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THE MICROBIOLOGICAL TRANSFORMATION OF SOME *ENT*-13-*EPI*-MANOYL OXIDE DITERPENES BY *GIBBERELLA FUJIKUROI*

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Key Word Index—*Gibberella fujikuroi*; microbiological transformation; diterpenes; *ent*-13-*epi*-manoyl oxide derivatives; varol; varodiol.

Abstract—Incubation of *ent*-19-hydroxy-13-*epi*-manoyl oxide with *Gibberella fujikuroi* afforded *ent*-12α,19-dihydroxy-13-*epi*-manoyl oxide and *ent*-7β,12α,19-trihydroxy-13-*epi*-manoyl oxide, the first of which was transformed by chemical methods into varol (*ent*-12α-hydroxy-13-*epi*-manoyl oxide). The incubation of *ent*-3β-hydroxy-13-*epi*-manoyl oxide (ribenol) with the same fungus gave the 12β-hydroxy, the 11β-hydroxy and the 11β,12β-dihydroxy derivatives of ribenol, the first of which was identical with varodiol.

INTRODUCTION

The gibberellins and the kaurenolides, isolated from the fungus *Gibberella fujikuroi*, are diterpenoid metabolites derived from *ent*-kaur-16-ene (**1**) [1]. During the past year we have been interested in the study of the microbiological transformations of natural and synthetic diterpenoids by this fungus. These studies have been directed to preparing modified gibberellins and to obtaining information on the substrate requirements of the enzymes involved in several steps of the biosynthesis of the gibberellins and kaurenolides. The results obtained in the incubations of *ent*-kaurene derivatives and other tetracyclic diterpenes have been reported [2–4].

RESULTS AND DISCUSSION

The fungus *G. fujikuroi* produces *ent*-13-*epi*-manoyloxide (**5**), a labdane diterpene which seems to be a final metabolite of a biosynthetic branch [5]. Its 19-hydroxy derivative (**6**) has been obtained from *Baccharis tola*, a plant endemic to Chile [6, 7]. This compound possesses a hydroxyl group in the same position as *ent*-19-hydroxykaur-16-ene (**2**), an intermediate in the biosynthesis of gibberellins. Thus, we thought it would be interesting to know if the alcohol **6** could be transformed by this fungus.

The results obtained in the feeding indicated that the alcohol **6** was not oxidized to the corresponding aldehyde, and then to the acid, as occurs with the *ent*-kaurene analogue **2**, which is transformed into **3**, and then to **4**, in the biosynthetic pathway of gibberellins [1]. However, a series of interesting hydroxylations on the molecule of **6**, did occur as described below.

The microbiological transformations were carried out in the presence of the *ent*-kaur-16-ene biosynthesis inhibitor, AMO 1618, to suppress the formation of the normal metabolites, thus facilitating the analysis of the products formed [8, 9]. The fermentations were harvested after eight days, and the broth and mycelium extracts were mixed and separated into neutral and acidic fractions. The incubations of *ent*-19-hydroxy-13-*epi*-manoyl oxide (**6**) gave the 12β-hydroxy derivative **7** and the 7α, 12β-dihydroxy derivative **15**. These metabolites were identified as follows.

The less polar compound isolated has the empirical formula $C_{20}H_{34}O_3$. Its ^{1}H NMR spectrum, when compared with that of the starting material **6**, showed the resonance of a new geminal proton to a hydroxyl group at δ4.08, as a broad singlet. The form of this signal is characteristic of an equatorial hydrogen at C-1, C-3, C-7 or C-12. In this spectrum the resonances of the hydrogens of the double bond also appear modified in comparison with those of **6**. Thus, considering that the conformation of ring C in these compounds is of the boat type, we assigned the location of the new alcoholic group to C-12(β). This was confirmed by the ^{13}C NMR spectrum, whose assignments are given in Table 1. The structure of this metabolite is therefore *ent*-12α,19-dihydroxy-13-*epi*-manoyl oxide (**7**).

