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THE BIOTRANSFORMATION OF SOME *ENT*-BEYERAN-19-OIC ACIDS BY *GIBBERELLA FUJIKUROI*

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Key Word Index—*Gibberella fujikuroi*; diterpenoid; beyeran-19-oic acids; gibberellins; biotransformation.

Abstract—The biotransformation of isostevic acid (*ent*-beyeran-19-oic acid), *ent*-16β-hydroxybeyeran-19-oic acid, and *ent*-16β-hydroxy-16α-methylbeyeran-19-oic acid by *Gibberella fujikuroi* has been shown to give ring A desoxybeyergibberellin analogues of GA_9, GA_{12} and GA_{25}. The *ent*-16β-hydroxybeyeran-19-oic acid also gave some hydroxylated beyeranes and the 7β-hydroxybeyeranolide.

INTRODUCTION

The flexibility of the gibberellin and kaurenolide biosynthetic pathways in the fungus *Gibberella fujikuroi* in transforming analogues of the natural substrates, has provided useful information on the stereochemical constraints of various enzymatic steps. This flexibility has extended to the biotransformation of tetracyclic diterpenoids possessing the beyerane skeleton [1, 2]. The transformation of isosteviol (**1**) [3] has been shown by GC-MS [1] to give the beyergibberellins, (**5–8**), together with the *ent*-7α-hydroxy (**2**) and *ent*-6α,7α-dihydroxy (**3**) derivatives and the beyeranolide (**4**). In a second study [2], incubation of *ent*-beyer-15-en-19-ol (**9**) gave the beyergibberellins A_4, A_7, A_9, A_{13} and A_{25}, (**10–14**), together with the 7β-hydroxy- (**15**) and 7β,18-dihydroxybeyerenolides (**16**). *seco*-Ring B metabolites related to fujenal were not detected in either study. Whereas in the first study [1], it was concluded that the structural changes in the C/D ring system had suppressed the 3-hydroxylation, this was clearly not the case in the second study [2]. The 3β-hydroxylation represents an important point at which the gibberellin biosynthetic pathways diverge in *Gibberella fujikuroi* [4]. Isosteviol (**1**) [3] is readily obtained from stevioside and hence we have been able to examine the biotransformation of some relatives with the object of shedding some further light on the effect of the structure of ring D on the biotransformations in *Gibberella fujikuroi*.

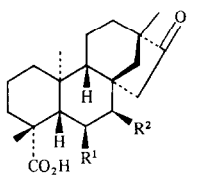
RESULTS AND DISCUSSION

In the biotransformation studies with *Gibberella fujikuroi*, the cultures were grown in the presence of chlorocholine chloride (CCC) which blocks *ent*-kaurene synthetase and thus limits the production of the endogenous metabolites [5]. This facilitated the detection and isolation of the metabolites of exogenous substrates. The crude fungal extracts obtained from the incubations were methylated and in some cases acetylated with acetic anhydride, to give material which was easier to separate and purify by chromatography.

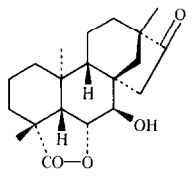
Isostevic acid (**17**) [3] (*ent*-beyeran-19-oic acid), obtained by the Wolff–Kishner reduction of isosteviol (**1**) was poorly metabolized and two metabolites were characterized as their methyl esters. The first was a gibberellin 19-10-γ-lactone (**18**) (ν_{max} 1767 cm^{-1}). Its ^1H NMR spectrum possessed only two –C(Me)– signals (δ1.01 and 1.13) and one methoxyl signal (δ3.71). The characteristic gibberellin H-5:H-6 doublets (δ2.45 and 2.59) possessed the smaller coupling constant ($J = 6.6$ Hz) typical of 8:13-isogibberellin 19-10-γ-lactones [6]. The second metabolite (**19**) also possessed only two –C(Me)– signals but had three methoxyl signals (δ3.59, 3.68 and 3.72) whilst the H-5:H-6 doublets (δ2.06 and 3.44, $J = 11.6$ Hz) were typical of a C_{20} gibberellin ester.

Incubation of the 16-alcohol (**20**) for five days gave an alcohol which was isolated as its methyl ester (**21**). Selective decoupling experiments based on irradiating the new CH(OH) signal (δ3.57) revealed that it was coupled ($J = 2.3$ and 3.0 Hz) to two protons (δ1.88 and 1.96). These were in turn geminally coupled ($J = 14$ Hz) and also coupled to a signal at δ2.18 ($J = 14$ and 3 Hz, respectively). The signals at δ2.18 and 1.96 showed NOE enhancements (2 and 8%, respectively) on irradiation of the H-18 signal (δ1.17) consistant with their assignment to H-5 and H-6β. Irradiation of the methyl signal (H-17) (δ0.95) gave NOE enhancements at δ3.88 (H-16) (8%) and δ1.32 (H-14) (2.5%). This latter signal was enhanced on irradiating both the CH(OH) signals (δ3.57 and 3.88). This can only be accommodated by the structure (**21**) with a C-7β hydroxyl group.

