PERGAMON

Phytochemistry 62 (2003) 67–70

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

The biotransformation of the diterpene 2β-hydroxy-*ent*-13-*epi*-manoyl oxide by *Gibberella fujikuroi*

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Received 19 June 2002; received in revised form 28 August 2002

Abstract

Incubation of the diterpene 2β-hydroxy-*ent*-13-*epi*-manoyl oxide with *Gibberella fujikuroi* afforded in good yield 2β,6β-dihydroxy-*ent*-13-*epi*-manoyl oxide, 2β,12β-dihydroxy-*ent*-13-*epi*-manoyl oxide and 2β,20-dihydroxy-*ent*-13-*epi*-manoyl oxide, confirming that although *ent*-13-*epi*-manoyl oxide is a final metabolite of a biosynthetic branch in this fungus, more polar derivatives of this compound can be transformed by this micro-organism.

Keywords: *Gibberella fujikuroi*; Diterpenes; 2β-Hydroxy-*ent*-13-*epi*-manoyl oxide

1. Introduction

The fungus *Gibberella fujikuroi* produces the gibberellins and kaurenolides, which are diterpenoids derived from *ent*-kaur-16-ene (MacMillan, 1997). In a previous work we have shown that although 13-*epi*-*ent*-manoyl oxide (**1**) is a final metabolite of a biosynthetic branch in this fungus (Cross et al., 1963), several of its derivatives, with greater polarity such as ribenol (**2**), 19-hydroxy-*ent*-13-*epi*-manoyl oxide (**4**) and ribenone (**3**) (Fraga et al., 1989, 1999), can be transformed by this micro-organism. In addition we have also shown in these works that in this type of compounds the oxidation at C-19, characteristic of the gibberellins, is inhibited. With the aim of confirming these earlier conclusions the microbiological transformation of 2β-hydroxy-*ent*-13-*epi*-manoyl oxide (**5**) with the fungus *G. fujikuroi* was carried out and the results are described herein.

2. Results and discussion

The substrate **5** was isolated for the first time from *Sideritis perfoliata* (Sezik et al., 1985) and later obtained by us from *S. nutans* (Fernández et al., 1986). The incubation with *G. fujikuroi* was carried out in the presence of AMO 1618, a product that inhibits the formation of *ent*-kaur-16-ene in this fungus, without influencing the post-kaurene metabolism, which facilitates the isolation of the new metabolites formed (Dennis et al., 1965; Cross and Myers, 1969). The fermentation flasks were harvested after six days, and the broth and mycelium extracts were combined. Chromatography of the extract gave the metabolites **6–8**.

The main metabolite obtained in this fermentation was **6**. In the high resolution MS the molecular ion was observed at 322.2516 in very low proportion (0.4%). However, the molecular formula was determined to be $C_{20}H_{34}O_3$. The ^{1}H NMR spectrum was similar to that of the substrate, with a new geminal proton to a hydroxyl group being observed at δ 4.11 (*t*, $J = 3.2$ Hz). The form of resonance was characteristic of equatorial protons at C-1(α), C-3(α), C-7(α) or C-12(α) in *ent*-13-*epi*-manoyl oxide (**1**), in which the three rings have a chair conformation. The presence of a 2β-hydroxyl group in **6** discarded the C-1 and C-3 positions, whilst the differences observed in the 16,17-double bond

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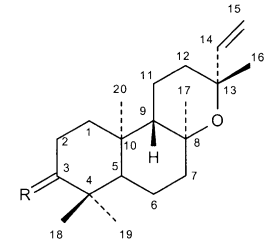
PII: S0031-9422(02)00436-3



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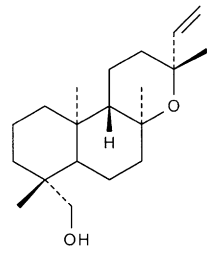
3 R = O



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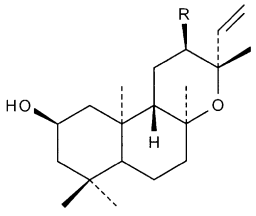

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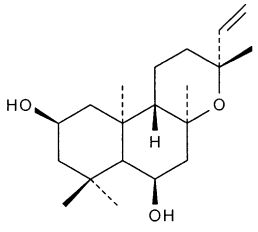
6 R = OH



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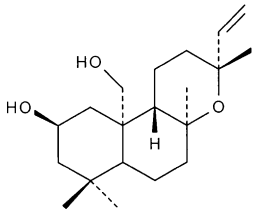

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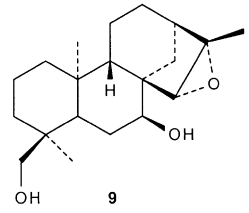

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resonance, in comparison with that of the substrate **5**, pointed to the C-12(β) position for the location of the hydroxyl group. This was confirmed by assignment of the proton and carbon NMR spectra using two-dimensional techniques (COSY, HMQC and HMBC). Therefore, the structure of this metabolite was determined as 2β,12β-dihydroxy-*ent*-13-*epi*-manoyl oxide (**6**).