The second product isolated from the fermentation showed a ^{1}H NMR spectrum similar to that of the less polar **7**. The chemical shift and the form of resonance of the geminal hydrogen to the alcohol at C-12, were identical with those of **7**, but the geminal hydrogen to a new alcohol appeared at δ3.55, as a double doublet (J = 11 and 4.5 Hz), indicating the presence of another equatorial hydroxyl. Thus, this function can be at C-1, C-3 or C-7. The study of its ^{13}C NMR spectrum (Table 1) led us to choose the C-7 position. Therefore the structure of *ent*-7β,12α,19-trihydroxy-13-*epi*-manoyl oxide (**15**) was assigned to this compound.

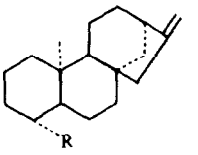
The formation of the diol **7** in the feeding prompted us to transform it by chemical procedures into varol (**14**), a labdane diterpene isolated from *Sideritis varoi* [10, 11]. Acetylation of **7** in the usual way gave the corresponding diacetate **8**. Partial hydrolysis of **8** afforded a mixture of the monoacetates **9** and **10**, inseparable by silica gel chromatography. Treatment of this mixture with triphenylphosphine–carbon tetrachloride gave the chloro-deriva-

tives **11** and **12**. The 19-chloride **12** was reduced with tri-*n*-butyltin hydride to give compound **13**. Hydrolysis of this with a saturated solution of sodium carbonate in methanol gave **14**, identical with varol [10]. The enantiomer of **14** has been isolated from Turkish tobacco [12].

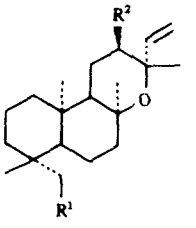
Continuing with this work we decided to incubate with the fungus the diterpene ribenol (**16**), a compound isolated from *Sideritis canariensis* [13] and *S. varoi* [10, 11], because it also possesses an *ent*-13-*epi*-manoyl oxide skeleton and a hydroxyl group at C-3(α), a spatial position similar to that of a C-19 hydroxyl. Thus, we thought that it could also be transformed into a 12β-hydroxy derivative such as **17**, a compound also isolated from *S. varoi* and named varodiol.

In fact, in the fermentation of ribenol (**16**) with *G. fujikuroi* we obtained varodiol (*ent*-3β,12α-dihydroxy-13-*epi*-manoyl oxide) (**17**) in addition to a further two compounds, **18** and **19**. Varodiol (**17**) was identified by its ^1H and ^{13}C NMR spectra, and by comparison with those obtained from an authentic sample [10]. The structures of compounds **18** and **19** were determined on the basis of the following data.

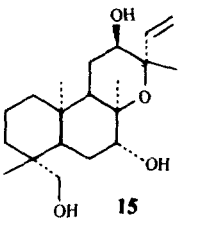
The ^1H NMR spectrum of substance **18** showed the geminal proton to C-3, and a further one at δ4.13 as a broad multiplet ($W_{1/2} = 26$ Hz) indicative of an equatorial alcohol at C-6 or C-11. The last position was assigned considering its ^{13}C NMR spectrum, and comparing it with that of ribenol (**16**) (Table 1). Therefore the structure of this compound is *ent*-3β,11α-dihydroxy-13-*epi*-manoyl oxide (**18**). The most polar compound (**19**) showed in its ^1H NMR spectrum the geminal hydrogen to the hydroxyl group at C-3, and further geminal protons to another two functions of this type at δ4.00 (*d*, $J = 4.5$ Hz) and 4.13 (*dd*, $J = 9.4$ and 4.5 Hz), which are coupled. The ^{13}C NMR spectrum of this compound compared to that of **17** indicated that the vicinal hydroxyl groups are on ring C at C-11 and C-12. In accordance