When the 16-alcohol (**20**) was incubated for a longer period of time (10 days) some further metabolites were isolated. In this experiment the products were separated by chromatography after methylation and acetylation. The first product to be eluted from the column was assigned the gibberellin structure (**24**). It was a dimethyl ester (δ3.63 and 3.69) and it possessed the characteristic H-5:H-6 doublets (δ1.89 and 3.30, $J = 11.6$ Hz) which were identified by decoupling studies. Irradiation of the H-20 signal (δ0.60) produced an NOE enhancement (8%)



	R^1	R^2
1	H	H
2	H	OH
3	OH	OH



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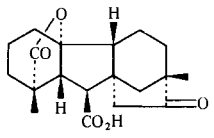

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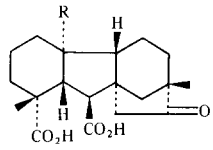
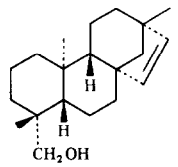


	R
6	Me
7	CHO
8	CO_2H



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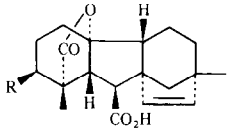
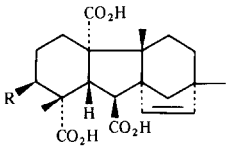
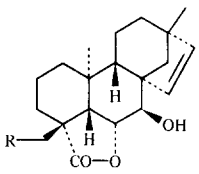


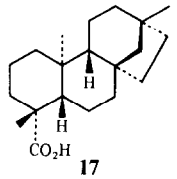
	R
10	OH
11	OH: $\Delta^{1,2}$
12	H



	R
13	OH
14	H



	R
15	H
16	OH



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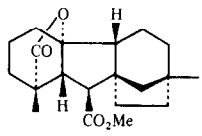

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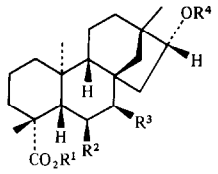
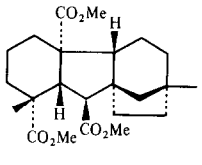


	R^1	R^2	R^3	R^4
20	H	H	H	H
21	Me	H	OH	H
22	Me	H	OAc	Ac
23	Me	OAc	OAc	Ac



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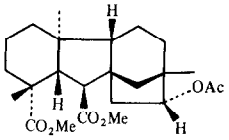

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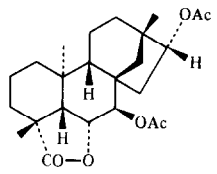

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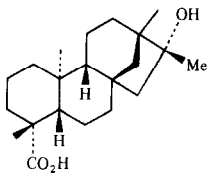

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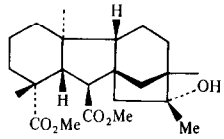

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of the H-6 resonance (δ3.30) whilst irradiation of the H-18 signal (δ1.02) produced NOE enhancements of both H-5 (δ1.89) (7%) and H-6 (6%). Irradiation at H-17 produced an 11% enhancement of the H-16 signal (δ4.64). The second product to be eluted from the column was identical to the diacetate (**22**) of the diol (**21**) which had been obtained previously. The third metabolite was isolated as the triacetate (**23**). ^{1}H NMR studies showed that it possessed a vicinal diacetate (δ4.92 and 5.28), one component (δ5.28) of which experienced a significant NOE enhancement (13%) on irradiation of H-20 (δ0.71). Irradiation of H-17 (δ0.83) led to an NOE enhancement of the H-16 signal (δ4.66, 10%). The final metabolite was assigned the beyeranolide structure (**25**). It possessed an IR absorption at 1769 cm^{-1} characteristic of a γ-lactone. ^{1}H NMR decoupling studies established the H-5 : H-6 : H-7 sequence (δ1.75, 4.60 and 5.38; $J_{5,6} = 6$ Hz, $J_{6,7} = 2.7$ Hz). Irradiation at δ1.27 (H-18) produced significant NOE enhancements of the H-5 (9%) and H-6 (12%) resonances whilst irradiation at δ0.94 (H-17) produced an NOE enhancement (10%) at H-16 (δ4.72). There was an NOE enhancement (9%) of the H-7 signal (δ5.38) on irradiation of H-20 (δ0.86) showing that ring B of this beyeranolide, like the kaurenolides [7], exists in a twisted boat conformation.

