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NEW POLYOXYGENATED *ENT*-MANOYL OXIDES OBTAINED BY BIOTRANSFORMATION WITH FILAMENTOUS FUNGI

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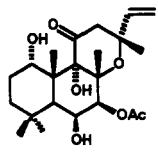
ABSTRACT.—Incubation of methyl (13*R*)-*ent*-16-hydroxy-8α,13-epoxylabd-14-en-18-oate [**4**] with *Curvularia lunata* yielded *ent*-1β-hydroxy [**6**] and *ent*-6β-hydroxy [**7**] derivatives, and that of methyl (13*S*)-*ent*-16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**5**] with the same organism gave *ent*-11β-hydroxy [**8**], *ent*-6β-hydroxy [**9**], and *ent*-6β,11β-dihydroxy [**10**] derivatives. The incubation of substrates **4** and **5** with *Fusarium moniliforme* afforded *ent*-1β-hydroxy derivatives (**6** and **14**, respectively). *Cunninghamella elegans* produced *ent*-3β-hydroxy, *ent*-1β-hydroxy and *ent*-1β,3β-dihydroxy derivatives, and led to epoxidation of the double bond of the substrates. In addition, *ent*-3β,11α-dihydroxy (as the acetoxy derivative **17**) and *ent*-3β,11β-dihydroxy [**12**] derivatives were isolated from incubations of substrates **4** and **5**, respectively. Compounds **7**, **9–11**, **14**, **16–18**, and **21** were characterized as new polyoxygenated *ent*-manoyl oxides.

In recent years, a group of diterpenoids with an 11-oxo-manoyl oxide basic skeleton has been isolated from the medicinal plant *Coleus forskohlii* Briq. (Lamiaceae) (1). The major product, forskolin [**1**], shows remarkable biological activity and has emerged as a very attractive target for synthesis (2,3), structure-activity relationship studies (4,5) and biomedical research (6). An additional new antihypertensive labdane diterpenoid, 13-epiforskolin, was also isolated from *Coleus forskohlii* (7).

Biotransformation processes with microorganisms can be used to introduce hydroxyl groups at difficult positions on the substrates (8). Chemical-microbiological methods constitute an alternative way to obtain new polyoxygenated products from abundant natural products. Thus, *ent*-8α-hydroxylabda-13(16),14-dien-18-oic acid [**2**], isolated from an Andalusian species of *Sideritis* (10), was transformed to its methyl ester [**3**]. Methyl (13*R*)-*ent*-16-hydroxy-8α,13-epoxylabd-14-en-18-oate [**4**] and methyl (13*S*)-*ent*-16-hydroxy-8α,13-epoxylabd-14-en-18-oate [**5**]) were then obtained from the methyl ester [**3**] (9). From substrates **4** and **5**, we have accomplished a series of biotransformations with filamentous fungi to study the influence of these microorganisms on the nature of the transformations, and to obtain new manoyl oxides of the *enantio*-series in order to determine their biological activity. In previous papers we have described the biotransformation of *ent*-16-hydroxymanoyl oxides with *R. nigricans* (9,11,12) and reported that the configuration at C-13 is important for the observed effect of the microorganism. An *ent*-11β-hydroxy derivative isolated from the biotransformation of substrate **5** was found to be an inhibitor of adenylate cyclase (12). To complete this study we have incubated substrates **4** and **5** with the fungi *Curvularia lunata*, *Fusarium moniliforme*, and *Cunninghamella elegans*, and determined the biotransformation products, which are described herein.

RESULTS AND DISCUSSION

Incubation of substrate **4** with *Curvularia lunata* for 7 days gave 76% recovered **4** together with the metabolites **6** and **7**. Both metabolites showed a molecular peak at *m/z* 366 in their cims (*m/z* 367 $[M+1]^+$), which indicated that the microorganism had introduced an oxygen atom into substrate **4** in both cases. Metabolite **6** exhibited nmr



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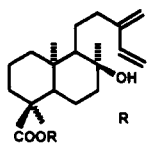

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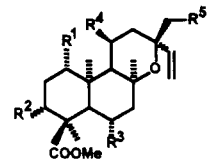
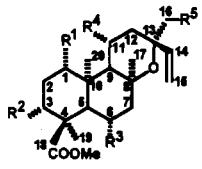
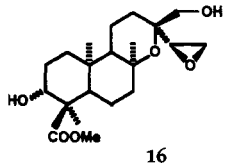


	R^1	R^2	R^3	R^4	R^5
4	H	H	H	H	OH
6	OH	H	H	H	OH
7	H	H	OH	H	OH
15	H	OH	H	H	OH
17	H	OAc	H	OAc	OAc
18	OAc	OAc	H	H	OAc



	R^1	R^2	R^3	R^4	R^5
5	H	H	H	H	OH
8	H	H	H	OH	OH
9	H	H	OH	H	OH
10	H	H	OH	OH	OH
11	H	OAc	H	OAc	OAc
12	H	OH	H	OH	OH
13	H	OH	H	H	OH
14	OH	H	H	H	OH
21	OH	OH	H	H	OH



