from *ent*-labda-13(16),14-diene compounds isolated from the genus *Sideritis*. Treatment of acid *ent*-8α-hydroxylabda-13(16),14-dien-18-oic (**2**) [7] with diazomethane–ether gave a methyl ester compound **3** [5] which, after treatment with MCPBA, gave the *ent*-manoyl oxide **4** (43%) and the *ent*-13-epi-manoyl oxide **5** (41%) [5]. Compound **5** was the starting material for a study of biotransformation with *Rhizopus nigricans* [5], aimed at elucidating the relationship between the structure of the substrate and the action of the microorganism. To complete this study, we have now incubated **4** with the same microorganism.

RESULTS AND DISCUSSION

Biotransformation of substrate **4** with *R. nigricans* for 4 days gave, in addition to the recovery of some of substrate **4** (6%), metabolites **6** (10%), **7** (36%), **8** (13%), **9** (4%) and **10** (10%). Metabolite **6** had a M_r (MS, 367 $[M + 1]^+$, CI) compatible with hydroxylation of substrate **4**. In its ^{1}H NMR spectrum a signal of a proton geminal to hydroxyl group at δ4.41 (1H, $J_1 = J_2 = 6.7$, $J_3 = 4.5$ Hz) was observed. This multiplicity was only compatible with the axial location of this hydroxyl group at C-11 or C-6. Taking into account that only two (not three) of the signals of methyl groups, and the hydroxymethylene group at C-16, were affected by the presence of this hydroxyl group, it must be located axially at C-11. These vicinal coupling constants, determined by double-resonance experiments, were in accordance with a dihedral angle H-9/H-11 of *ca* 40° and dihedral angles H-11/H-12β and H-11/H-12α of *ca* 30° and 150°, respectively. Its ^{13}C NMR spectrum indicated the presence of a new oxygenated carbon at δ64.1 (C-11). β-Effects on C-9 ($\Delta\delta = +3.1$) and C-12 ($\Delta\delta = +11.4$) and deshielding on C-17 ($\Delta\delta = +1.6$) and C-20 ($\Delta\delta = +2.1$) were observed by comparison of the ^{13}C NMR spectra of metabolite **6** and substrate **4**. The oxidation of metabolite **6** with ruthenium perruthenate [8] gave lactone **11**. In the



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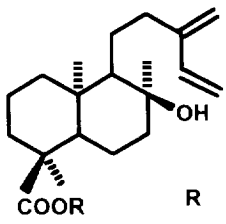
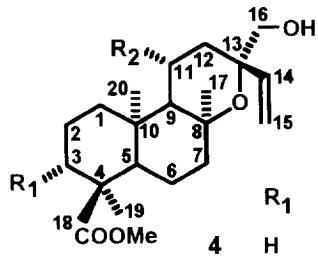
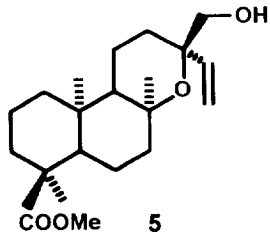


	R
2	H
3	Me



	R_1	R_2
4	H	H
6	H	OH
7	OH	H
8	OH	OH



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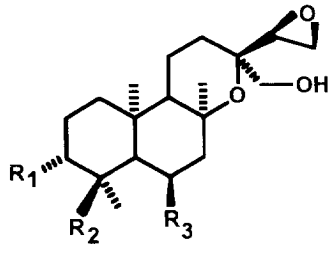
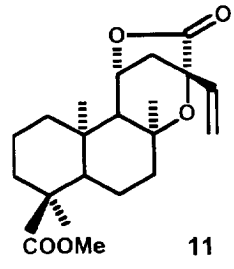


	R_1	R_2	R_3
9	OH	COOMe	H
12	H	CH_2OAc	OAc



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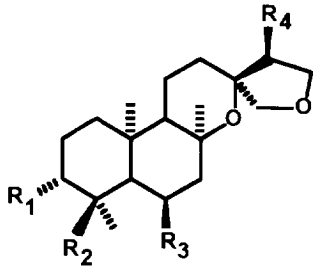
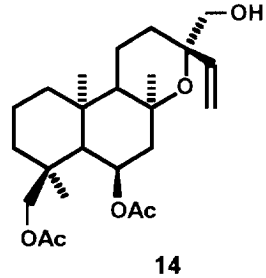


	R_1	R_2	R_3	R_4
10	OH	COOMe	H	OH
13	H	CH_2OAc	OAc	OH
15	AcO	COOMe	H	OAc



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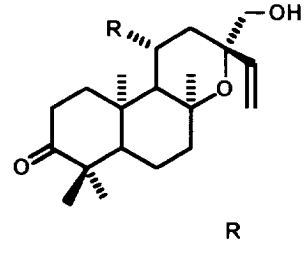
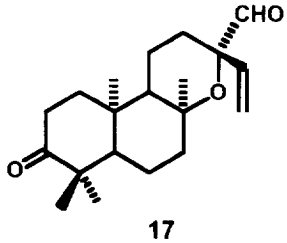


