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THE MICROBIOLOGICAL TRANSFORMATION OF SOME *ENT*-BEYERENE DITERPENOIDS BY *GIBBERELLA FUJIKUROI* TO BEYERGIBBERELLINS AND BEYERENOLIDES

CARMEN E. DIAZ, BRAULIO M. FRAGA, ANTONIO G. GONZALEZ, JAMES R. HANSON,* MELCHOR G. HERNANDEZ and AURELIO SAN MARTIN

Instituto de Productos Naturales Organicos del CSIC, Carretera La Esperanza 2, La Laguna, Tenerife, Spain; *School of Molecular Sciences, University of Sussex, Brighton BN1 9QJ, U.K.

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Key Word Index—*Gibberella fujikuroi*; gibberellins; *ent*-beyer-15-ene; beyergibberellins, beyerenolides

Abstract—The microbiological transformation by *Gibberella fujikuroi* of *ent*-beyer-15-ene into the beyergibberellins A_9 and A_{13}, 7β-hydroxy- and 7β,18-dihydroxybeyerenolides, and of *ent*-beyer-15-en-19-ol into beyergibberellins A_4, A_7, A_9, A_{13} and A_{25}, and 7β-hydroxy- and 7β,18-dihydroxybeyerenolides is described. In contrast, *ent*-beyer-15-en-18-ol gave *ent*-7α,18,19-trihydroxybeyer-15-ene, 7β,18-dihydroxybeyerenolide and *ent*-7α,18-dihydroxybeyer-15-en-19-oic acid again revealing the inhibitory effect of an 18-hydroxyl group on oxidative transformations at C-6β by *Gibberella fujikuroi*.

INTRODUCTION

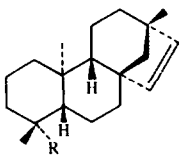
The gibberellin plant hormones are biosynthesized by the fungus *Gibberella fujikuroi* via the tetracyclic diterpenoid hydrocarbon, *ent*-kaurene [1]. Although there are several structurally similar families of polycyclic diterpenoid, all the naturally-occurring gibberellin plant hormones which have been isolated so far belong to this series. Amongst the tetracyclic diterpenoids, those with the *ent*-beyer-15-ene carbon skeleton are a large class second in number only to those with an *ent*-kaurenoid skeleton. Despite the fact that *ent*-beyer-15-ene diterpenoids are known with a hydroxylation pattern which is reminiscent of gibberellin biosynthetic intermediates, no beyergibberellins, i.e. gibberellins with the beyerene arrangement of rings C and D, have hitherto been isolated. Nevertheless, studies on gibberellin biosynthesis [2, 3] have not revealed any features which might preclude the formation of these compounds. In examining the skeletal requirements for gibberellin biosynthesis by *Gibberella fujikuroi*, we have shown that the 'wild-type' fungus will metabolize substrates with the trachylobane [4] and atiserene [5] skeleta to afford the corresponding trachyloba- and atisagibberellins. In this paper [6] we report on the transformation of the hydrocarbon, *ent*-beyer-15-ene (**1**), the 19-alcohol (**2**) and the corresponding 18-alcohol (**10**) [7, 8]. In these compounds the unsaturation on ring D is on the α-face of the molecule, in contrast to the kaurenes. This difference might affect the way in which the beyerenes bind to the oxidative enzymes and, hence, the hydroxylation pattern of the metabolites. The *ent*-beyer-15-en-18- and 19-alcohols which were required for this work were isolated from *Baccharis tola* [9]. The 19-alcohol was converted to the hydrocarbon by oxidation to the 19-aldehyde followed by Wolff–Kishner (Huang–Minlon) reduction. Previous studies [10] have shown that a mutant of *G. fujikuroi* can accept isosteviol (*ent*-16-oxobeyeran-19-oic acid) to afford some 8,13-isogibberellin 16-ketones.