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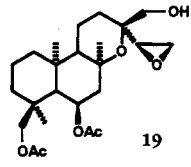

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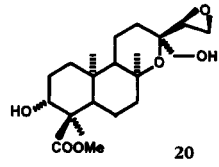

20

and cims data identical to those of methyl (13*R*)-*ent*-1β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate, obtained in a previous biotransformation from substrate **4** with *R. nigricans* (9). The ^{1}H-nmr spectrum of metabolite **7** showed a signal at 4.00 ppm (1H, ddd, $J_1=J_2=3.2$ Hz, $J_3=2.4$ Hz) due to an equatorial proton, geminal to a hydroxy group on C-6. Comparison of the ^{1}H-nmr spectra of substrate **3** and metabolite **7** indicated that the ^{1}H-nmr spectrum of metabolite **7** showed deshielding effects of the protons at C-17 (δ 1.49, Δδ +0.34), C-19 (δ 1.47, Δδ +0.43) and C-20 (δ 1.11, Δδ +0.43), compatible with the presence of an axial hydroxy group at C-6. Analysis of the ^{13}C-nmr spectra of metabolite **7** and substrate **4** confirmed that this hydroxylation occurred at C-6.

The biotransformation of substrate **5** with *C. lunata* for 7 days left 21% of substrate **5** unaltered and produced metabolites **8** (26%), **9** (18%), **10** (3%), and a polar mixture that was acetylated to yield triacetate **11** (3%). The spectroscopic data of metabolite **8** were nearly identical to those of the *ent*-11β-hydroxy derivative isolated in a previous incubation of substrate **5** with *R. nigricans* (12). The cims of metabolite **9** had a molecular peak of *m/z* 366 (*m/z* 367 $[M+1]^+$). Its ^{1}H-nmr spectrum showed a signal at 3.99 ppm (1H, ddd, $J_1=J_2=3.2$ Hz, $J_3=2.7$ Hz) owing to an equatorial proton, geminal to a hydroxy group on C-6. Comparison of the ^{13}C-nmr data of substrate **5** and metabolite **9** confirmed that **9** was methyl (13*S*)-*ent*-6β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate.

The cims spectrum of metabolite **10** exhibited a molecular peak at *m/z* 383, compatible with being a dihydroxylated derivative of substrate **5**. Its ^{1}H-nmr spectrum showed a signal at 4.51 ppm identical to that observed in the ^{1}H-nmr spectrum of metabolite **8**. This signal was due to an equatorial proton, geminal to a hydroxy group on C-11. Another signal appeared at 4.00 ppm owing to a proton geminal to an axial hydroxy group at C-6. The ^{1}H-nmr spectrum of **10**, compared with that of substrate **5**, showed deshielding of the protons of the hydroxymethylene group at C-16 as well as of the protons at C-17 and C-20 (Δδ +0.57 and +0.58, respectively). Analysis of the ^{13}C-nmr spectra of metabolite **10** and substrate **5**

showed variations in chemical shift similar to those observed in the ^{13}C-nmr spectra of metabolites **8** and **9**. Thus, metabolite **10** was assigned as methyl (13*S*)-*ent*-6β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate.

After acetylation of the mixture of polar metabolites, triacetate **11** was isolated. The nmr and cims data for **11** were identical to those of methyl (13*S*)-*ent*-3β,11β,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate obtained by acetylation from methyl (13*S*)-*ent*-3β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate [**12**] isolated from the incubation of substrate **5** with *R. nigricans* (12).

Incubation of **5** with *R. nigricans* yielded the *ent*-3β-hydroxy derivative [**13**, 36%] (12). Incubation of this product [**13**] with *C. lunata* for 10 days gave the trihydroxy derivative [**12**] (22%), which was also isolated from the incubation of substrate **5** with *R. nigricans* (12).

When substrate **4** was incubated with *Fusarium moniliforme* for 14 days, metabolite **6** was obtained. The incubation of substrate **5** with *F. moniliforme* for 14 days gave metabolite **14** (14%) with nmr and cims data very similar to those of metabolite **6**. Its ^{1}H-nmr spectrum showed a signal at δ 3.45 (1H, dd, J_1=9.3 Hz, J_2=5.6 Hz), due to an axial proton, geminal to the hydroxyl group on C-1. The ^{13}C-nmr spectrum of **14**, compared with that of substrate **5**, indicated that **14** was methyl (13*S*)-*ent*-1β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate.