Another compound obtained from this biotransformation was **7**. Its structure was given on the basis of the following considerations: The ion of the highest mass in the HRMS was at *m*/*z* 307.2274, which is formed from the molecular ion by loss of the C-16 methyl group, which is easily lost due to its location over a carbon bearing oxygen. Thus, the molecular formula of this metabolite was $C_{20}H_{34}O_3$. The new oxygen introduced in the molecule must be in the form of an alcoholic group, because in the ^{1}H NMR spectrum a new proton geminal to a hydroxyl group appears at δ 3.83 as a triple doublet with coupling constants of 11 and 3.8 Hz. The form of resonance indicated that this hydrogen must be located at C-6(α) or C-11(β), and consequently the corresponding alcohol at C-6(β) or C-11(α), respectively. The relatively low value of resonance of the corresponding new hydroxylated carbon at δ 68.8 was also characteristic of these positions. Finally, the C-6(β) location was chosen by assignment of its ^{1}H- and ^{13}C-NMR spectra using 2D-NMR techniques. Another characteristic of the 1H NMR spectrum of **7** was the resonance of the H-18 (methyl), which appears at δ 1.21 in comparison with δ 0.93 in the substrate. This difference in the chemical shifts was due to the interaction between the equatorial 18-methyl and the equatorial 6β-hydroxyl in **7**.

The compound obtained in the least amount was **8**. It was isomeric with the other two metabolites isolated from this feeding, $C_{20}H_{34}O_3$, but as in the case of **7** the molecular ion was not observed in the MS. A fragment at *m*/*z* 307 is produced by the loss of the C-16 methyl group. Its ^{1}H NMR, in comparison with that of the substrate, showed the disappearance of a methyl group and the new presence of a $-CH_2OH$ group, which resonates as a pair of doublets at δ 3.74 and 3.76 ($J = 11.6$ Hz). This indicated that a methyl group had been oxidized to a hydroxymethylene during the incubation. Since, in the biosynthetic route of gibberellin, ent-kaur-16-ene is first hydroxylated at C-19, it was logical to assume that our compound was also oxidized at this position. However, we have previously shown that in the biotransformation of *ent*-manoyl oxide derivatives by *G. fujikuroi* an inhibition of this oxidation takes place. In this way a study of the 2D-NMR data of this compound was carried out, which permitted us to assign its ^{1}H- and ^{13}C NMR spectra and to locate the new alcohol at C-20. In the HMBC spectrum, correlations were observed between H-18,C-19, H-19,C-18, H-16,C-13 and H-17,C-18, which indicated the presence in the molecule of the 16-,17-,18- and 19-methyl, and located the new hydroxyl group at C-20. This fact was confirmed by other correlations observed, such as for example, between H-1,C-20, H-20,C-9 and H-20,C-10. Thus, the structure 2β,20-dihydroxy-*ent*-13-*epi*-manoyl oxide (**8**) was given to this product.

Several conclusions can be deduced from this biotransformation:

1. The results confirmed that although *ent*-13-*epi*-manoyl oxide (**1**) is a final metabolite of a biosynthetic branch in *G. fujikuroi*, an increase in polarity, produced in **5** by a new hydroxyl group at C-2(β), can lead the fungus to transform this type of substrates.
2. In the incubation of compound **5** the oxidation at C-19 is inhibited. We have reported that the same occurs in the biotransformation of other three *ent*-13-*epi*-manoyl oxide derivatives (Fraga et al., 1989, 1999). We think that this was due to the presence in these substances of the oxygen atom in the ring C, considering that an atom of this type, situated on the α-face of ring D in *ent*-kaurane

derivatives, such as **9**, also inhibits the oxidation of C-19 (Fraga et al., 1992, 1993). In both cases, the substrates possess an oxygen atom in the same spatial region.

