BIO-TRANSFORMATION OF GIBBERELLIN 20,19-LACTONES BY *RHIZOPUS STOLONIFER*

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Abstract—In a study of the stereo-electronic requirements for microbiological hydroxylation by *Rhizopus stolonifer*, *ent*-19-hydroxygibberell-16-en-20-oic acid 20,19-lactone, *ent*-7,19-dihydroxygibberell-16-en-20-oic acid 20,19-lactone and *ent*-19-hydroxygibberell-16-en-7,20-dioic acid 20,19-lactone 7-methyl ester are shown to be hydroxylated at the 15 α -position.

INTRODUCTION

Some directing effects in the microbiological hydroxylation of steroids have been rationalized [1-4] in terms of hydroxylation within the general framework of a triangular site arrangement (1) in which the directing group occupied one of the apices and the hydroxyl groups were introduced at the other positions. This framework was constructed in terms of the steroids in which the directing group, commonly a carbonyl group, occupied a skeletal position. The steroids that were used, typically 5 α -androstanes, were relatively flat molecules and thus there were limitations to the extent to which the three-dimensional structure of the active site of the hydroxylase could be mapped. The tetracyclic diterpenoids are rigid molecules which, whilst bearing some resemblance to the steroids, possess functionality on pendant groups attached to the carbon skeleton. Thus they allow a greater opportunity for the molecule to rotate within the active site and to provide a three-dimensional mapping. There have been a number of studies using various tetracyclic diterpenoids [5] but hitherto no systematic pattern has emerged. The availability of some lactones from gibberellin A₁₃ (2) [6, 7] enabled us to make some systematic studies with these compounds as substrates with an organism, *Rhizopus stolonifer* (*niqricans*) which has been widely used in microbiological hydroxylations.

RESULTS AND DISCUSSION

The substrates 3, 10, 12 and 14 were prepared from gibberellin A₁₃ [7]. Gibberellin A₁₃ trimethyl ester was converted to gibberellin A₁₃ trimethyl ester by hydrogenolysis of the 3-methyldithiocarbonate. This trimethyl ester was then reduced with lithium aluminium hydride in tetrahydrofuran to afford the δ -lactone (10). The other

substrates 3, 12 and 14, respectively, were obtained from the δ -lactone-7-alcohol by (i) hydrogenolysis of the corresponding 7-alkyl chloride, (ii) oxidation to the 7-carboxylic acid and methylation with diazomethane and (iii) catalytic reduction of the 16-ene.

In this series the possible directing groups are on pendant groups attached to the carbon skeleton. The results of the biotransformation by *R. stolonifer* are shown in Scheme 1. Some of the metabolites were difficult to separate and were purified as their acetates. There were no detectable metabolites of the 7-carboxylic acid obtained during the preparation of the 7-methyl ester (12). The location of the hydroxyl group at C-15 in the metabolites 5, 11 and 13 was established by changes in the ¹³C NMR spectra (see Table 1). In particular the ¹³C NMR signals assigned to C-15 and the adjacent C-8 and C-16 had moved downfield whilst C-13 and C-14 showed γ -shielding effects. The stereochemistry of the hydroxyl group at C-15 was confirmed by oxidation of the 7-alcohol (10) and the 7-ester (12) with hydrogen peroxide and selenium dioxide. This oxidation is known [8] to give the 15 α -alcohols. In both cases this afforded a 15 α -alcohol identical to the biotransformation product.

There was some additional hydroxylation of the double bond by *R. stolonifer* in the case of the 7-desoxy-20,19-lactone (3). The structure of these products followed from their ¹H and ¹³C NMR spectra. The alkene ¹³C

Scheme 1.

In the presence of the 16-ene hydroxylation takes place at the allylic 15 α -position. This contrasts with a previous observation by Bearder *et al.* [9] that hydroxylation of gibberellin A₉ methyl ester by this organism takes place at the 15 β -position. Examination of molecular models shows that by placing the lactone carbonyl (C-20) over the C-3 carbonyl of a steroid model, C-15 and C-16 of the gibberellins lie relatively close to C-11 of the steroids, a typical site of hydroxylation by this organism. The isolation of the diol (6) and the 15,16-epoxy-17-alcohol (8) from the transformation of the 16-ene (3) and the 16-alcohol (20) from the transformation of the dihydro-compound (14) is interesting. Firstly, this represents a modification of the general observation [10] that an epoxide may be formed from the biotransformation of an alkene where the corresponding sp³ centre undergoes biohydroxylation. Secondly, the isolation of the 15,16-epoxy-17-alcohol (8) which could be formed via the 15 α -alcohol (4) might suggest that the microbial oxidation of a double bond is a two-step process involving electron-abstraction and then carbon-oxygen bond formation. Hydroxylation at the 16-position [11] and epoxidation of a 16-ene [12] have been observed in the biotransformation of some *ent*-kauranols and *ent*-kaurenes by *Rhizopus nigricans*.