Incubation of substrate **4** with *Cunninghamella elegans* for five days gave metabolites **15**, **16**, and a mixture of polar metabolites which was acetylated to give triacetates **17** and **18**. Spectroscopic data for metabolite **15** were identical to those of the *ent*-3β-hydroxy derivative isolated from the incubation of substrate **4** with *R. nigricans* (9). Metabolite **16** was the result of epoxidation of the vinyl moiety together with an equatorial hydroxylation at C-3. The structure of **16** was deduced from its nmr and cims data and from comparison with the spectroscopic data for (13*S*,14*R*)-*ent*-6α,18-diacetoxy-16-dihydroxy-8α,13:14,15-diepoxylabdane [**19**], described previously (11). Thus, metabolite **16** was the result of epoxidation of the double bond of metabolite **15** from the Re-face, to give methyl (13*S*,14*R*)-*ent*-3β,16-dihydroxy-8α,13:14,15-diepoxylabdan-18-oate. The ^{1}H-nmr spectra of the triacetoxy derivatives **17** and **18** indicated that two of the acetoxy groups were at C-3 (equatorial) and C-16 in both products. The third acetoxy group showed an equatorial disposition at C-11 in product **17** (geminal proton at δ 5.21, ddd, J_1=9.1 Hz, J_2=5.5 Hz, J_3=4.2 Hz) and an equatorial disposition at C-1 in product **18** (geminal proton at δ 4.80, dd, J_1=11.3 Hz, J_2=4.7 Hz). ^{13}C-Nmr data confirmed the positions of these functionalizations. Thus, products **17** and **18** were assigned as methyl (13*R*)-*ent*-3β,11α,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate and methyl (13*R*)-*ent*-1β,3β,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate, respectively.

The incubation of substrate **5** with *C. elegans* for five days gave two metabolites, the spectroscopic data of which were identical to those **12** and **13** isolated in a previous incubation of substrate **5** with *R. nigricans* (12). Furthermore, two new metabolites [**20** and **21**] were isolated. Metabolite **20** was characterized as methyl (13*R*,14*S*)-*ent*-3β,16-dihydroxy-8α,13:14,15-diepoxylabd-14-en-18-oate, also isolated from the incubation of substrate **5** with *R. nigricans* (12). The spectroscopic data of metabolite **21** indicated that the microorganism had introduced two hydroxy groups into substrate **5**. Its ^{1}H-nmr spectrum showed two signals for equatorial protons, geminal to a hydroxy group at C-1 and C-3, respectively. The ^{13}C-nmr data were also compatible with the structure, methyl (13*S*)-*ent*-1β,3β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate [**21**].

Analysis of the incubations of substrates **4** and **5** (epimers at C-13) allowed one to observe the influence of this chiral center in the biotransformation processes of these diterpenoids with filamentous fungi. Thus, *C. lunata* biotransformed more efficiently substrate **5** than

substrate **4**. *F. moniliforme* biotransformed both substrates in low yields. However, *C. elegans* biotransformed substrate **5** (73%) more efficiently than substrate **4** (50%).

ent-1β-Hydroxy and *ent*-6β-hydroxy derivatives were obtained in the incubation of substrate **4** with *C. lunata*. The incubation of substrate **5** with this microorganism gave rise to *ent*-11β-hydroxylation followed by *ent*-6β-hydroxylation and small quantities of *ent*-6β,11β-dihydroxy and *ent*-3β,11β-dihydroxy derivatives. We observed that 11-hydroxylation is characteristic of substrates that belong to the 13*S* configuration (13-*normal*). *C. lunata* produced 6-hydroxy derivatives in both incubations, suggesting that this type of hydroxylation is characteristic of this microorganism regardless of the configuration at C-13.

The action of *F. moniliforme* was similar in both substrates and *ent*-1β-hydroxy derivatives were obtained. The effects of *C. elegans* were also independent of the configuration at C-13 since, in both cases, *ent*-3β-hydroxylation and *ent*-1β,3β-dihydroxylation were observed. The microorganism also led to epoxidation of the double bond in both substrates. The only difference in the action of *C. elegans* on substrates **4** and **5** was the functionalization at C-11 of the *ent*-3β-hydroxy derivatives. Thus, in the incubation of substrate **4**, the action occurred on the *ent*-α face to give an equatorial hydroxylation. However, in the incubation of substrate **5**, the reaction occurred on the *ent*-β face to give an axial hydroxylation at C-11.

These incubations have provided a wide range of new polyoxygenated manoyl oxides. The incubation of substrate **5** with *C. lunata* improved the yield of an *ent*-11β-hydroxy derivative [**8**, 26%], obtained in a previous incubation from substrate **5** with *R. nigricans* (10%) (12). Compound **8** is known to inhibit the activity of the adenylate cyclase enzyme (12). The *ent*-6β-hydroxy derivatives were also obtained by incubations of other *ent*-13-*epi*-manoyl oxides with *C. lunata*, and may also mediate the activity of the adenylate cyclase enzyme (13,14). In view of these results, it has been observed that the use of different microorganisms can facilitate greatly the accumulation of new diterpene oxides, which can then be tested for possible biological activity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The nmr spectra (300 MHz, ^{1}H and 75.4 MHz, ^{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 DEPT techniques using a flip angle of 135°. Ir spectra were obtained on a Perkin-Elmer 983 G spectrometer, and cims spectra on a Hewlett-Packard 5988 A spectrometer in CH_4. Specific rotations were measured in $CHCl_3$ (1 dm tube) with a Jasco DIP-370 polarimeter at 20°. Cc employed Merck 60 Si gel (230–400 mesh ASTM), with CH_2Cl_2 and increasing amounts of Me_2CO as eluent. Tlc was performed using Si gel (Merck G), with visualization by spraying with H_2SO_4/HOAc/H_2O followed by heating at 120°.