3. In the microbiological transformations of 2β-hydroxy-*ent*-13-*epi*-manoyl oxide (**4**) a preference exists for hydroxylation at C-12(β). The same occurred in previous work with ribenol (**1**) and ribenone (**2**), which have a 3α-OH and a 3-oxo substituent in ring A, respectively (Fraga et al., 1989, 1999).
4. The hydroxylation of **4** at C-20 was also interesting. Thus, considering that ribenol (**1**) and ribenone (**2**) were hydroxylated at C-11(β) and C-1(α),C-11(β), respectively, by *G. fujikuroi*, it is probable that the same enzyme was responsible for the functionalization of these three carbons, C-1(β), C-11(β) and C-20, situated relatively near in the same spatial region.

3. Experimental

3.1. General procedures

Mps were determined with a Reichert Thermovar apparatus and are uncorrected. ^{1}H NMR spectra were recorded in $CDCl_3$ solutions at 200.13 and 500.13 MHz, with a Bruker AC-200 or a Bruker AMX2–500 spectrometer, respectively. ^{13}C NMR spectra (Table 1) were run in $CDCl_3$ at 50.32 MHz, with a Bruker AC-200. Chemical shifts are given in ppm (δ). Mass spectra were taken at 70 eV (probe) in a Micromass Autospec spectrometer. Conformations of minimum energy were determined by computational methods employing the *Hyperchem* program from Hypercube. Dry column chromatographies were made on Si gel Merck 0.02–0.063 mm. The fungal strain was *Gibberella fujikuroi* IMI 58289. The substances were crystallised from petrol–EtOAc except where otherwise indicated.

3.2. Incubation procedure and isolation of products

G. fujikuroi, inhibited with 5×10^{-5} M AMO 1618, was grown in shake culture at 25 °C for 2 days in 40 conical flasks (250 ml), each containing a sterile medium (50 ml) (see Hanson et al., 1972). The main fermentation was inoculated with seed (1 ml) grown on the foregoing medium for 4–5 days. The substrate **5** (125 mg) in EtOH (7 ml) was distributed equally between the flasks and the incubation allowed to continue for a further 6 days. The broth was filtered, adjusted to pH 2 with dil HCl, and extracted with EtOAc. The extracts were separated into acidic and neutral fractions with $NaHCO_3$. The acidic fraction was methylated with diazomethane, but no metabolites were isolated therefrom. The neutral fraction was chromatographed on silica gel, eluting with petrol–EtOAc mixtures, giving starting material (73 mg), 2β,20-dihydroxy-*ent*-13-*epi*-manoyl oxide (**8**) (2 mg), 2β,6β-dihydroxy-*ent*-13-*epi*-manoyl oxide (**7**) (9 mg) and 2β,12β-dihydroxy-*ent*-13-*epi*-manoyl oxide (**6**) (22 mg).

3.3. 2β,12β-Dihydroxy-ent-13-epi-manoyl oxide (6)

Colourless prisms, mp: 184–186 °C; [M]+ at *m/z* 322.2516. $C_{20}H_{34}O_3$ requires 322.2508; 1H NMR (500 MHz): δ 0.76 (3H, *s*, H-20), 0.81 (3H, *s*, H-19), 0.82 (1H, *t*, *J* = 11.6, H-1β), 0.90 (3H, *s*, H-18), 1.01 (1H, *dd*, *J* = 12.4 and 2 Hz, H-5), 1.03 (1H, *t*, *J* = 12 Hz, H-3β), 1.16 (3H, *s*, H-16), 1.21 (3H, *s*, H-17), 1.26 (1H, *m*, H-6α), 1.40 (1H, *td*, *J* = 13 and 3.6 Hz, H-7β), 1.67 (3H, *m*, H-6β, H-9, and H-11α), 1.77 (2H, *m*, H-3α and H-7α), 1.83 (1H, *td*, *J* = 13.5 and 2.6 Hz, H-11β), 1.99 (1H, *br d*, *J* = 10 Hz, H-1α), 3.89 (1H, *br m*, H-2), 4.11 (1H, *t*, *J* = 3.2, H-12), 4.93 (1H, *d*, *J* = 18.2 Hz, H-15), 4.94 (1H, *d*, *J* = 11.3 Hz, H-15), 6.04 (1H, *dd*, *J* = 18.2 and 11.3 Hz, H-14); EIMS *m/z* (rel. int.): 322 [M]+ (0.4), 307 (2), 289 (1), 279 (23), 271 (4), 208 (23), 190 (100), 175 (88), 147 (45), 135 (11).