RESULTS

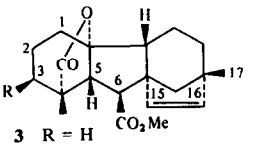
The fermentations were performed in the presence of the *ent*-kaur-16-ene biosynthesis inhibitor, AMO 1618, to suppress the formation of the normal metabolites and facilitate analysis of the products [11, 12]. The transformations were relatively inefficient although the incorporation of the natural substrate, *ent*-kaur-16-ene, into gibberellic acid is usually only of the order of a few per cent. The fermentations were harvested after 5–6 days and the metabolites were isolated. The acidic fractions were methylated with diazomethane and the resultant methyl esters separated chromatographically.

ent-Beyer-15-ene (**1**) gave the methyl esters of beyergibberellin A_9 (**3**) and beyergibberellin A_{13} (**4**) in the methylated acid fraction together with 7β-hydroxybeyerenolide (**5**) and 7β,18-dihydroxybeyerenolide (**6**) in the neutral fraction. *ent*-Beyer-15-en-19-ol (**2**) was examined in the hope that it might be more efficiently transformed. It gave beyergibberellins A_4 (**7**), A_9 (**3**), A_{13} (**4**) and A_{25} (**8**) which were isolated as their methyl esters. On one occasion the beyergibberellin A_4 fraction contained an inseparable amount of *ca* 20% beyergibberellin A_7 methyl ester (**9**). The beyerenolides **5** and **6** were again obtained from the neutral fraction. On the other hand, *ent*-beyer-15-en-18-ol (**10**) gave *ent*-7α,18,19-trihydroxybeyer-15-ene (**11**), the corresponding 19-acid (isolated as its methyl ester, **12**) and 7β,18-dihydroxybeyerenolide (**6**).

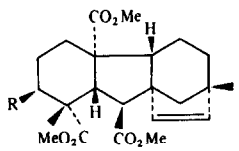
The metabolites were identified as follows. The ^{1}H NMR spectrum of the methyl ester of beyergibberellin A_4 (**7**) contained C–Me signals at δ1.08 and 1.25 together with the characteristic gibberellin AB double doublet (δ2.71 and 3.14, J = 6 Hz) assigned to H-5 and H-6 and a C<u>H</u>(OH) signal at δ3.85 (cf. gibberellin A_4, δ1.15, 2.71, 3.22 and 3.85) [13]. The ring D olefinic proton resonances appeared at δ5.45 and 5.50. The methyl ester of beyergibberellin A_9 (**3**) contained a similar pattern of signals with, as anticipated, the C-5 and C-18 proton resonances at



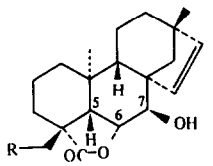
1 R = Me
2 R = CH_2OH



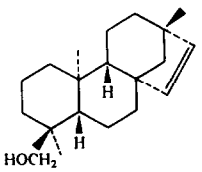
3 R = H
7 R = OH
9 R = OH, $\Delta^{1,2}$



4 R = OH
8 R = H



5 R = H
6 R = OH



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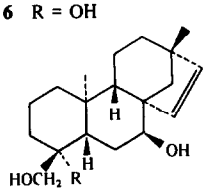

11 R = CH_2OH
12 R = CO_2Me

higher field (δ2.46 and 1.15, respectively). In both cases the metabolites were identical to material prepared synthetically [14] from gibberellic acid. A small amount of beyergibberellin A_7 methyl ester (**9**) was also present in the beyergibberellin A_4 fraction. It contained the typical ring A ^{1}H NMR olefinic signals [δ6.39 (*d*, J = 9 Hz, H-1), 5.90, (*dd*, J = 5 and 9 Hz, H-2)] whilst the H-3 resonance was at δ4.20 (cf. gibberellin A_7 methyl ester) [13]. The two C_{20} beyergibberellins were identified as beyergibberellins A_{13} (**4**) and A_{25} (**8**) on the basis of their mass spectra which showed fragmentation patterns comparable to the natural gibberellins [15, 16]. Their ^{1}H NMR spectra contained three methoxyl signals (δ3.60, 3.61 and 3.63 in **4**; 3.60, 3.63 and 3.68 in **8**) and the low field H-6 NMR signal (δ3.77) characteristic of C-20 gibberellins bearing ester groups at C-19 and C-20.