	R
16	H
18	OH



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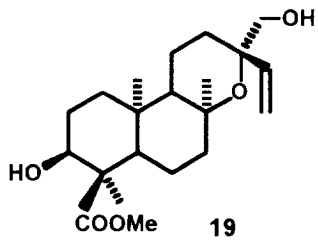

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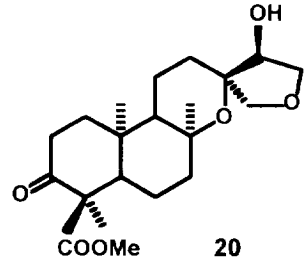

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^{1}H NMR spectrum of this lactone **11** a signal of δ5.07 (*ddd*, $J_1 = 5.91$, $J_2 = J_3 = 0.0$ Hz) of proton geminal to lactone group at C-11 was observed. In this case, the dihedral angles between H-9/H-11 and H-11/H-12β were nearly 90° and the dihedral angle H-9/H-12α was nearly 45°. Moreover, in the ^{13}C NMR spectrum of lactone **11** a signal at δ176.5 (C-16) was observed. Thus, metabolite **6** was methyl (13*S*)-*ent*-11β, 16-dihydroxy-8α, 13-epoxylabd-14-en-18-oate.

The main metabolite **7** isolated from this incubation had a M_r (MS, 367 $[M + 1]^+$, CI). The spectroscopic data of this metabolite **7** indicated that *R. nigricans* had introduced a new hydroxyl group into substrate **4**. In its ^{1}H NMR spectrum, multiplicity of the geminal proton to the new hydroxyl group (δ3.97, *dd*, $J_1 = 11.5$, $J_2 = 5$ Hz) indicated that this hydroxyl group was situated at C-1, C-3 or C-12. Comparison of the ^{13}C NMR spectra of metabolite (**7**) and substrate **4** indicated a new oxygenated carbon at δ75.5 (C-3). β-Effects on C-2 ($\Delta\delta = +8.8$) and C-4 ($\Delta\delta = +6.2$) and γ-effects on C-1 ($\Delta\delta = -1.4$), C-18 ($\Delta\delta = -1.3$) and C-19 ($\Delta\delta = -5.7$) were also observed. These effects are compatible with the *ent*-3β-hydroxylation of substrate **4**. Therefore, metabolite **7** was methyl (13*S*)-*ent*-3β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate.

The mass spectrum of the third metabolite **8** isolated from this incubation indicated that two new hydroxyl groups had been introduced into substrate **4**, their geminal protons coincided with that described above for the hydroxylation of metabolite **6** at C-11 (δ4.40, $J_1 = J_2 = 6.6$, $J_3 = 4.3$ Hz) and the hydroxylation of metabolite (**7**) at C-3 (δ3.97, $J_1 = 11.4$, $J_2 = 5.0$ Hz). The ^{13}C NMR data (see Table 1) also confirmed that metabolite **8** was methyl (13*S*)-*ent*-3β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate.

The biotransformation of substrate **4** with *R. nigricans* to give metabolite **9** was different from those described for metabolites **6**–**8**. In this case, biotransformation of substrate **4** gave metabolite **9** resulting from epoxidation of the vinyl moiety and also an equatorial hydroxylation at C-3. This structure was deduced from its spectroscopic data and from comparison with the spectroscopic data of (13*R*,14*S*)-*ent*-6α,18-diacetoxy-16-hydroxy-8α,13; 14,15-diepoxylabdane **12** previously obtained by us [6]. Thus, metabolite **9** was the result of epoxidation of the double bond of substrate **4** from the *si*-face, to give methyl (13*R*,14*S*)-*ent*-3β,16-dihydroxy-8α,13; 14, 15-diepoxylabdan-18-oate. This structure was also confirmed by the isolation of metabolite **10** from the mixture of biotransformation products. It was the result of an intramolecular attack of the hydroxyl group of C-16 on the epoxy group in metabolite **9**. Determination of the structure of metabolite **10** was useful to elucidate the configuration of the epoxy compound **9** at C-14, because cyclization of **9** to give spiran **10** maintained the configuration at this carbon. The structure of spiran **10** was deduced by comparison with the spectroscopic data of (13*R*,14*S*)-*ent*-6α,18-diacetoxy-14-hydroxy-8α,13;15,16-diepoxylabdane (**13**) a similar spiran previously described by us [6]. Thus, the comparison of the chemical shifts of C-9, C-11, C-16, C-17 and mainly C-12 of substrate **4** and spiran **10**, together with the corresponding chemical shifts for *ent*-6α,18-diacetoxy-16-hydroxymanoyl oxide **14** and spiran **13** [6], clearly indicated the presence of a 14(*S*)-hydroxyl