ISOLATION OF STARTING MATERIAL AND PREPARATION OF SUBSTRATES **4**, **5**, AND **13**.—The starting material used in this study was *ent*-8α-hydroxylabd-13(16),14-dien-18-oic acid [**2**, 6-deoxyandalusoic acid] isolated from *Sideritis varoi* subsp. *cuatrecasassii* (10). Methyl (13*R*)-*ent*-16-hydroxy-8α,13-epoxylabd-14-en-18-oate [substrate **4**] and methyl (13*S*)-*ent*-16-hydroxy-8α,13-epoxylabd-14-en-18-oate [substrate **5**] were obtained from acid **2**, via methyl ester **3**, as reported previously (12). Substrate **13** [methyl (13*S*)-*ent*-3β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate] was obtained previously by biotransformation of substrate **5** with *Rhizopus nigricans* (12).

ORGANISMS, MEDIA, AND CULTURE CONDITIONS.—*Curvularia lunata* CECT 2130, *Fusarium moniliforme* CECT 2152, and *Cunninghamella elegans* CECT 2123 were obtained from the Colección Española de Cultivos Tipo (CECT), Departamento de Microbiología, Universidad de Valencia, Spain. The bacterial culture was stored in YEPGA medium containing 1% yeast extract, 1% peptone, 2% glucose, and 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. Erlenmeyer flasks (250 ml) containing 80 ml of medium were inoculated with a suspension of *R. nigricans*. Incubations were maintained at 28° with gyratory shaking (150 rpm) for 6 days, after which the substrates **4**, **5**, and **13** 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 $MgSO_4$, and evaporated at 40° *in vacuo*. Mixtures of products obtained were chromatographed on Si gel columns.

BIOTRANSFORMATION OF SUBSTRATE **4** WITH *C. LUNATA*.—Substrate **4** (200 mg) was dissolved in EtOH (5 ml) and the solution distributed among five Erlenmeyer flask cultures. These were incubated for seven days and the metabolites were recovered and chromatographed on a Si gel column to obtain the starting product **4** [153 mg, 76%], methyl (13*R*)-*ent*-1β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**6**, 6 mg, 3%] (9), and methyl (13*R*)-*ent*-6β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**7**, 17 mg, 8%].

*Methyl (13*R*)-ent-6β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate* [**7**].—Gum; $[\alpha]_D$ −10° (*c*=1, $CHCl_3$); ir ν max 3489 (OH), 3088 (C=C), 1718, 1250 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.91 (1H, dd, X part of an ABX system, $J_{AX}+J_{BX}$=28.3 Hz, H-14), 5.08 (2H, AB part of an ABX system, H_2-15), 4.00 (1H, ddd, $J_1=J_2$=3.1 Hz, J_3=2.4 Hz, H-6), 3.67 (3H, s, MeOCO-), 3.29 and 2.96 (2H, AB system, *J*=10.8 Hz, H_2-16), 1.49, 1.47 (3H each, s, Me-17, Me-19), 1.11 (3H, s, Me-20); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 179.12 (C-18), 144.11 (C-14), 113.37 (C-15), 76.12 (C-13), 76.03 (C-8), 71.02 (C-6), 69.71 (C-16), 59.14 (C-9), 52.74 (C-5), 52.06 (COO*Me*), 49.95 (C-7), 48.24 (C-4), 40.72 (C-1), 38.83 (C-3), 36.86 (C-10), 28.47 (C-12), 25.12 (C-17), 18.79 (C-19), 17.88 (C-2), 17.35 (C-20), 15.15 (C-11); cims (methane) *m/z* 367 ($[M+1]^+$, 11), 349 ($[M+1-H_2O]^+$, 100), 331 ($[M+1-2H_2O]^+$, 92).

BIOTRANSFORMATION OF SUBSTRATE **5** WITH *C. LUNATA*.—Substrate **5** (300 mg) was dissolved in EtOH (10 ml) and the solution distributed among ten Erlenmeyer flask cultures. These were incubated for seven days and the metabolites were recovered and chromatographed on a Si gel column to obtain the starting product [**5**] (62 mg, 21%), methyl (13*S*)-*ent*-11β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**8**, 81 mg, 26%] (12), methyl (13*S*)-*ent*-6β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**9**, 55 mg, 18%], methyl (13*S*)-*ent*-6β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate [**10**, 10 mg, 3%], and a mixture of polar metabolites which was acetylated with Ac_2O/pyridine (1:2 ml) for 24 h at room temperature to give methyl (13*S*)-*ent*-3β,11β,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate [**11**, 12 mg, 3%].