The hydroxy-γ-lactones 7β-hydroxybeyerenolide (**5**) (IR ν_{max} cm^{-1}: 3600, 1760) and 7β,18-dihydroxybeyerenolide (**6**) (IR ν_{max} cm^{-1}: 3600 and 1750) were also identified by their ^{1}H NMR spectra. 7β-Hydroxybeyerenolide possessed three C–Me resonances (δ0.76, 1.05 and 1.21) whilst 7β,18-dihydroxybeyerenolide showed two C–Me resonances (δ0.76 and 1.06) and a two-proton signal (δ3.65) attributed to a primary alcohol. Furthermore, these two compounds showed the resonances associated with the CHCH(OCO)CH(OH)C moiety on ring B [δ1.77, 4.66 and 4.17 ($J_{5,6}$ = 5 Hz, $J_{6,7}$ = 2 Hz)] similar to the kaurenolides [17]. However, the magnitude of the $J_{6,7}$ value is smaller, suggesting that ring B may exist in a conformation closer to that of a chair. Comparison of the position of the C–Me signals with the data for the kaurenolides and for beyerenes [9] shows that the dihydroxybeyerenolide (**6**) possessed, as anticipated, the primary alcohol at C-18.

The ^{1}H NMR spectrum of *ent*-7α,18,19-trihydroxybeyer-15-ene (**11**) contained signals attributed to two primary alcohols [AB doublets, $\delta_{pyridine}$ 4.00, 4.07 and 4.31 (2H, J = 11 Hz)] and a secondary alcohol (δ3.97). It readily gave a triacetate [δ_{CDCl_3} 2.01, 2.02, 2.03 (each 3H, 3 × OAc), δ3.85, 3.94, 4.00 and 4.27 (AB doublets, J = 11 Hz, 2 × CH_2OAc), 4.84 [*t*, J = 2 Hz, CH(OAc)]. This spectral data is comparable to that of *ent*-7α,18,19-trihydroxykaurene. Methyl *ent*-7α,18-dihydroxybeyer-15-en-19-oate showed the H-18 resonance at δ3.71 as a broad singlet (cf. methyl *ent*-7α,18-dihydroxykaur-16-en-19-oate, δ3.61) and that of H-7 at δ3.74. The Me-10 resonance was at a higher field than in the corresponding kaurene series due to the shielding effect of the 15-double bond.

DISCUSSION

These transformations further illustrate the ability of *G. fujikuroi* to metabolize other diterpenoid skeleta along the gibberellin pathway. Apart from making available novel gibberellins by analogue biosynthesis, it does suggest the possibility of finding beyergibberellins as natural products. By comparison with the natural series of *Gibberella* metabolites, there is a higher proportion of 3-desoxy metabolites. One implication of this, which has been observed with other 'false substrates', is that the 3-hydroxylation of gibberellin A_{12} 7-aldehyde is rather more structure specific than some of the other steps in the pathway. The inhibitory action of an 18-hydroxyl group on the ring-contraction to form gibberellins has been noted previously [2]. These results suggest that the position of the double bond on ring D does not have a major determining effect on biosynthetic transformations in *G. fujikuroi*.

EXPERIMENTAL

Incubation experiments Gibberella fujikuroi (ACC 917) inhibited with 5 × 10^{-5} M AMO 1618, was grown in shake culture at 25° for 1–2 days in 80–100 conical flasks (250 ml) each containing sterile medium (50 ml) [18]. The substrate (see below) in EtOH (16–20 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 extract was separated into acidic and neutral fractions with aq. $NaHCO_3$. The acidic fraction was methylated with CH_2N_2. The fractions were chromatographed on silica gel in petrol–EtOAc. *ent*-Beyer-15-ene (**1**) (280 mg) gave the methyl esters of beyergibberellins A_9 (**3**) (9 mg) and A_{13} (**4**) (8 mg) in the methylated acid fraction and 7β-hydroxybeyerenolide (**5**) (8 mg) and 7β,18-dihydroxybeyerenolide (**6**) (6 mg) in the neutral fraction. *ent*-Beyer-15-en-19-ol (**2**) (320 mg) gave the methyl esters of beyergibberellins A_9 (**3**) (11 mg), A_{25} (**8**) (4 mg), A_{13} (**4**) (5 mg) and A_4 (**7**) (10 mg) in the methylated acid fraction. The neutral fraction contained 7β-hydroxybeyerenolide (**5**) (6 mg) and 7β,18-dihydroxybeyerenolide (**6**) (4 mg) In another expt the beyergibberellin A_4 methyl ester contained (*ca* 20% by NMR) beyergibberellin A_7 methyl ester (**9**).