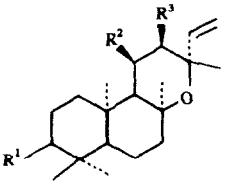
1 R = Me
2 R = CH_2OH
3 R = CHO
4 R = CO_2H



5 $R^1 = R^2 = H$
6 R^1 = OH, R^2 = H
7 $R^1 = R^2$ = OH
8 $R^1 = R^2$ = OAc
9 R^1 = OAc, R^2 = OH
10 R^1 = OH, R^2 = OAc
11 R^1 = OAc, R^2 = Cl
12 R^1 = Cl, R^2 = OAc
13 R^1 = H, R^2 = OAc
14 R^1 = H, R^2 = OH



15



16 R^1 = OH, $R^2 = R^3$ = H
17 $R^1 = R^3$ = OH, R^2 = H
18 $R^1 = R^2$ = OH, R^3 = H
19 $R^1 = R^2 = R^3$ = OH

Table 1. ^{13}C NMR (50.3 MHz) spectral data for compounds **6**, **7**, **14**–**19**

C	6	7	14	15	16*	17	18	19
1	39.58	39.21	39.08	39.12	37.81	37.45	39.51	38.86
2	18.38	18.25	18.54	18.28	27.42	27.34	27.52	27.42
3	35.92	35.91	42.19	35.97	78.91	78.96	78.63	78.70
4	38.75	38.71	33.34	38.71	38.93	39.00	39.31	39.23
5	57.22	57.26	56.68	55.03	55.39	55.53	55.53	55.46
6	20.30	20.35	20.03	27.40	19.51	19.75	19.64	19.81
7	43.58	43.22	42.87	80.91	43.00	42.83	40.70	43.68
8	76.01	76.18	76.36	79.56	75.92	76.20	76.39	76.25
9	58.74	49.48	49.22	47.88	58.42	49.74	63.24	58.70
10	36.86	36.40	36.50	36.72	36.25	36.27	38.41	38.08
11	16.15	23.84	23.72	23.50	16.01	23.85	65.63	66.75
12	34.92	69.15	69.22	69.10	34.96	69.21	44.47	71.85
13	73.47	76.23	75.93	†	73.35	76.30	73.87	75.57
14	147.74	147.27	147.33	146.87	147.45	147.25	147.82	146.19
15	109.71	110.74	110.68	111.21	109.80	110.83	110.17	112.10
16	32.84	27.04	26.96	26.77	32.73	27.09	31.86	27.09
17	23.91	24.28	24.45	18.96	24.13	24.36	25.95	25.03
18	27.04	27.04	33.34	27.16	28.02	28.12	28.31	28.28
19	65.43	65.35	21.29	65.69	15.15	15.30	15.45	15.42
20	16.39	16.31	15.85	16.41	15.97	16.02	16.54	16.57

*Data from ref. [11].
†Not observed.

with the coupling constants of their geminal protons both alcoholic groups have the β-stereochemistry. Thus the structure of this metabolite was determined as *ent*-3β, 11α, 12α-trihydroxy-13-*epi*-manoyl oxide (**19**). This triol can be formed in the incubation, alternatively, by hydroxylation of **17** or **18**.

These feedings indicated that although *ent*-13-*epi*-manoyl oxide (**5**) may be a final metabolite in *G. fujikuroi*, an increase in polarity, produced by new hydroxyl groups, may lead the fungus to transform these compounds. However, as occurs with compound **5**, in these substances the oxidations at C-19 are also inhibited. Another conclusion that can be reached from the incubations with these substrates is that there is a preference for hydroxylation at C-12 (β).

EXPERIMENTAL

Mps: uncorr. IR spectra were taken for solns in $CHCl_3$ and ^1H and ^{13}C NMR spectra were determined for solns in $CDCl_3$. Silica gel Merck (0.065–0.2 mm) was used for column chromatography.