Table 1. ^{13}C NMR spectral data of compounds **4**, **6**–**11** and **16**–**20** ($CDCl_3$)

C	4*	6	7	8	9	10	11	16	17	18	19	20
1	38.1	38.2[a]	36.7	37.0	37.0	37.2	38.4	36.9	37.4	36.9	31.1	37.3
2	17.5	17.4	26.3	26.3	26.5	26.5	17.5	34.1	34.2	34.2	24.7	34.2
3	36.9	36.7	75.5	75.4	75.5	78.7	37.0	210.4	210.2	218.4	72.5	210.2
4	47.5	47.5	53.7	53.7	53.7	53.7	47.4	61.1	61.1	61.2	—	61.1
5	51.0	51.4	51.3	51.6	50.8	51.0	51.5	50.9	50.6	51.9	45.3	50.6
6	22.8	22.9	22.4	22.6	22.2	22.4	23.2	22.3	22.3	22.6	22.6	22.3
7	43.2	44.2	43.0	44.1	42.2	41.6	43.5	42.3	41.2	43.6	43.2	40.9
8	76.3	75.8[b]	75.5[a]	75.9[a]	75.3	76.2	79.9	76.4	76.3	76.0	76.4[a]	78.9
9	52.9	56.0	52.6	55.6	55.7	57.7	61.0	52.2	56.4	55.2	52.5	57.1
10	36.7	37.5	36.9	37.3	36.2	36.1	37.2	36.0	35.8	38.9	36.8	
11	14.4	64.1	14.7	64.3	15.2	15.8	74.3	14.6	16.3	64.3	14.5	16.1
12	27.3	38.7[a]	27.2	38.7	28.2	33.1	45.7	27.3	30.5	36.9	27.0	33.2
13	75.5	75.5[b]	75.3[a]	75.3[a]	72.3		76.8	74.9	81.8	75.0	75.5[a]	75.9
14	143.9	143.4	143.7	143.3	58.0	75.5	133.8	143.6	136.6	143.1	143.7	78.7
15	114.1	114.2	114.3	114.4	44.5	73.1[a]	118.8	114.3	117.2	114.7	114.4	73.0[a]
16	68.5	68.5	68.4	68.5	66.2	72.1[a]	176.5	68.3	201.5	68.6	68.6	72.0[a]
17	25.7	27.3	25.6	27.3	24.1	22.7	26.1	25.2	22.7	27.2	26.0	22.4
18	179.2	179.2	177.9	177.9	177.7	177.7	178.8	173.5	173.4	173.7	177.8	173.4
19	16.4	16.4	10.7	10.7	10.6	10.5	17.5	14.6	15.1	16.5[a]	16.8	15.2
20	15.5	17.6	15.7	17.7	16.0	16.3	15.8	16.6	16.5	16.6[a]	15.6	16.5
CO_2Me	51.9	52.0	52.3	52.3	52.3	52.3	52.1	52.6	52.7	52.7	52.2	52.7

*Data from ref. [5]. [a, b] values bearing the same supercript may be interchanged.
Not observed.

group in spiran **10**. Moreover, acetylation of spiran **10** gave the diacetate **15**, which showed a clearer ^{1}H NMR spectrum in which the most important proton signals could be assigned by double resonance experiments. Thus we concluded that the structure of metabolite **10** is methyl (13*R*,14*S*)-*ent*-3β,14-dihydroxy-8α,13;15,16-diepoxylabdan-18-oate.

Compounds **4**–**8** were tested to evaluate their action on the activity of adenylatecyclase enzyme (AC enzyme). Compound **5** was a mild activator of the AC enzyme (30 times less active than forskolin **1**). Compound **6** (carbomanoyl [9]) showed an inhibitory action (40%) on the activity of AC enzyme previously stimulated by **1** or fluorides and did not inhibit AC activity previously activated by glucagon. Compounds **4**, **7** and **8** were inactive.

Compounds **4**–**7** were tested to evaluate their activity against *Leishmania donovani*. Compounds **5** and **7** were inactive, whereas compounds **4** and **6** inhibited the growth at 24 hr (15 and 5%, respectively). Moreover, compound **4** inhibited the growth of *L. donovani* at 48 hr (40%) and 72 hr (63%).