The related tertiary alcohol (**26**) which was obtained by the addition of methyl lithium to isosteviol (**1**), was poorly metabolized and only one metabolite could be purified as its methyl ester by column and thin layer chromatography. This was assigned the gibberellin structure (**27**) on the basis of its ^{1}H NMR spectrum which revealed the presence of four C–Me groups (δ0.60, 0.81, 1.00 and 1.16), two methoxyl groups (δ3.62 and 3.67) and the characteristic 5-H : 6-H doublets (δ1.86 and 3.22, $J = 11.6$ Hz).

In conclusion the gibberellin metabolites that were obtained from these bio-transformations were those related to the ring A desoxy gibberellins A_{12}, A_{25} and A_9. This suggests that it is the modification of ring D in this series which leads to C-15 being an sp^3 centre which makes the substrates a poor fit for the 3β-hydroxylase and thus prevents the formation of the ring A hydroxylated metabolites. Once C-7 of gibberellin A_{12} 7-aldehyde has been oxidized from an aldehyde to a carboxylic acid, there is little crossover [8] between the 3-desoxy and 3-hydroxy pathways. Although both 7β-hydroxybeyeranolides and 6β,7β-dihydroxylated beyeranes were formed, there was also an absence of fujenal-like ring B seco metabolites and 18-hydroxylated beyeranolides. However, hydroxylation at C-18 occurred with the beyer-15-enolide [2].

EXPERIMENTAL

General experimental details have been described previously [9]. ^{1}H NMR spectra were determined in $CDCl_3$ unless otherwise stated whilst IR spectra were determined as nujol mulls. *Gibberella fujikuroi* was cultured as described previously [10]. Metabolites were detected by comparison by TLC with control fermentations grown in parallel. Isostevic acid (**17**), prepared by the method of ref. [3], had mp 182–184° (ref. [3] 183–187°).

Preparation of ent-16*β-hydroxybeyeran*-19*-oic acid* (**20**). Isosteviol (600 mg) in MeOH (50 ml) was treated with $NaBH_4$ (200 mg) for 2 hr at room temp. The soln was diluted with 10% aq. HCl. The MeOH was evapd and the product recovered in EtOAc. The extract was washed with H_2O and dried over Na_2SO_4. The solvent was evapd to give *ent*-16*β*-hydroxybeyeran-19-oic acid (**20**) (560 mg), needles, mp 193°. (Found: C, 72.9; H, 10.2. $C_{20}H_{32}O_3$. 0.5 H_2O requires C, 73.35; H, 10.2%). IR ν_{max} cm^{-1}: 3470–3250 (br), 1703; ^{1}H NMR (CD_3OD): δ0.85 (3H, *s*, H-20), 0.89 (3H, , H-17), 1.16 (3H, *s*, H-18), 3.79 (1H, *dd*, J = 10.7 and 4.7 Hz, H-16). The methyl ester had mp 170–171°. (Found: C, 75.4; H, 10.4. $C_{21}H_{34}O_3$ requires C, 75.4; H, 10.2%). IR ν_{max} cm^{-1}: 3520, 1730; ^{1}H NMR: δ0.71 (3H, *s*, H-20), 0.90 (3H, *s*, H-17), 1.16 (3H, *s*, H-18), 3.62 (3H, *s*, OMe), 3.85 (1H, *dd*, J = 10.6 and 4.7 Hz, H-16).

Preparation of ent-16*β-hydroxy*-16*α-methylbeyeran*-19*-oic acid* (**26**). Isosteviol (600 mg) in dry THF (40 ml) was treated with methyl lithium (1.6 M, 3.5 ml) in Et_2O at −5° under N_2 for 20 min. The soln was poured into 10% NH_4Cl (30 ml) and the THF evapd. The product was recovered in CH_2Cl_2, washed with H_2O, and dried. The solvent was evapd to give a gum which was chromatographed on silica gel. Elution with EtOAc gave *ent*-16*β*-hydroxy-16*α*-methylbeyeran-19-oic acid (**26**) (550 mg) as needles, mp 254–256°. (Found: C, 73.1; H, 10.3. $C_{21}H_{34}O_3$. 0.5 H_2O requires C, 73.4; H, 10.3%). IR ν_{max} cm^{-1}: 3468 (br), 1700; ^{1}H NMR (C_5D_5N): δ0.95 (3H, *s*, H-20), 1.15, 1.33 and 1.36 (each 3H, *s*, H-17, H-18 and H-21). The methyl ester, had mp 184–185°. (Found: C, 75.8; H, 10.5. $C_{22}H_{36}O_3$ requires C, 75.8; H, 10.4%). IR ν_{max} cm^{-1}: 3393, 1704; ^{1}H NMR: δ0.72 (3H, *s*, H-20), 0.80 (3H, *s*, H-18), 1.16 and 1.17 (each 3H, *s*, H-17 and H-21), 3.61 (3H, *s*, OMe).