Incubation experiments. Gibberella fujikuroi (ACC 917), inhibited with 5×10^{-5} M AMO 1618, was grown in shake cultures at 25° for 1 day in 75 conical flasks (250 ml), each containing sterile medium (50 ml) [14]. The substrate (see below) in EtOH (16–20 ml) was distributed equally among the flasks, and the incubation was allowed to continue for a further 8 days. The broth was filtered, adjusted to pH 2 with diluted HCl and extracted with EtOAc. The mycelium was treated with liquid N_2, crushed with a mortar and extracted with EtOAc. The two extracts were combined and separated into acidic and neutral fractions with $NaHCO_3$.

The neutral fraction of the incubation of *ent*-19-hydroxy-13-*epi*-manoyl oxide (**6**) (275 mg) was chromatographed on silica gel. Elution with mixtures of petrol–EtOAc gave starting material (150 mg), *ent*-12α,19-dihydroxy-13-*epi*-manoyl oxide (**7**) (45 mg), and *ent*-7β,12α,19-trihydroxy-13-*epi*-manoyl oxide (**15**) (4 mg). The acidic fraction was methylated with CH_2N_2, but in the chromatography of the residue no acidic diterpenes were detected.

The incubation of *ent*-3β-hydroxy-13-*epi*-manoyl oxide (**16**) (320 mg) gave starting material (190 mg), *ent*-3β, 11α-dihydroxy-13-*epi*-manoyl oxide (**18**) (22 mg), *ent*-3β, 12α-dihydroxy-13-*epi*-manoyl oxide (varodiol) (**17**) (20 mg) and *ent*-3β, 11α, 12α-trihydroxy-13-*epi*-manoyl oxide (**19**) (15 mg). These compounds were separated by chromatography on silica gel of the neutral fraction, eluting with petrol–EtOAc mixtures. The acidic fraction did not contain diterpenoid metabolites.

ent-12α, 19-*Dihydroxy*-13-epi-*manoyl oxide* (**7**). Mp 175–178°. (Found: $[M-Me]^+$ at *m/z* 307.2239. $C_{19}H_{31}O_3$ requires $[M-Me]^+$ at 307.2273). ^1H NMR (200 MHz): δ 0.73, 0.96, 1.19 and 1.22 (each 3H, *s*), 3.44 and 3.67 (each 1H, *d*, *J* = 11 Hz, 19-H), 4.08 (1H, *br s*, 12-H), 4.95 and 4.97 (each 1H, *d*, *J* = 18 and 11 Hz, H-15), 6.06 (1H, *dd*, *J* = 18 and 11 Hz, H-14); MS *m/z* (rel. int.): 307 $[M-Me]^+$ (11), 289 (4), 279 (5), 271 (5), 221 (6), 208 (62), 178 (43), 107 (64).

ent-7β, 12α, 19-*Trihydroxy*-13-epi-*manoyl oxide* (**15**). Gum (Found: $[M]^+$ at *m/z* 338.2471. $C_{20}H_{34}O_4$ requires $[M]^+$ at 338.2457). ^1H NMR (200 MHz): δ 0.75, 0.98, 1.18 and 1.20 (each 3H, *s*), 3.45 and 3.67 (each 1H, *d*, *J* = 11 Hz, H-19), 3.55 (1H, *dd*, *J* = 11 and 4.5 Hz, H-7), 4.08 (1H, *br s*, H-12), 4.96 and 4.99 (each 1H, *d*, *J* = 18 and 11 Hz, H-15), 6.03 (1H, *dd*, *J* = 18 and 11 Hz, H-14); MS *m/z* (rel. int.): 338 $[M]^+$ (0.2), 323 (1), 287 (1), 267 (5), 250 (3), 231 (2), 224 (3), 207 (7), 189 (6), 163 (11), 123 (29), 109 (71).