Because of the high yield of metabolite **7** in the biotransformation of substrate **4**, we oxidized this metabolite **7** to give ketone **16** and ketoaldehyde **17**. The biotransformation of ketone **16** with *R. nigricans* for 4 days afforded metabolites **18** (26%), **19** (11%) and **20** (8%). Unaltered substrate **16** (20%) was also isolated from this incubation. The structures of metabolites **18**–**20** were easily determined by comparison of the spectroscopic data with those indicated above for metabolites **6**, **7** and **10**. *Rhizopus nigricans* introduced an *ent*-11β-hydroxyl group in metabolite **18**, reduced the keto group at C-3 of substrate **16**, to give an *ent*-3α-hydroxy group (epimer at C-3 of metabolite **7**) and epoxidized the double bond, with subsequent cyclization, to give 14(*S*)-hydroxyspiran compound **20**, similar to that described for metabolite **10**.

We compared the influence of the configuration at C-13 on **4** and on the previously reported **5** in the biotransformations by *R. nigricans* [5]. In the biotransformation of **5**, hydroxylation occurs at C-20 (12%), C-1 (5%) and C-3 (7%) with 57% of **5** recovered unaltered. In the biotransformation of **4**, an epimer at C-13 of **5**, hydroxylation occurs at C-3 (metabolite **7**, 36%) or at C-11 (metabolite **6**, 10%) and dihydroxylation at C-3 and C-11 (metabolite **8**, 13%). Two products from epoxidation of the double bond [metabolites **9** (4%) and **10** (10%)] were then isolated from biotransformation of substrate **4**. As can be observed, the configuration at C-13 is important in the behaviour of *R. nigricans*. The presence of a keto group at C-3 (**16**) did not modify the action of the microorganism, because such action occurred at C-11 (metabolite **18** in higher yield, 26%) and on the double bond of the substrate (metabolite **20**, 8%). In fact, the action on C-3 was similar (via the *ent*-β-face), although in this case the result was an *ent*-3α-hydroxyl group (metabolite **19**, 11%). The increased yield in the hydroxylation at C-11 is interesting, because we have shown that metabolite **6** is a notable inhibitor of the activity of the adenylatecyclase enzyme. At the moment, the biological activity of **18** has not been tested.

EXPERIMENTAL

Mps: uncorr. The NMR spectra (300 MHz ^{1}H and 75.47 MHz ^{13}C) were determined in $CDCl_3$ soln (which also provided the lock signal) on a Bruker AM-300 spectrometer. Assignments of ^{13}C chemical shifts were made with the aid of distortionless enhancement by polarization transfer (DEPT) using a flip angle of 135°. CI (methane) MS. CC: silica gel SDS 60 A CC, with CH_2Cl_2 and increasing amounts of Me_2CO as the eluent. TLC: silica gel (Merck G), visualization by spraying with H_2SO_4–HOAc–H_2O, followed by heating at 120°.

Isolation of starting materials. The acid *ent*-8α-hydroxylabda-13(16),14-dien-18-oic (**2**, acid 6-deoxyandalusoic) used in this study was isolated from *Sideritis varoi* subsp. *cuatrecasassii* [7].

Formation of methyl ester of acid **2**. Acid **2** (4 g) was dissolved in Et_2O (25 ml), and a solution of CH_2N_2 in Et_2O was added [10]. After CC, 3.6 g of methyl *ent*-8α-hydroxylabda-13(16),14-dien-18-oate (**3**, 86%) was isolated [5].

Cyclization of methyl ester **3**. Me ester **3** (3.5 g) was dissolved in $CHCl_3$ (25 ml) and MCPBA (1.4 g) was added. After 24 hr at 0° the mixture was diluted with $CHCl_3$ (75 ml) and washed with aq. $FeSO_4$, $NaHCO_3$ and H_2O. The organic layer was dried with dry $MgSO_4$, concd *in vacuo* and chromatographed on a silica gel column to give methyl (13*S*)-*ent*-hydroxy-8α,13-epoxylabd-14-en-18-oate (**4**, 1580 mg, 43%) and methyl (13*R*)-*ent*-16-hydroxy-8α,13-epoxylabd-14-en-18-oate (**5**, 1510 mg, 41%) [5].

Organism, media and culture conditions. Rhizopus nigricans CECT 2072 (ATCC 10404) was obtained from the Colección Española de Cultivos Tipo (CECT), Departamento de Microbiología, Universidad de Valencia, Spain. The bacterial culture was stored in medium YEPGA containing 1% yeast extract, 1% peptone, 2% glucose, 2% agar, at pH 5. A medium composed of peptone (0.1%), yeast extract (0.1%), beef extract (0.1%) and glucose (0.5%) at pH 5.7 in H_2O was used in all transformation experiments. Conical flasks (250 ml) containing 80 ml of medium were inoculated with a dense suspension of *R. nigricans*. Incubations were maintained at 28° with gyratory shaking (150 rpm) for 6 days, after which the substrates **4** and **16** in EtOH were added.

Recovery and purification of metabolites. Cultures were filtered and pooled, cells were washed with H_2O and the liquid was saturated with NaCl and extracted with CH_2Cl_2. These extracts were mixed, dried over dry $MgSO_4$ and at 40° under red. pres. The mixture of products obtained was chromatographed on a silica gel column.