*Methyl (13*S*)-ent-6β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate* [**9**].—Gum; $[\alpha]_D$ −11° (*c*=0.5, $CHCl_3$); ir ν max 3480 (OH), 3087 (C=C), 1715, 1246 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.78 (1H, dd, J_1=17.4 Hz, J_2=10.9 Hz, H-14), 5.20 (1H, dd, J_1=17.4 Hz, J_2 =1.5 Hz), 5.08 (1H, J_1=10.8 Hz, J_2=1.5 Hz) (H_2-15), 3.99 (1H, ddd, $J_1=J_2$=3.2 Hz, J_3=2.7 Hz, H-6), 3.67 (3H, s, MeOCO-), 3.32, 3.26 (2H, AB system, *J*=11.0 Hz, H_2-16), 1.51, 1.48 (3H each, s, Me-17, Me-19), 1.18 (3H, s, Me-20); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 179.20 (C-18), 144.03 (C-14), 113.99 (C-15), 76.29 (C-13), 74.87 (C-8), 70.85 (C-6), 68.50 (C-16), 53.53 (C-5), 52.74 (C-9), 52.07 (COO*Me*), 50.74 (C-7), 48.13 (C-4), 40.37 (C-1), 38.75 (C-3), 37.12 (C-10), 27.27 (C-12), 26.96 (C-17), 18.85 (C-19), 17.74 (C-2), 16.61 (C-20), 14.30 (C-11); cims (methane) *m/z* 367 ($[M+1]^+$, 11), 349 ($[M+1-H_2O]^+$, 100), 331 ($[M+1-2H_2O]^+$, 92).

*Methyl (13*S*)-ent-6β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate* [**10**].—Gum; ir ν max 3425 (OH), 3080 (C=C), 1717, 1248 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.78 (1H, dd, J_1=17.4 Hz, J_2=10.8 Hz, H-14), 5.21 (1H, dd, J_1=17.4 Hz, J_2=1.3 Hz), 5.10 (1H, dd, J_1=10.8 Hz, J_2=1.3 Hz) (H_2-15), 4.51 (1H, ddd, $J_1=J_2$=6.8 Hz, J_3=4.4 Hz, H-11), 4.00 (1H, ddd, $J_1=J_2$=3.2 Hz, J_3=2.7 Hz, H-6), 3.67 (3H, s, MeOCO-), 3.54, 3.35 (2H, AB system, *J*=11.1 Hz, H_2-16), 1.80 (3H, s, Me-17), 1.58 (3H, s, Me-20), 1.53 (3H, s, Me-19); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 179.16 (C-18), 143.57 (C-14), 114.32 (C-15), 75.82 (C-13), 74.99 (C-8), 70.85 (C-6), 68.62 (C-16), 64.25 (C-11), 56.40 (C-9), 53.09 (C-5), 52.22 (C-7), 52.12 (COO*Me*), 48.26 (C-4), 40.80 (C-1), 38.75 (C-3, C-12), 28.81 (C-17), 18.98, 18.92 (C-19, C-20), 17.71 (C-2); cims (methane) *m/z* 383 ($[M+1]^+$, 51), 365 ($[M+1-H_2O]^+$, 100), 347 ($[M+1-2H_2O]^+$, 62), 329 ($[M+1-3H_2O]^+$, 39).

*Methyl (13*S*)-ent-3β,11β,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate* [**11**].—Gum; $[\alpha]_D$ −56° (*c*=1, $CHCl_3$); ir ν max 3083 (C=C), 1740, 1239 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.81 (1H, dd, J_1=17.3 Hz, J_2=10.8 Hz, H-14), 5.39 (1H, ddd, $J_1=J_2$=6.6 Hz, J_3=4.5 Hz, H-11), 5.26 (1H, dd, J_1=17.3 Hz, J_2=1.3 Hz), 5.10 (1H, dd, J_1=10.8 Hz, J_2=1.3 Hz) (H_2-15), 5.11 (1H, dd, J_1=11.8 Hz, J_2=5.0 Hz, H-3), 4.16, 4.00 (2H, AB system, *J*=11.1 Hz, H_2-16), 3.60 (3H, s, MeOCO-), 2.06, 2.03, 1.95 (3H each, s, 3×AcO), 1.52 (3H, s, Me-17), 1.15 (3H, s, Me-20), 1.03 (3H, s, Me-19); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 176.25 (C-18), 170.82, 170.07, 170.03 (3×Me*C*OO), 142.35 (C-14), 114.41 (C-15), 74.89, 73.53 (C-8, C-13), 68.70 (C-16), 66.34 (C-11), 55.44 (C-9), 52.44 (COO*Me*), 51.90 (C-4), 51.72 (C-5), 43.73 (C-7), 36.83 (C-10), 36.55 (C-1), 35.94 (C-12), 26.80 (C-17), 22.83, 22.23 (C-2, C-6), 21.75, 21.09, 21.04 (3×*Me*COO), 17.07 (C-20), 11.69 (C-19); cims (methane) *m/z* 509 ($[M+1]^+$, 21), 449 ($[M+1-AcOH]^+$, 100), 389 ($[M+1-2AcOH]^+$, 37).