Diacetate **8**. Acetylation of **7** with Ac_2O and pyridine in the usual way gave **8**, mp 122–123°. (Found: $[M-Me]^+$ at *m/z* 391.2487. $C_{23}H_{35}O_5$ requires $[M-Me]^+$ at 391.2485). ^1H NMR (200 MHz): δ 0.73, 0.96, 1.10 and 1.23 (each 3H, *s*), 2.04 and 2.13 (each 3H, *s*), 4.15 and 3.87 (each 1H, *d*, *J* = 11 Hz, H-19), 5.00 and 5.15 (each 1H, *d*, *J* = 18 and 11 Hz, H-15), 5.35 (1H, *br s*, H-12), 6.02 (1H, *dd*, *J* = 18 and 11 Hz, H-14); MS *m/z* (rel. int.): 391 $[M-Me]^+$ (1), 346 (1), 331 (3), 276 (3), 271 (2), 250 (20), 190 (78), 177 (68), 147 (15).

Partial hydrolysis of **8**. A soln of **8** (40 mg) in C_6H_6 (minimum quantity) was treated with 2% methanolic KOH (4 ml) leaving the mixt. at room temp. for 2 hr. Usual work-up and subsequent dry CC, eluting with petrol–EtOAc (23:2), gave starting material (4 mg), an unseparable mixture of the monoacetates **9** and **10** (16 mg), and the diol **7** (14 mg).

Chloration of **9** *and* **10**. The mixture of monoacetates (16 mg) and Ph_3P (40 mg) in CCl_4 (2 ml) and pyridine (0.5 ml) were heated under reflux for 12 hr. The ppt. was separated by filtration, and washed with EtOAc. The solvents were evapd and the residue chromatographed on silica, eluted with petrol–EtOAc (97:3), to afford impure 12-chloro derivative and ent-12α-*acetoxy*-19-*chloro*-13-epi-*manoyl oxide* (**12**) (9 mg), mp 137–139°. (Found: $[M-AcOH]^+$ at *m/z* 324.2007. $C_{20}H_{31}O^{37}Cl$ requires $[M-AcOH]^+$ at 324.2034. Found: $[M-AcOH]^+$ at *m/z* 322.2063. $C_{20}H_{31}O^{35}Cl$ requires $[M-AcOH]^+$ at 322.2063). ^1H NMR (200 MHz): δ 0.73, 1.04, 1.10 and 1.23 (each 3H, *s*), 2.14 (3H, *s*), 3.39 and 3.77 (each 1H, *d*, *J* = 11 Hz, 19-H), 5.00 and 5.06 (each 1H, *d*, *J* = 18 and 11.5 Hz, 15-H), 5.35 (1H, *br s*, 12-H), 6.02 (1H, *dd*, *J* = 18 and 11.5 Hz, 14-H); MS *m/z* (rel. int.): 324 $[M-AcOH]^+$ (0.5), 322 (1.5), 309 (1), 307 (3), 277 (7), 228 (18), 226 (51), 211 (22), 177 (100), 135 (22), 123 (38).

Reduction of **12**. The chloro derivative **12** (8 mg) in dry toluene (3 ml) was refluxed with tri-*n*-butyltin hydride (56 μl) and azobis-isobutyronitrile (traces) under N_2 for 7 hr. The solvent was evapd and the residue dissolved in Et_2O and stirred with water satd with KF for 15 min. The solution was filtered, dried and the solvent evapd to give a gum which was chromatographed on silica. Elution with petrol–EtOAc (49:1) gave ent-12α-*acetoxy*-13-epi-*manoyl oxide* (*varol acetate*) (**13**) (3 mg). ^1H NMR (200 MHz): δ 0.72, 0.79, 0.87, 1.11 and 1.25 (each 3H, *s*), 2.14 (3H, *s*), 5.00 and 5.06 (each 1H, *d*, *J* = 18 and 11 Hz, 15-H), 5.35 (1H, *t*, *J* = 3 Hz, 12-H), 6.04 (1H, *dd*, *J* = 18 and 11 Hz, 14-H); MS *m/z* (rel. int.): 333 $[M-Me]^+$ (1), 273 (4), 192 (96), 177 (89), 137 (18).