Biotransformation of substrate **4**. Substrate **4** (1.3 g) was dissolved in EtOH (20 ml) and the soln distributed among 20 conical flask cultures. These were incubated for 4 days and the metabolites were recovered and chromatographed on a silica gel column to obtain starting product **4** (77 mg, 6%), methyl (13*S*)-*ent*-11β,16-dihydroxy-8α,

13-epoxylabd-14-en-18-oate (**6**, 135 mg, 10%), methyl (13*S*)-*ent*-3β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate (**7**, 490 mg, 36%), methyl (13*S*)-*ent*-3β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate (**8**, 185 mg, 13%), methyl (13*R*,14*S*)-*ent*-3β,16-dihydroxy-8α,13;14,15-diepoxylabdan-18-oate (**9**, 55 mg, 4%) and methyl (13*R*,14*S*)-*ent*-3β,14-dihydroxy-8α,13;15,16-diepoxylabdan-18-oate (**10**, 142 mg, 10%).

Methyl (13S)-ent-11β,16-*dihydroxy*-8α,13-*epoxylabd*-14-*en*-18-*oate* (**6**). Mp 56–58°; $[\alpha]_D$ − 29° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3460 (OH), 3090 (C=C), 1716 and 1248 (CO_2). ^1H NMR (300 MHz): δ5.76 (1H, *dd*, J_1 = 17.4, J_2 = 10.7 Hz, H-14); 5.21 (1H, *dd*, J_1 = 17.4, J_2 = 1.3 Hz) and 5.08 (1H, J_1 = 10.7, J_2 = 1.3 Hz) (H_2-15); 4.41 (1H, *ddd*, $J_1 = J_2 = 6.7$, J_3 = 4.5 Hz, H-11); 3.62 (3H, *s*, MeOCO group); 3.52 and 3.35 (2H, AB system, *J* = 11.1 Hz, H_2-16); 1.52 (3H, *s*, 3H-17); 1.48 (1H, *d*, *J* = 4.5 Hz, H-9); 1.17 (3H, *s*, H_3-20) and 1.12 (3H, *s*, H_3-19). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 367 ([M + 1]$^+$, 100%); 349 ([M + 1 − H_2O]$^+$, 48); 33 ([M + 1 − 2H_2O]$^+$, 34).

Methyl (13S)-ent-3β,16-*dihydroxy*-8α,13-*epoxylabd*-14-*en*-18-*oate* (**7**). Mp 118–120°; $[\alpha]_D$ − 34° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3428 (OH), 3085 (C=C), 1719 and 1248 (COO). ^1H NMR (300 MHz): δ5.77 (1H, *dd*, J_1 = 17.4, J_2 = 10.8 Hz, H-14); 5.23 (1H, *dd*, J_1 = 17.4, J_2 = 1.5 Hz) and 5.11 (1H, *dd*, J_1 = 10.8, J_2 = 1.5 Hz) (H_2-15); 3.97 (1H, *dd*, J_1 = 11.5, J_2 = 5 Hz, H-3); 3.70 (3H, *s*, MeOCO group); 3.32 and 3.26 (2H, AB system, *J* = 11.0 Hz, H_2-16); 1.24 (3H, *s*, H_3-17); 1.09 (3H, *s*, H_3-19); 0.80 (3H, *s*, H_3-20). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 367 ([M + 1]$^+$, 48%); 349 ([M + 1 − H_2O]$^+$, 100); 331 ([M + 1 − 2H_2O]$^+$, 57).

Methyl (13S)-ent-3β,11β,16-*trihydroxy*-8α,13-*epoxylabd*-14-*en*-18-*oate* (**8**). Mp 130–132°; $[\alpha]_D$ − 43° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3424 (OH), 3089 (C=C), 1713 and 1248 (CO_2). ^1H NMR (300 MHz): δ5.75 (1H, *dd*, J_1 = 17.4, J_2 = 10.8 Hz, H-14); 5.23 (1H, *dd*, J_1 = 17.4, J_2 = 1.4 Hz) and 5.09 (1H, *dd*, J_1 = 10.8, J_2 = 1.4 Hz) (H_2-15); 4.40 (1H, *ddd*, $J_1 = J_2 = 6.6$, J_3 = 4.3 Hz, H-11); 3.97 (1H, *dd*, J_1 = 11.4, J_2 = 5.0 Hz, H-3); 3.70 (3H, *s*, MeOCO group); 3.51 and 3.36 (2H, AB system, *J* = 11.2 Hz, H_2-16); 1.52 (3H, *s*, H_3-17); 1.37 (1H, *d*, *J* = 4.3 Hz, H-9); 1.18 (3H, *s*, H_3-20); 1.11 (3H, *s*, H_3-19). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 383 ([M + 1]$^+$, 43%); 365 ([M + 1 − H_2O]$^+$, 100); 347 ([M + 1 − 2H_2O]$^-$, 59); 329 ([M + 1 − 3H_2O]$^+$, 32).