ent-Beyer-15-en-18-ol (**10**) (300 mg) gave the methyl ester of *ent*-7α,18-dihydroxybeyer-15-en-19-oic acid (**12**) (20 mg) in the methylated acid fraction. The neutral fraction contained the starting material (140 mg), 7β,18-dihydroxybeyerenolide (**6**) (22 mg) and *ent*-7α,18,19-trihydroxybeyer-15-ene (**11**) (17 mg).

Beyergibberellin A_9 methyl ester (**3**). Gum ([M]$^+$, 330.1831. Calc. for $C_{20}H_{26}O_4$, 330.1831); ^{1}H NMR (200 MHz, $CDCl_3$): δ1.06 (3H, *s*, H-17), 1.15 (3H, *s*, H-19), 2.46 and 2.73 (each 1H, *d*, J = 6 Hz, H-5 and H-6), 3.68 (3H, *s*, OMe), 5.44 and 5.47 (each 1H, *d*, J = 2 Hz); MS *m/z* (rel. int.): 330 [M]$^+$ (8), 298 (26), 284 (9), 270 (21), 243 (3), 225 (11).

Beyergibberellin A_{25} methyl ester (**8**). Gum ($[M]^+$, 404.2181. Calc. for $C_{23}H_{32}O_6$, 404.2196); ^{1}H NMR (200 MHz, $CDCl_3$): δ0.98 and 1.05 (each 3H, *s*), 2.27 and 3.77 (each 1H, *d*, J = 10 Hz, H-5 and H-6), 3.60, 3.63 and 3.68 (each 3H, *s*), 5.43 and 5.52 (each 1H, *d*, J = 5 Hz, H-15 and H-16); MS *m/z* (rel. int.): 404 $[M]^+$ (16), 372 (9), 312 (73), 284 (53), 225 (42).

Beyergibberellin A_{13} methyl ester (**4**). Gum ($[M]^+$, 420.2124. Calc. for $C_{23}H_{32}O_7$, 420.2148); MS *m/z* (rel. int.): 420 $[M]^+$ (1), 388 (7), 360 (2), 328 (7), 300 (6); ^{1}H NMR (200 MHz, $CDCl_3$): δ0.96, 1.18, 3.60, 3.61 and 3.63 (each 3H, *s*), 5.76 (2H, *br s*).

Beyergibberellin A_4 methyl ester (**7**). Gum ($[M]^+$, 346.1780. Calc. for $C_{20}H_{26}O_5$, 346.1779); ^{1}H NMR (360 MHz, $CDCl_3$): δ1.08 (3H, *s*, H-17), 1.25 (3H, *s*, H-19), 2.71 and 3.14 (each 1H, *d*, J = 6 Hz), 3.69 (3H, *s*, OMe), 3.88 (1H, *br s*, H-3), 5.45 and 5.50 (each 1H, *d*, J = 5 Hz, H-15 and H-16); MS *m/z* (rel. int.): 346 $[M]^+$ (6), 328 (3), 314 (24), 286 (27), 225 (29).

Beyergibberellin A_7 methyl ester (**9**) ^{1}H NMR (360 MHz, $CDCl_3$): δ1.09 and 1.24 (each 3H, *s*), 2.82 and 3.17 (each 1H, *d*, J = 6 Hz), 3.72 (3H, *s*, OMe), 4.20 (1H, *br d*, H-3), 5.45 and 5.50 (each 1H, *d*, J = 5 Hz, H-15 and H-16), 5.90 (1H, *q*, J = 5 and 9 Hz, H-2), 6.39 (1H, *d*, J = 9 Hz, H-1); MS *m/z* (rel. int.): 344 $[M]^+$, 326 (1), 312 (6), 298 (4), 284 (34).