Varol (ent-12α-*hydroxy*-13-epi-*manoyl oxide*) (**14**). The alcohol **14** was obtained by hydrolysis of **13** (2 mg) with a saturated soln of Na_2CO_3 in MeOH (2 ml) at reflux for 2 hr. (Found: $[M-Me]^+$ at *m/z* 291.2336. $C_{19}H_{31}O_2$ requires $[M-Me]^+$ at 291.2324). ^1H NMR (200 MHz): δ 0.74, 0.79, 0.86, 1.20 and 1.24 (each 3H, *s*), 4.07 (1H, *br s*, 12-H), 4.96 and 4.98 (each 1H, *d*, *J* = 18 and 11 Hz, 15-H), 6.08 (1H, *dd*, *J* = 18 and 11 Hz, 14-H); MS *m/z* (rel. int.): 291 $[M-Me]^+$ (0.1), 273 (1), 192 (74), 177 (100), 137 (12).

ent-3β, 11α-*Dihydroxy*-13-epi-*manoyl oxide* (**18**). Mp 80–82°. (Found: $[M-Me]^+$ at *m/z* 307.2308. $C_{19}H_{31}O_3$ requires $[M-Me]^+$ at 307.2273). ^1H NMR (200 MHz): δ 0.78, 0.92, 0.99, 1.23 and 1.27 (each 3H, *s*), 3.24 (1H, *t*, *J* = 7 Hz, 3-H), 4.13 (1H, *m*, 11-H), 4.93 (1H, *dd*, *J* = 11.5 and 0.8 Hz), 5.08 (1H, *dd*, *J* = 18 and 0.8 Hz), 5.98 (1H, *dd*, *J* = 18 and 11 Hz); MS *m/z* (rel. int.): 307 $[M-Me]^+$ (25), 289 (12), 271 (13), 253 (6), 237 (5), 219 (4), 201 (17), 191 (5), 175 (10), 157 (11), 149 (13), 135 (49).

Varodiol (ent-3β, 12α-*dihydroxy*-13-epi-*manoyl oxide* (**17**). (Found: $[M-Me]^+$ at *m/z* 307.2269. $C_{19}H_{31}O_3$ requires $[M-Me]^+$ at 307.2273). ^1H NMR (200 MHz): 0.75 (6H, *s*), 0.97, 1.19 and 1.24 (each 3H, *s*), 3.21 (1H, *dd*, *J* = 10 and 5 Hz, 3-H), 4.08 (1H, *t*, *J* = 3 Hz, 12-H), 4.95 (1H, *d*, *J* = 18 Hz, H-15), 4.97 (1H, *d*, *J* = 11.5 Hz, 15-H), 6.07 (1H, *dd*, *J* = 18 and 11 Hz, 14-H);

MS *m/z* (rel. int.): 307 $[M]^+$ (1), 279 (4), 208 (23), 193 (10), 190 (63), 175 (74), 147 (38), 135 (19).

ent-3β, 11α, 12α-*Trihydroxy*-13-epi-*manoyl oxide* (**19**). (Found: $[M]^+$ at *m/z* 338.2380. $C_{20}H_{34}O_4$ requires $[M]^+$ at 338.2455). ^{1}H NMR (200 MHz): δ 0.78, 0.92, 0.99, 1.25 and 1.27 (each 3H, *s*), 3.24 (1H, *dd*, $J = 10$ and 6 Hz, 3-H), 4.00 (1H, *d*, $J = 4.5$ Hz, 12-H), 4.13 (1H, *dd*, $J = 9.4$ and 4.5 Hz, 11-H), 5.04 (1H, *dd*, $J = 11$ and 0.7 Hz, 15-H), 5.15 (1H, *d*, $J = 18$ Hz, 15-H), 6.04 (1H, *dd*, $J = 18$ and 11 Hz, 14-H); MS *m/z* (rel. int.): 338 $[M]^+$ (0.2), 323 (2), 307 (2), 289 (3), 268 (3), 250 (6), 191 (21), 175 (16), 135 (23).

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