Methyl (13R, 14S)-ent-3β,16-*dihydroxy*-8α,13; 14,15-*diepoxylabdan*-18-*oate* (**9**). Gum; $[\alpha]_D$ − 29° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3440 (OH), 1719 and 1246 (CO_2) and 1046 (OC). ^1H NMR (300 MHz): δ4.00 (1H, *dd*, J_1 = 11.7, J_2 = 4.5 Hz, H-3); 3.69 (3H, *s*, MeOCO group); 3.69 and 3.35 (2H, AB system, *J* = 11.6 Hz, H_2-16); 2.85 (1H, *dd*, J_1 = 4.0, J_2 = 2.8 Hz); 2.81 (1H, *dd*, J_1 = 5.4, J_2 = 2.8 Hz) and 2.71 (1H, *dd*, J_1 = 5.4, J_2 = 4.0 Hz) (H-14 and H_2-15); 1.28 (3H, *s*, H_3-17); 1.08 and 0.77 (3H each, *s*, H_3-19 and H_3-20). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 383 ([M + 1]$^+$, 15%); 365 ([M + 1 − H_2O]$^+$, 100).

Methyl (13R,14S)-ent-3β,14-*dihydroxy*-8α,13;15,16-*diepoxylabdan*-18-*oate* (**10**). Gum; $[\alpha]_D$ − 6° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3436 (OH), 1718 and 1246 (CO_2). ^1H NMR (300 MHz): δ4.0 (1H, *dd*, J_1 = 11.7, J_2 = 4.5 Hz, H-3); 3.70 (3H, *s*, MeOCO); 3.90 (1H, *dd*, J_1 = 9.7, J_2 = 4.6 Hz, part C of an ABC system) and 3.71–3.60 (4H, part AB of an ABC system) (H-14, H_2-15 and H_2-16); 1.13 and 1.09 (3H each, *s*, H_3-17 and H_3-19) and 0.78 (3H, *s*, H_3-20). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 383 ([M + 1]$^+$, 36%); 365 ([M + 1 − H_2O]$^+$, 100); 347 ([M + 1 − 2H_2O]$^+$, 26).

Lactonization of metabolite **6**. Metabolite **6** (15 mg) was dissolved in dry CH_2Cl_2 (2 ml) and 4-methyl morpholine *N*-oxide (NMO) (12 mg) and activated powdered molecular sieves (12 mg) were added. The solution was stirred in an argon atmosphere at room temp. and then tetrapropylammonium perruthenate (TPAP) (2 mg) was added [8]. After 3 hr at room temp., the mixture was chromatographed over a silica gel column to give (13*S*)-*ent*-3β-hydroxy-4α-metoxicarbonil-8α,13-epoxy-18-nor-labd-14-en-11β,16-olide (**11**, 10 mg, 67%): gum; $[\alpha]_D$ − 31° ($CHCl_3$; *c* 0.5); IR ν_{max} cm^{-1}: 3450 (OH), 1785 (OCO), 1722 and 1247 (CO_2). ^1H NMR (300 MHz): δ5.95 (1H, *dd*, J_1 = 17.5, J_2 = 10.8 Hz, 14-H); 5.44 (1H, *dd*, J_1 = 17.5, J_2 = 0.9 Hz) and 5.38 (1H, *dd*, J_1 = 10.8, J_2 = 0.9 Hz) (H_2-15); 5.07 (1H, *ddd*, J_1 = 5.9, $J_2 = J_3$ = 0.0 Hz, H-11); 3.65 (3H, *s*, MeOCO group); 2.43 (1H, *dd*, J_1 = 11.8, J_2 = 5.9 Hz, H_{eq}-12); 2.02 (1H, *d*, *J* = 11.8 Hz, H_{ax}-12); 1.60 (3H, *s*, H_3-17); 1.14 and 1.04 (3H each, *s*, H_3-19 and H_3-20). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 363 ([M + 1]$^+$, 100%); 345 ([M + 1 − H_2O]$^+$, 16).

Cyclization of the epoxide **9**. Epoxide **9** (40 mg) was dissolved in $CHCl_3$ (15 ml) and Al_2O_3 (50 mg) was added. After the mixture had been heated under reflux for 12 hr it was filtered and subjected to CC to give metabolite **10** (30 mg, 75%).