BIOTRANSFORMATION OF SUBSTRATE **13** WITH *C. LUNATA*.—Substrate **13** (50 mg) was dissolved in

EtOH (2 ml) and the solution distributed between two Erlenmeyer flask cultures. These were incubated for ten days and the metabolites were recovered and chromatographed on a Si gel column to obtain the starting product [**5**] (30 mg, 60%) and methyl (13*S*)-*ent*-3β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate [**12**, 12 mg, 22%] (12).

ACETYLATION OF METABOLITE **12**.—Metabolite **12** (10 mg) was acetylated with Ac_2O-pyridine (0.5:1) for 24 h at room temperature to yield 8 mg of triacetate **11** (60 %).

BIOTRANSFORMATION OF SUBSTRATE **4** WITH *F. MONILIFORME*.—Substrate **4** (100 mg) was dissolved in EtOH (2 ml) and the solution distributed between two Erlenmeyer flask cultures. These were incubated for 14 days and the metabolites were recovered and chromatographed on a Si gel column to obtain the starting product [**4**] (59 mg, 59%) and metabolite **6** (24 mg, 23%) (9).

BIOTRANSFORMATION OF SUBSTRATE **5** WITH *F. MONILIFORME*.—Substrate **5** (100 mg) was dissolved in EtOH (2 ml) and the solution distributed between two Erlenmeyer flask cultures. These were incubated for 14 days and the metabolites were recovered and chromatographed on a Si gel column to obtain the starting product [**5**] (61 mg, 61%) and methyl (13*S*)-*ent*-1β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**14**, 15 mg, 14%]: gum; $[\alpha]_D$ −10° (*c*=0.7, $CHCl_3$); ir ν max 3452 (OH), 3085 (C=C), 1725, 1246 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.81 (1H, dd, J_1=17.4 Hz, J_2=10.8 Hz, H-14), 5.29 (1H, dd, J_1=17.4 Hz, J_2=1.5 Hz), 5.13 (1H, J_1=10.8 Hz, J_2=1.5 Hz) (H_2-15), 3.66 (3H, s, MeOCO-), 3.45 (1H, dd, J_1=9.3 Hz, J_2=5.6 Hz, H-1), 3.26 (2H, s, H_2-16), 1.25, 1.12, 0.88 (3H each, s, 3×Me); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 178.83 (C-18), 143.83 (C-14), 114.39 (C-15), 79.05 (C-1), 76.41 (C-8), 75.40 (C-13), 68.83 (C-16), 52.95 (C-9), 52.15 (COO*Me*), 50.21 (C-5), 47.35 (C-4), 43.11 (C-7), 42.58 (C-10), 34.99 (C-3), 28.40 (C-12), 26.73 (C-2), 26.13 (C-13), 22.65 (C-6), 17.98 (C-11), 16.09 (C-19), 11.49 (C-20); cims (methane) *m/z* 367 ($[M+1]^+$, 100), 349 ($[M+1-H_2O]^+$, 57), 331 ($[M+1-2H_2O]^+$, 94).

BIOTRANSFORMATION OF SUBSTRATE **4** WITH *C. ELEGANS*.—Substrate **4** (100 mg) was dissolved in EtOH (2 ml) and the solution distributed between two Erlenmeyer flask cultures. These were incubated for five days and the metabolites were recovered and chromatographed on a Si gel column to obtain the starting product [**4**] (2 mg, 2%), methyl (13*R*)-*ent*-3β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**15**, 17 mg, 15%] (9), methyl (13*S*,14*R*)-*ent*-3β,16-dihydroxy-8α,13;14,15-diepoxylabdan-18-oate [**16**, 35 mg, 29%], and a mixture of polar metabolites which was acetylated with Ac_2O-pyridine (1:2 ml) for 24 h at room temperature to give methyl (13*R*)-*ent*-3β,11α,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate [**17**, 12 mg, 3%] and methyl (13*R*)-*ent*-1β,3β,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate [**18**, 5 mg, 3%].