EXPERIMENTAL

Fermentation details. *Rhizopus stolonifer* (IMI 57761) was maintained on malt agar slopes. The incubations were carried out in shake culture at 25° in 250 ml conical flasks (100 ml medium per flask) using the following medium (per l): glucose (20 g), peptone (5 g), yeast extract (3 g), potassium dihydrogen phosphate (5 g). The incubations were carried out as follows: (a) *ent*-19-hyd-

Table 1. ^{13}C NMR signals of the gibberellin 20-19 lactones

C	Compounds									
	5	6*	7	9	10	11	12	13	14	19
1	40.8	40.8	40.9	41.0	40.9	40.8	40.4	40.5	40.7	40.8
2	21.3	22.5	21.3	20.8	21.4	21.3	21.1	21.1	21.4	21.3
3	38.4*	39.1	39.4	37.8	38.0	38.3	37.3	37.4	38.1	38.2
4	33.7	34.8	33.8	33.7	33.7	33.8	33.0	33.3	33.7	33.7
5	51.4	56.3	55.0	51.6	51.8	51.8	54.5*	52.2	52.0	53.2
6	38.0	45.8*	45.5*	37.8	46.4	48.1	50.2*	49.0	46.9	43.7
7	19.5	20.7	20.3	19.1	64.3	65.3	175.1	177.0	64.5	66.2
8	54.3*	52.5*	50.6	51.8*	50.8*	56.3	51.4*	57.2	50.6*	51.3*
9	60.0	60.9	59.9	59.0	54.9	53.7	54.7	56.3	56.8	55.8
10	51.0*	51.9*	51.4	51.6*	50.5*	50.6	51.2*	51.7	50.3*	50.4*
11	17.5	18.1	17.0	16.9	17.3	17.5	17.1	17.2	17.0	16.7
12	30.9	24.7	23.6	22.6	30.9	30.6	30.9	30.6	23.9	29.7
13	39.5	40.1*	38.8*	34.5	41.6	39.5	41.7	39.8	38.4	48.0
14	38.5*	41.8	38.2	32.1	41.4	38.4	42.9	39.2	40.6	40.8
15	30.4	46.6	46.1	60.4	42.8	80.4	49.0	79.2	43.9	50.2
16	155.1	82.9	80.1	62.0	155.0	159.7	153.4	158.6	34.0	79.5
17	114.9	67.6	69.1	62.2	105.1	109.8	106.0	110.6	16.5	25.5
18	24.0	24.4	24.1	24.4	24.2	24.1	23.2	23.0	23.9	24.0
19	75.3	77.1	75.6	75.9	75.5	75.3	75.6	75.6	75.3	75.2
20	175.4	179.4	176.0	175.1	176.3	175.5	175.1	175.1	176.3	176.2
OMe	21.7		20.9	21.2			51.7	51.9		
OAc	170.4		171.2	170.9						

^a These assignments may be interchanged.^b Determined in MeOD.

roxygibberell-16-en-20-oic acid 20-19-lactone (3) (600 mg) in EtOH (30 ml) was evenly distributed between 60 flasks of *R. stolonifer* and incubated on shake culture for 10 days. The broth was filtered and the metabolites recovered in EtOAc and chromatographed on silica gel. Elution with 10% EtOAc-petrol gave unchanged substrate (130 mg) followed by a mixt. of 2 compounds (vide infra). Elution with 50% EtOAc-petrol gave *ent*-16,17,19-trihydroxygibberell-20-oic acid 20,19-lactone (6) (66 mg) which crystallized from MeOH as prisms, mp 195°. (Found: C, 70.3; H, 8.9. $\text{C}_{20}\text{H}_{30}\text{O}_4 \cdot 0.5 \text{H}_2\text{O}$ requires C, 69.9; H, 9.1%). IR ν_{max} cm^{-1} : 3388, 1718. ^1H NMR (CD_3OD): δ 0.95 (3H, s, H-18), 1.03 (3H, d, $J = 6.8$ Hz, H-7), 3.63 and 3.74 (each 1H, d, $J = 11.3$ Hz, H-17), 4.11 (1H, dd, $J = 11.6$ and 1.6 Hz) and 4.38 (1H, dd, $J = 11.6$ and 1.8 Hz) (each H-19). The acetate (7), prepared with Ac_2O -pyridine, crystallized from EtOAc as needles, mp 158°. (Found: C, 66.3; H, 8.3. $\text{C}_{22}\text{H}_{32}\text{O}_5 \cdot \text{H}_2\text{O}$ requires C, 66.9; H, 8.7%). IR ν_{max} cm^{-1} : 3301, 1717, 1659. ^1H NMR (500 MHz): δ 0.94 (3H, s, H-18), 1.02 (3H, d, $J = 6.8$ Hz, H-7), 2.12 (3H, s, OAc), 4.07 (1H, dd, $J = 11.5$ and 1.7 Hz) and 4.32 (1H, dd, $J = 11.5$ and 2.3 Hz) (each H-19), 4.20 and 4.30 (each 1H, d, $J = 11.2$ Hz, H-17). The mixt. described above was acetylated with Ac_2O in pyridine at room temp. overnight and chromatographed on silica gel. Elution with 5% EtOAc-petrol gave *ent*-15 β -acetoxyl-19-hydroxygibberell-16-en-20-oic acid 20,19-lactone (5) (20 mg) which crystallized from EtOAc as needles, mp 168°. (Found: C, 73.9; H, 8.6. $\text{C}_{22}\text{H}_{30}\text{O}_4$ requires C, 73.7; H, 8.4%). IR ν_{max} cm^{-1} : 1736, 1718. ^1H NMR (500 MHz): δ 0.88 (3H, s, H-18), 0.97 (3H, d, $J = 6.7$ Hz, H-7), 1.97 (3H,