Acetylation of metabolite **10**. Metabolite **10** (20 mg) was dissolved in pyridine–Ac_2O (1:0.5 ml) and the solution set aside at room temp. for 12 hr. The mixture was chromatographed on a silica gel column to give methyl (13*R*,14*S*)-*ent*-3β,14-diacetoxy-8α,13;15,16-diepoxylabdan-18-oate (**15**, 15 mg, 61%): gum; $[\alpha]_D$ − 15° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 1736 and 1246 cm^{-1} (COO). ^1H NMR (300 MHz): δ5.13 (1H, *dd*, J_1 = 11.8, J_2 = 4.7 Hz, H-3); 4.97 (1H, *dd*, J_1 = 5.4, J_2 = 2.7 Hz, H-14); 4.03 (1H, *dd*, J_1 = 10.5, J_2 = 5.4 Hz) and 3.63 (1H, *dd*, J_1 = 10.5, J_2 = 2.7 Hz) (H_2-15); 3.82 and 3.52 (2H, AB system, *J* = 8 Hz, H_2-16); 3.65 (3H each, *s*, MeOCO group); 2.05 and 1.96 (3H each, *s*, AcO group); 1.14 and 1.08 (3H each, *s*, H_3-17 and H_3-19) and 0.79 (3H, *s*, H_3-20). CI-MS *m/z* (rel. int.): 467 ([M + 1]$^+$, 46%); 407 ([M + 1 − HOAc]$^+$ 100).

Oxidation of metabolite **7**. Metabolite **7** (400 mg) was dissolved in dry CH_2Cl_2 (20 ml), and pyridinium dichromate (PDC) (308 mg) was added [11]. After the mixture had been stirred for 30 min at room temp. it was filtered and chromatographed on a silica gel column to give starting product **7** (95 mg, 24%), methyl (13*S*)-*ent*-16-hydroxy-3-oxo-8α,13-epoxylabd-14-en-18-oate (**16**, 145 mg, 36%) and methyl (13*S*)-*ent*-3,16-dioxo-8α,13-epoxylabd-14-en-18-oate (**17**, 52 mg, 13%).

Methyl (13S)-ent-16-*hydroxy*-3-*oxo*-8α,13-*epoxylabd*-14-*en*-18-*oate* (**16**). Mp 98–100°; $[\alpha]_D$ − 33° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3468 (OH), 3084 (C=C), 1702 (CO), 1737 and 1243 (COO). ^1H NMR (300 MHz): δ5.8 (1H, *dd*, J_1 = 17.4, J_2 = 10.8 Hz, H-14); 5.23 (1H, *dd*, J_1 = 17.4, J_2 = 1.5 Hz) and 5.10 (1H, *dd*, J_1 = 10.8, J_2 = 1.5 Hz) (H_2-15); 3.69 (3H, *s*, MeOCO group); 3.34 and 3.27 (2H, AB system, *J* = 11.0 Hz, H_2-16); 1.29 and 1.28 (6H, H_3-17 and H_3-19) and 0.94 (3H, *s*, H_3-20). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 365 ($[M + 1]^+$, 100%); 347 ($[M + 1 - H_2O]^+$, 27).

Methyl (13S)-ent-3,16-*dioxo*-8α,13-*epoxylabd*-14-*en*-18-*oate* (**17**). Mp 63–65°; $[\alpha]_D$ − 140° ($CHCl_3$; *c* 0.5); IR ν_{max} cm^{-1}: 3090 (C=C), 2788 and 1700 (HCO), 1727 and 1245 (COO). ^1H NMR (300 MHz): δ9.39 (1H, *s*, H-16); 5.48 (1H, part X of an ABX system, $J_{AX} + J_{BX}$ = 27.7 Hz, H-14); 5.34–5.20 (2H, part AB of an ABX system, H_2-15); 3.70 (3H, *s*, MeOCO group); 1.30, 1.22 and 0.87 (3H each, *s*, methyl groups). ^{13}C NMR: Table 1. *m/z* 363 ($[M + 1]^+$, 100%).

Biotransformation of substrate **16**. Substrate **16** (100 mg) was dissolved in EtOH (2 ml) and the soln distributed between two conical flask cultures. These were incubated for 4 days to give a mixture (90 mg) which was then chromatographed on silica gel column to obtain starting product **16** (20 mg, 20%), methyl (13*S*)-*ent*-11β,16-dihydroxy-3-oxo-8α,13-epoxylabd-14-en-18-oate (**18**, 25 mg, 26%), methyl (13*S*)-*ent*-3α,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate (**19**, 10 mg, 11%) and methyl (13*R*,14*S*)-ent-14-hydroxy-3-oxo-8α,13;15,16-di-epoxylabdan-18-oate (**20**, 8 mg, 8%).