*Methyl (13*S*,14*R*)-ent-3β,16-dihydroxy-8α,13; 14,15-diepoxylabdan-18-oate* [**16**].—Gum; $[\alpha]_D$ −26° (*c*=0.9, $CHCl_3$); ir ν max 3449 (OH), 1718, 1246 (COO), 1051 (CO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 3.92 (1H, dd, J_1=11.5 Hz, J_2=4.4 Hz, H-3), 3.64 (3H, s, MeOCO), 3.43, 3.19 (2H, AB system, *J*=10.9 Hz), (H_2-16), 2.86 (1H, dd, J_1=J_2=3.0 Hz), 2.77 (1H, J_1=J_2=4.7 Hz), 2.71 (1H, dd, J_1=4.7 Hz, J_2=3.0 Hz), (H-14 and H_2-15), 1.20, 1.03, 0.71 (3H each, s, 3×Me), ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 177.76 (C-18), 75.18 (C-3, C-8), 73.22 (C-13), 68.21 (C-16), 57.02 (C-9), 54.95 (C-14), 53.67 (C-4), 52.10 (COO*Me*), 51.00 (C-5), 45.93 (C-15), 41.93 (C-7), 37.10 (C-1), 35.96 (C-10), 26.28 (C-2), 24.22 (C-12), 23.43 (C-17), 22.13 (C-6), 16.02 (C-20), 15.95 (C-11), 10.46 (C-19); cims (methane) *m/z* 383 ($[M+1]^+$, 25), 365 ($[M+1-H_2O]^+$, 100), 347 ($[M+1-2H_2O]^+$, 37).

*Methyl (13*R*)-ent-3β,11α,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate* [**17**].—Gum; $[\alpha]_D$ −15° (*c*=0.6, $CHCl_3$); ir ν max 3090 (C=C), 1735, 1244 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.84 (1H, dd, J_1=17.5 Hz, J_2=10.9 Hz, H-14), 5.29 (1H, dd, J_1=17.5 Hz, J_2=1.1 Hz), 5.10 (1H, dd, J_1=10.9 Hz, J_2=1.1 Hz), (H_2-15), 5.21 (1H, ddd, J_1=9.1 Hz, J_2=5.5 Hz, J_3=4.2 Hz, H-11), 5.13 (1H, dd, J_1=11.7 Hz, J_2=4.6 Hz, H-3), 4.03, 3.92 (2H, AB system, *J*=10.8 Hz, H_2-16), 3.66 (3H, s, MeOCO-), 2.30 (1H, dd, J_1=15.4 Hz, J_2=5.5 Hz, H-12a), 2.04, 2.00, 1.96 (3H each, s, 3×AcO), 1.88 (1H, dd, J_1=15.4 Hz, J_2=4.2 Hz, H-12), 1.77 (1H, d, *J*=9.1 Hz, H-9), 1.25, 1.15, 0.89 (3H each, s, 3×Me); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 176.24 (C-18), 170.63, 170.48, 170.12 (3×Me*C*OO), 142.43 (C-14), 114.07 (C-15), 76.82 (C-3), 75.53 (C-8), 73.81 (C-13), 69.58 (C-16), 66.94 (C-11), 58.67 (C-9), 52.47 (COO*Me*), 52.04 (C-4), 50.75 (C-5), 42.66 (C-7), 37.24 (C-10), 36.85 (C-1), 34.45 (C-12), 26.51 (C-17), 22.97 (C-6), 21.99 (C-2), 21.72, 21.14, 21.06 (3×*Me*COO), 16.83 (C-20), 11.59 (C-19); cims (methane) *m/z* 509 ($[M+1]^+$, 12), 449 ($[M+1-AcOH]^+$, 100), 389 ($[M+1-2AcOH]^+$, 51), 329 ($[M+1-3×AcOH]^+$, 19).

*Methyl (13*R*)-*ent*-1β,3β,16-triacetoxy-8α,13-epoxylabd-14-en-18-oate* [**18**].—Gum; $[\alpha]_D$ −26° (*c*=0.5, $CHCl_3$); ir ν max 3088 (C=C), 1741, 1246 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.88 (1H, dd, X part of an ABX system, J_{AX}+J_{BX}=29.0 Hz, H-14), 5.26 (1H, dd, J_1=12.6 Hz, J_2=4.7 Hz, H-3), 5.12–5.06 (2H, AB part of an ABX system, H_2-15), 4.80 (1H, dd, J_1=11.3 Hz, J_2=4.7 Hz, H-1), 3.79, 3.72 (2H, AB system, *J*=11.0 Hz, H_2-16), 3.65 (3H, s, MeOCO-), 2.05, 2.01, 1.95 (3H each, s, 3×AcO), 1.21, 1.14, 0.93 (3H each, s, 3×Me); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 175.89 (C-18), 170.96, 170.01, 169.51

(3×MeCOO), 143.04 (C-14), 113.97 (C-15), 78.53 (C-1), 75.77 (C-8), 74.38 (C-13), 73.10 (C-3), 70.83 (C-16), 57.63 (C-9), 52.59 (COO*Me*), 51.89 (C-4), 48.59 (C-5), 42.00 (C-7), 40.73 (C-10), 29.78 (C-2), 29.40 (C-12), 24.19 (C-17), 21.73 (*Me*COO), 21.67 (C-6), 21.16, 20.91 (2×*Me*COO), 17.54 (C-11), 13.37 (C-20), 11.17 (C-19); cims (methane) *m/z* 509 ($[M+1]^+$, 4), 449 ($[M+1-AcOH]^+$, 16), 389 ($[M+1-2AcOH]^+$, 26), 329 ($[M+1-3AcOH]^+$, 52), 311 (100).