Incubation of isostevic acid (**17**). Isostevic acid (**17**) (240 mg) in EtOH (12 ml) was incubated with *G. fujikuroi* (2.4 l) containing CCC (50 mg l^{-1}) on shake culture for 14 days. The metabolites were recovered in EtOAc and methylated with CH_2N_2. The product was chromatographed on silica gel. Elution with 5% EtOAc–petrol gave *ent*-20-norbeyergibberell-7,19-dioic acid 19-10-lactone 7-methyl ester (**18**) (5 mg) as a gum. IR ν_{max} cm^{-1}: 1767, 1728; ^{1}H NMR: δ1.01 (3H, *s*, H-17), 1.13 (3H, *s*, H-18), 2.45 (1H, *d*, J = 6.6 Hz, H-6), 2.59 (1H, *d*, J = 6.6 Hz, H-5), 3.71 (3H, *s*, OMe); MS *m/z*: 288 $[M-CO_2]^+$, 272, 259, 229, 199. Further elution gave *ent*-beyergibberell-7,19,20,-trioic acid 7,19,20-trimethyl ester (**19**) (10 mg) as a gum. (Found: M, 406.237. $C_{23}H_{34}O_6$ requires M, 406.235). IR ν_{max} cm^{-1}: 1727; ^{1}H NMR: δ0.95 (3H, *s*, H-17), 1.09 (3H, *s*, H-18), 2.06 (1H, *d*, J = 11.6 Hz, H-5), 3.44 (1H, *d*, J = 11.6 Hz, H-6), 3.59, 3.68 and 3.72 (each 3H, *s*, OMe).

Incubation of ent-16*β-hydroxybeyeran*-19*-oic acid* (**20**). (a) The acid (**20**) (300 mg) in EtOH (10 ml) was incubated with *G. fujikuroi* (2 l) on shake culture for 5 days. The metabolites were recovered in EtOAc, methylated with CH_2N_2 and chromatographed on silica gel. Elution with EtOAc–petrol (2:3) gave the methyl ester of the substrate (76 mg) followed by methyl *ent*-7*α*,16*β*-dihydroxybeyeran-19-oate (**21**) (7 mg) as a gum. IR ν_{max} cm^{-1}: 3515, 1728; ^{1}H NMR: δ0.73 (3H, *s*, H-20), 0.95 (3H, *s*, H-17), 1.17 (3H, *s*, H-18), 1.32 (H-14), 1.88 (1H, *td*, J = 14.0, 14.0 and 2.3 Hz, H-6*α*), 1.96 (1H, *dt*, J = 14.0, 3.0 and 3.0 Hz, H-6*β*), 2.18 (1H, *dd*, J = 14 and 3 Hz, H-5), 3.57 (1H, *dd*, J = 2.3 and 3.0 Hz, H-7) 3.63 (3H, *s*, OMe), 3.88 (1H, *dd*, J = 10.6 and 4.8 Hz, H-16); MS *m/z*: 350 $[M]^+$, 332, 314, 301, 273.