ent-6β,7α-*Dihydroxybeyer-15-en-19-oic acid* 19-6β-*lactone* (7β-*hydroxybeyerenolide*) (**5**). Gum ($[M]^+$, 316.2021. Calc. for $C_{20}H_{28}O_3$, 316.2036); ^{1}H NMR (200 MHz, $CDCl_3$): δ0.76, 1.05 and 1.21 (each 3H, *s*), 1.77 (1H, *d*, J = 5 Hz, H-5), 4.17 (1H, *d*, J = 2 Hz, H-7), 4.66 (1H, *dd*, J = 2 and 5 Hz, H-6), 5.53 (2H, *s*, H-15 and H-16). Irradiation at δ1.77 collapsed the double-doublet at δ4.66 to a doublet. MS *m/z* (rel. int.): 316 $[M]^+$ (1), 298 (29), 283 (2), 255 (2), 239 (2), 165 (6), 159 (26), 137 (32), 131 (100).

ent-6β,7α,18-*Trihydroxybeyer-15-en-19-oic acid* 19-6β-*lactone* (7β,18-*dihydroxybeyerenolide*) (**6**). Gum ($[M-18]^+$, 314.1901. Calc. for $C_{20}H_{26}O_3$, 314.1882); ^{1}H NMR (200 MHz, $CDCl_3$): δ0.76 and 1.06 (each 3H, *s*), 1.76 (1H, *d*, J = 5 Hz, H-5), 3.65 (2H, *br s*, H-18), 4.30 (1H, *d*, J = 2 Hz, H-7), 4.73 (1H, *dd*, J = 2 and 5 Hz, H-6), 5.51 and 5.54 (each 1H, *d*, J = 4 Hz, H-15 and H-16); MS *m/z* (rel. int.): 332 $[M]^+$ (1), 314 (15), 296 (2), 271 (5), 254 (13), 239 (2), 181 (13), 149 (32), 131 (94).

ent-7α,18,19-*Trihydroxybeyer-15-ene* (**11**). Mp 235–238° ($[M-H_2O]^+$, 302.225. $C_{20}H_{30}O_2$ requires, 302.225); ^{1}H NMR (200 MHz, pyridine-d_5): δ0.90 and 1.05 (each 3H, *s*), 3.97 (1H, *br s*, H-7), 4.00 and 4.31 (each 1H, *d*, J = 11 Hz), 4.07 and 4.31 (each 1H, *d*, J = 11 Hz, H-18 and H-19), 5.57 and 5.70 (each 1H, *d*, J = 5 Hz, H-15 and H-16); MS *m/z* (rel int.): 320 $[M]^+$ (0.3), 302 (1), 284 (6), 272 (5), 271 (5), 254 (11), 253 (9), 241 (7), 225 (4), 206 (7), 167 (12), 149 (100) The triacetate, prepared with Ac_2O in pyridine was a gum ^{1}H NMR (200 MHz, $CDCl_3$): δ0.78 and 0.98 (each 3H, *s*), 2.01, 2.02 and 2.03 (each 3H, *s*), 3.85 and 3.94 (each 1H, *d*, J = 11 Hz), 4.00 and 4.27 (each 1H, *d*, J = 11 Hz), 4.84 (1H, *t*, J = 2 Hz), 5.53 and 5.58 (each 1H, *d*, J = 2 Hz); MS *m/z* (rel. int.): 404 $[M-42]^+$ (0.3), 3.86 (0.9), 358 (5), 326 (0.8), 266 (8), 253 (3).

ent-7α,18-*Dihydroxybeyer-15-en-19-oic acid methyl ester* (**12**). Mp 248–250° ($[M]^+$, 348.229. $C_{21}H_{32}O_4$ requires, 348.230); ^{1}H NMR (200 MHz, $CDCl_3$): δ0.58 and 1.03 (each 3H, *s*), 3.66 (3H, *s*), 3.71 (2H, *br s*, H-18), 3.74 (1H, *br s*, H-7), 5.51 (2H, *br s*, H-15 and H-16); MS *m/z* (rel. int.): 348 $[M]^+$ (0.1), 330 (1), 312 (20), 300 (13), 285 (3), 284 (2), 270 (8), 268 (5), 253 (3), 241 (7), 239 (4), 195 (3), 167 (5), 154 (9).

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