BIOTRANSFORMATION OF SUBSTRATE **5** WITH *C. ELEGANS*.—Substrate **5** (100 mg) was dissolved in EtOH (2 ml) and the solution distributed between two Erlenmeyer flask cultures. These were incubated for five days and the metabolites were recovered and chromatographed on a Si gel column to obtain the starting product **5** (3 mg, 3%), methyl (13*S*)-*ent*-3β,11β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate [**12**, 26 mg, 21%] (12), methyl (13*S*)-*ent*-3β,16-dihydroxy-8α,13-epoxylabd-14-en-18-oate [**13**, 32 mg, 29%] (12), methyl (13*R*,14*S*)-*ent*-3β,16-dihydroxy-8α,13;14,15-diepoxylabdan-18-oate [**20**, 14 mg, 19%] (12), and methyl (13*S*)-*ent*-1β,3β,16-trihydroxy-8α,13-epoxylabd-14-en-18-oate [**21**, 5 mg, 4%]: gum; $[\alpha]_D$ −19° (*c*=0.3, $CHCl_3$); ir ν max 3425 (OH), 3090 (C=C), 1722, 1257 (COO) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 5.80 (1H, dd, J_1=17.4 Hz, J_2=10.8 Hz, H-14), 5.20 (1H, dd, J_1=17.4 Hz, J_2=1.5 Hz), 5.13 (1H, J_1=10.8 Hz, J_2=1.5 Hz) (H_2-15), 4.04 (1H, dd, J_1=12.3 Hz, J_2=4.5 Hz, H-3), 3.72 (3H, s, MeOCO-), 3.53 (1H, dd, J_1=11.3 Hz, J_2=4.5 Hz, H-1), 3.26 (2H, s, H_2-16), 1.24, 1.10, 0.88 (3H each, s, 3×Me), ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 177.61 (C-18), 143.68 (C-14), 114.55 (C-15), 77.34 (C-1), 75.90, 75.22 (C-8, C-13), 72.67 (C-3), 68.77 (C-16), 53.71 (C-4), 52.62 (C-9), 52.47 (COO*Me*), 49.10 (C-5), 42.94 (C-7), 42.24 (C-10), 36.93 (C-2), 26.57 (C-12), 26.04 (C-17), 22.09 (C-6), 17.80 (C-11), 11.58 (C-20), 10.34 (C-19); cims (methane) *m/z* 383 ($[M+1]^+$, 12), 365 ($[M+1-H_2O]^+$, 100), 347 ($[M+1-2H_2O]^+$, 86), 329 ($[M+1-3H_2O]^+$, 56).

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LITERATURE CITED

1. S.V. Bhat, *Prog. Chem. Org. Nat. Prod.*, **63**, 1 (1993).
2. M.I. Colombo, J. Zinczuk, and E.A. Rúveda, *Tetrahedron*, **48**, 963 (1992).
3. G. Jordine, S. Bick, U. Möller, P. Welzel, B. Daucher, and G. Maas, *Tetrahedron*, **50**, 139 (1994).
4. K.B. Seamon, J.W. Daly, H. Metzger, N.J. de Souza, and N.J. Reden, *J. Med. Chem.*, **26**, 436 (1983).
5. J.D. Robbins, A. Laurenza, R.W. Kosley, G.J. O'Malley, B. Spahl, and K.B. Seamon, *J. Med. Chem.*, **34**, 3204 (1991).
6. K.B. Seamon and J.W. Daly, *Adv. Cyclic Nucl. Res.*, **20**, 1 (1986).
7. J.S. Tandon, R. Roy, S. Balachandran, and R.A. Vishwakarma, *Bioorg. Med. Chem. Lett.*, **2**, 249 (1992).
8. J.R. Hanson, *Nat. Prod. Rep.*, 139 (1992).
9. J.M. Arias, A. García-Granados, M.B. Jiménez, A. Martínez, F. Rivas, and M.E. Onorato, *J. Chem. Res. (S)*, 277 *(M)*, 2064 (1988).
10. A. García-Granados, A. Martínez, A. Molina, M.E. Onorato, M. Rico, A. Sáenz de Buruaga, and J.M. Sáenz de Buruaga, *Phytochemistry*, **24**, 1789 (1985).
11. A. García-Granados, A. Martínez, J.P. Martínez, M.E. Onorato, and J.M. Arias, *J. Chem. Soc., Perkin Trans. I*, 1261 (1990).
12. A. García-Granados, E. Liñán, A. Martínez, M.E. Onorato, A. Parra, and J.M. Arias, *Phytochemistry*, **38**, 287 (1995).
13. A. García-Granados, A. Martínez, M.B. Jiménez, M.E. Onorato, F. Rivas, and J.M. Arias, *J. Chem. Res., (S)*, 94 (1990).
14. A. García-Granados, M.B. Jiménez, A. Martínez, A. Parra, F. Rivas, and J.M. Arias, *Phytochemistry*, **37**, 741 (1994).

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