(b) *ent*-16*β*-Hydroxybeyeran-19-oic acid (**20**) (550 mg) in EtOH (11 ml) was incubated with *G. fujikuroi* (4.4 l) on shake culture for 10 days. The metabolites were recovered in EtOAc, methylated with CH_2N_2 and chromatographed on silica gel. Elution with 5% EtOAc–petrol gave the methyl ester of the starting material (260 mg). Further elution gave a mixture of compounds which were combined and treated with Ac_2O (3 ml) in pyridine (6 ml) overnight. The soln, was poured into H_2O, recovered in EtOAc and the extract washed with aq. $CuSO_4$, H_2O and dried over Na_2SO_4. The solvent was evapd and the residue chromatographed on silica gel. Elution with 1% EtOAc–petrol gave *ent*-16*β*-acetoxybeyergibberell-7,19-dioic acid 7,19-dimethyl ester (**24**) (20 mg) as a gum. (Found: M, 420.250. $C_{24}H_{36}O_6$ requires M, 420.251). IR ν_{max} cm^{-1}: 1734; ^{1}H NMR: δ0.60 (3H, *s*, H-20), 0.93 (3H, *s*, H-17), 1.02 (3H, *s*, H-18), 1.89 (1H, *d*, J = 11.6 Hz, H-5), 3.30 (1H, *d*, J = 11.6 Hz, H-6), 3.63 and 3.69 (each 3H, *s*, OMe), 4.64 (1H, *dd*, J = 11.0, 4.2 Hz, H-16). Further elution with 4% EtOAc–petrol gave *ent*-7*α*,16*β*-diacetoxybeyeran-19-oic acid 19-methyl ester (**22**) (3 mg) as a gum. IR ν_{max} cm^{-1}: 1733; ^{1}H NMR: δ0.70 (3H, *s*, H-20), 0.92 (3H, *s*, H-17), 1.08 (3H, *s*, H-18), 2.06 and 2.07 (each 3H, *s*, OAc), 3.61 (3H, *s*, OMe), 4.71 (1H, *dd*, J = 10 and 4.2 Hz, H-16), 4.79 (1H, *dd*, J = 3 and 2.3 Hz, H-7). The product was identical (^{1}H NMR) to that obtained by acetylation of **21**. Elution with 7% EtOAc–petrol gave *ent*-6*α*,7*α*,16*β*-triacetoxybeyeran-19-oic acid 19-methyl ester (**23**) (80 mg) which crystallized as prisms, mp 122°. (Found: C, 65.65; H, 8.15. $C_{27}H_{40}O_8$ requires C, 65.8; H, 8.2%). IR ν_{max} cm^{-1}: 1739; ^{1}H NMR: δ0.71 (3H, *s*, H-20), 0.83 (3H, *s*, H-17), 1.20 (3H, *s*, H-18), 1.87, 1.98 and 2.02 (each 3H, *s*, OAc), 3.51 (3H, *s*, OMe), 4.66 (1H, *dd*, J = 10 and 4.2 Hz, H-16), 4.92 (1H, *d*, J = 2 Hz, H-7), 5.28 (1H, *dd*, J = 11 and 2 Hz, H-6). Elution with 10% EtOAc–petrol gave *ent*-7*α*,16*β*-diacetoxy-6*β*-hydroxybeyeran-19-oic acid 19→6-lactone (**25**) (55 mg) which crystallized as prisms, mp 196°. (Found: C, 67.6; H, 8.0. $C_{24}H_{34}O_6$. 0.5 H_2O requires C, 67.4; H, 8.25%). IR ν_{max} cm^{-1}: 1769, 1745; ^{1}H NMR: δ0.86 (3H, *s*, H-20), 0.94 (3H, *s*, H-17), 1.27 (3H, *s*, H-18), 1.75 (1H, *d*, J = 6 Hz, H-5), 2.05 and 2.12 (each 3H, *s*, OAc), 2.36 (1H, *dd*, J = 14.9 and 10.3 Hz, H-15), 4.60 (1H, *dd*, J = 6.0 and 2.7 Hz, H-6), 4.72 (1H, *dd*, J = 10.3 and 4.4 Hz, H-16), 5.38 (1H, *d*, J = 2.7 Hz, H-7).

Incubation of ent-16*β-hydroxy*-16*α-methylbeyeran*-19*-oic acid* (**26**). The acid (**26**) (500 mg) in EtOH–DMSO (2:1, 25 ml) was incubated with *G. fujikuroi* (5 l) for 14 days. The metabolites were recovered in EtOAc, methylated with CH_2N_2, and chromatographed on silica gel. Elution with 5–7% EtOAc–petrol gave the methyl ester of the starting material (270 mg), identified by its ^{1}H NMR spectrum. Further elution with 8% EtOAc–petrol gave a mixture which was further purified by TLC on silica gel in EtOAc–petrol (1:1) to give *ent*-16*β*-hydroxy-16*α*-methylbeyergibberell-7,19-dioic acid 7,19-dimethyl ester (**27**) (25 mg) as a gum. (Found: M 392.256. $C_{23}H_{36}O_5$ requires M 392.256). IR ν_{max} cm^{-1}: 3398, 1739; ^{1}H NMR: δ0.60 (3H, *s*, H-20), 0.81 (3H, *s*, H-18), 1.00 (3H, *s*, H-17), 1.16 (3H, *s*, H-21), 1.86 (1H, *d*, J = 11.6 Hz, H-5), 3.22 (1H, *d*, J = 11.6 Hz, H-6), 3.62 and 3.67 (each 3H, *s*, OMe).

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