3.4. 2β,6β-Dihydroxy-ent-13-epi-manoyl oxide (7)

Colourless needles, mp: 156–158 °C; [M–CH_3]+ at *m/z* 307.2274. $C_{19}H_{31}O_3$ requires 307.2273; 1H NMR (500 MHz): δ 0.77 (1H, *t*, *J* = 11.5 Hz, H-1β), 0.79 (3H, *s*, H-20), 1.02 (3H, *s*, H-19), 1.03 (1H, *d*, *J* = 11 Hz, H-5), 1.12 (3H, *s*, H-16), 1.16 (1H, *t*, *J* = 12.6 Hz, H-3β), 1.21 (3H, *s*, H-18), 1.22 (1H, *d*, *J* = 10 Hz, H-9), 1.26 (3H, *s*, H-17), 1.45 (1H, *t*, *J* = 12 Hz, H-12α), 1.48 (1H, *t*, *J* = 11 Hz, H-7β), 1.69 (1H, *ddd*, *J* = 12, 4.4 and 2.3 Hz, H-3α),

Table 1
^{13}C NMR data of compounds **5–8**

Carbon	5	6	7	8
1	48.5	48.2	48.3	50.9
2	65.3	65.2	64.9	65.1
3	51.3	51.1	53.0	43.3
4	34.8	34.8	35.8	34.5
5	55.9	56.1	61.4	56.4
6	19.5	19.5	68.8	19.2
7	42.9	42.6	54.4	43.2
8	75.9	76.1	75.1	75.8
9	58.4	49.5	57.8	59.0
10	38.5	38.0	39.4	43.8
11	16.1	23.9	16.3	19.0
12	34.7	68.8	34.5	36.0
13	73.4	76.1	73.6	73.7
14	147.5	147.1	147.2	147.0
15	109.6	110.6	109.8	110.0
16	32.6	27.0	32.5	32.6
17	24.0	24.3	25.5	23.6
18	33.4	33.3	36.6	34.0
19	22.1	22.0	22.4	22.3
20	16.9	16.7	17.9	62.7

2.02 (1H, *ddd*, $J=12.1$, 4.4 and 2.3 Hz, H-1α), 2.11 (1H, *dt*, $J=11$ and 3.8, H-7α), 2.22 (1H, *dd*, $J=10.5$ and 3.5 Hz, H-12β), 3.83 (1H, *td*, $J=11$ and 3.8, H-6), 3.89 (1H, br m, H-2), 4.90 (1H, *d*, $J=11$ Hz, H-15), 4.96 (1H, *d*, $J=17.9$ Hz, H-15), 5.97 (1H, *dd*, $J=17.9$ and 11 Hz, H-14); EIMS *m*/*z* (rel. int.): $[M–CH_3]+$ (47), 289 (14), 271 (100), 253 (24), 206 (35), 201 (46), 199 (17), 191 (13), 177 (14), 173 (28), 163 (12), 159 (14).

3.5. 2β,20-Dihydroxy-ent-13-epi-manoyl oxide (**8**)

A gum; [M–CH3]+ at *m*/*z* 307.2263. $C_{19}H_{31}O_3$ requires 307.2273; ^{1}H NMR (500 MHz): δ 0.72 (1H, *t*, $J=11.6$ Hz, H-1), 0.72 (3H, *s*, H-19), 0.95 (3H, *s*, H-18), 1.14 (3H, *s*, H-16), 1.35 (3H, *s*, H-17), 2.20 (*dt*, $J=13$ and 2.9 Hz, H-12), 2.53 (1H, *dt*, $J=12$ and 2.4 Hz, H-1), 3.74 and 3.76 (each 1H, *d*, $J=11.6$ Hz, H-20), 4.02 (1H, br m, H-2), 4.93 (1H, *d*, $J=17.8$, H-15), 4.97 (1H, *d*, $J=11.1$; H-15), 6.01 (1H, *dd*, $J=17.8$ and 11.1, H-14); EIMS *m*/*z* (rel. int.): 307 $[M–CH_3]+$ (26), 204 (2), 289 (100), 271 (20), 259 (8), 241 (15), 201 (16), 189 (20), 187 (13), 175 (24), 173 (12), 161 (13).

Acknowledgements

This work has been supported by the SEUID, Ministry of Education and Culture, Spain (PB98-0540). S. Suárez thanks the University of La Laguna and Santander Bank for a fellowship.

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