Methyl (13S)-ent-11β,16-*dihydroxy*-3-*oxo*-8α,13-*epoxy-labd*-14-*en*-18-*oate* (**18**). Gum; $[\alpha]_D$ − 30° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3456 (OH), 3090 (C=C), 1703 (CO), 1730 and 1247 (COO). ^1H NMR (300 MHz): δ5.79 (1H, *dd*, J_1 = 17.5, J_2 = 10.7 Hz, H-14); 5.25 (1H, *dd*, J_1 = 17.5, J_2 = 1.2 Hz) and 5.12 (1H, *dd*, J_1 = 10.7, J_2 = 1.2 Hz); (H_2-15); 4.46 (1H, *m*, H-11); 3.70 (3H, *s*, MeOCO group); 3.56 and 3.39 (2H, AB system, *J* = 11.1 Hz, H_2-16); 1.60 (3H, *s*, H_3-17); 1.35 and 1.34 (3H each, *s*, H_3-19 and H_3-20). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 381 ($[M + 1]^+$, 100%); 363 ($[M + 1 - H_2O]^+$, 18); 345 ($[M + 1 - 2H_2O]^+$, 9).

Methyl (13S)-ent-3α,16-*dihydroxy*-8α,13-*epoxylabd*-14-*en*-18-*oate* (**19**). Gum; $[\alpha]_D$ − 12° ($CHCl_3$; *c* 1); IR ν_{max} cm^{-1}: 3456 (OH), 3086 (C=C), 1723 and 1243 (COO). ^1H NMR (300 MHz): δ5.79 (1H, *dd*, J_1 = 17.4, J_2 = 10.7 Hz, H-14); 5.24 (1H, *dd*, J_1 = 17.4, J_2 = 1.2 Hz) and 5.11 (1H, *dd*, J_1 = 10.7, J_2 = 1.2 Hz) (H_2-15); 3.77 (1H, *dd*, J_1 = 2.9, J_2 = 2.6 Hz, H-3); 3.71 (3H, *s*, MeOCO group); 3.30 (2H, *s*, H_2-16); 1.27 (3H, *s*, H_3-17); 1.16 (3H, *s*, H_3-19) and 0.84 (3H, *s*, H_3-20). ^{13}C NMR: Table 1. CI-MS *m/z* (rel. int.): 367 ($[M + 1]^+$, 87%); 349 ($[M + 1 - H_2O]^+$, 100); 331 ($[M + 1 - 2H_2O]^+$, 89).

Methyl (13R,14S)-ent-14-*hydroxy*-3-*oxo*-8α,13;15,16-*diepoxylabdan*-18-*oate* (**20**). Gum; $[\alpha]_D$ − 7° ($CHCl_3$; *c* 0.5); IR ν_{max} cm^{-1}: 3511 (OH), 1703 (CO), 1737 and 1250 (CO_2). ^1H NMR (300 MHz): δ3.92 (1H, *dd*, part C of an ABC system, $J_{AC} + J_{BC}$ = 14.35 Hz) and 3.80–3.62 (4H, part AB of an ABC system and AB system) (H-14, H_2-15 and H_2-16); 3.71 (3H, *s*, MeOCO group); 1.32 (3H, *s*, H_3-17); 1.20 (3H, *s*, H_3-19) and 0.93 (3H, *s*, H_3-20). ^{13}C NMR: Table 1. CIMS *m/z* (rel. int.): 381 ($[M + 1]^+$, 100%); 363 ($[M + 1 - H_2O]^+$, 15).

Adenylatecyclase assay. The standard incubation mixture contained 50 mM Tris–HCl buffer, pH 7.4, 0.2 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP, 2.5 mM $MgCl_2$, 0.5 mM [α − ^{32}P]ATP (specific activity 200 dpm $pmol^{-1}$), 25 μM, 2 mM phosphocreatine, 0.2 mg creatine kinase, and about 200 μg rat liver membranes protein. The compounds to be tested were added (1 mM) to a final vol. of 0.1 ml. Triplicate samples were incubated at 37° for 10 min. Reactions were stopped and cyclic AMP was purified and counted for radioactivity as described in ref. [12]. Compounds **4**–**8** were tested. Compound **5** was 30 times less active than forskolin **1** in activating the AC enzyme. Compound **6** showed an inhibitory action (40%) on the activity of AC enzyme, previously stimulated by forskolin **1** or fluorides at the same percentage and it did not inhibit the activity of AC enzyme previously activated by glucagon. Compounds **4**, **7** and **8** were inactive.

Anti-leishmania assay. The promastigote forms of *Leishmania donovani* were cultivated as indicated in ref. [13]. The compounds to be tested were dissolved in DMSO. Screening *in vitro* was carried out in order to determine the leishmanicide effects of compounds **4**–**7**. The concn assayed was 100 μg ml^{-1} and the parasites were counted in the chamber haemocytometer after 24 hr. Compounds **4** and **6** showed growth inhibition of 15 and 5%, respectively. Compound **4** was tested at 48 and 72 hr in triplicate samples. Growth inhibition of *L. donovani* at 48 hr (40%) and 72 hr (63%) was observed.

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