s, OAc), 2.60 (1H, br s, H-13), 4.01 (1H, dd, $J = 11.5$ and 1.8 Hz) and 4.25 (1H, dd, $J = 11.5$ and 2.2 Hz) (each H-19), 5.00 and 5.32 (each 1H, s, H-17) and 5.37 (1H, br s, H-15). Further elution afforded *ent*-17-acetoxyl-15 β ,16 β -epoxy-19-hydroxygibberell-20-oic acid 20,19-lactone (9) (15 mg) as a gum. (Found: M 374.210 $\text{C}_{22}\text{H}_{30}\text{O}_5$ requires M 374.209.) IR ν_{max} cm^{-1} : 1723, 1151. ^1H NMR (500 MHz): δ 0.97 (3H, s, H-18), 1.24 (3H, d, $J = 6.8$ Hz, H-7), 2.10 (3H, s, OAc), 3.13 (1H, s, H-15), 4.08 (1H, dd, $J = 11.6$ and 1.5 Hz) and 4.28 (1H, dd, $J = 11.6$ and 2.3 Hz) (each H-19), 4.28 and 4.60 (each 1H, d, $J = 12$ Hz, H-17). MS m/z : 374 [M]⁺, 332, 314, 304, 286, 269, 258, 227. (b) *ent*-7,19-Dihydroxygibberell-16-en-20-oic acid 20,19-lactone (10) (600 mg) in EtOH (30 ml) was evenly distributed between 60 flasks and incubated in shake culture for 10 days. The broth was filtered and the metabolites were recovered in EtOAc and chromatographed on silica gel. Elution with 30% EtOAc-petrol gave the unchanged substrate (80 mg). Further elution with 45% EtOAc-petrol gave *ent*-7,15 β ,19-trihydroxygibberell-16-en-20-oic acid 20,19-lactone (11) (35 mg) which crystallized from EtOAc as needles, mp 190°. (Found: C, 71.8; H, 8.8. $\text{C}_{20}\text{H}_{30}\text{O}_4$ requires C, 72.3; H, 8.5%). IR ν_{max} cm^{-1} : 3329, 1718, 1654 cm^{-1} . ^1H NMR: δ 0.99 (3H, s, H-18), 3.50 (1H, dd, $J = 12.1$, 1.6 Hz) and 3.75 (1H, dd, $J = 12.1$ and 8.2 Hz) (each H-7), 4.07 (1H, dd, $J = 11.6$ and 1.7 Hz) and 4.38 (1H, dd, $J = 11.6$ and 2.3 Hz) (each H-19), 4.35 (1H, d, $J = 1.7$ Hz, H-15), 5.09 and 5.29 (each 1H, s, H-17). (c) *ent*-19-Hydroxygibberell-16-en-7,20-dioic acid 20,19-lactone 7-methyl ester (12) (500 mg) in EtOH (11 ml) was distributed between 22 flasks and incubated in shake

culture for 10 days. The broth was filtered and the metabolites recovered in EtOAc and chromatographed on silica gel. Elution with 15% EtOAc-petrol gave unchanged substrate (60 mg). Further elution with 20% EtOAc-petrol gave *ent*-15 β ,19-dihydroxygibberell-16-en-7,20-dioic acid 20,19-lactone 7-methyl ester (13) (100 mg) which crystallized from CHCl₃ as prisms, mp 163°. (Found: C, 66.5; H, 7.75. C₂₂H₃₂O₈ · H₂O requires C, 66.6; H, 8.0%). IR $\nu_{\max}$ cm⁻¹: 3489, 1716, 1650. ¹H NMR: δ 0.86 (3H, s, H-18), 2.59 (1H, d, *J* = 10.2 Hz, H-6), 3.68 (3H, s, OMe), 4.14 (1H, dd, *J* = 11.8 and 1.5 Hz) and 4.36 (1H, dd, *J* = 11.8 and 2.2 Hz) (each H-19), 4.17 (1H, s, H-15), 5.11 and 5.29 (each, 1H, s, H-17). (d) *ent*-7,19-Dihydroxygibberell-20-oic acid 20,19-lactone (14) (500 mg) in EtOH (20 ml) was evenly distributed between 40 flasks and incubated in shake culture for 8 days. The broth was filtered and the metabolites were recovered in EtOAc and chromatographed on silica gel. Elution with 40% EtOAc-petrol gave the unchanged substrate (52 mg). Further elution gave a mixt. A (18 mg) and then elution with EtOAc gave mixt. B (31 mg). Each mixt. was acetylated (Ac₂O-pyridine) and the products were chromatographed on silica gel in EtOAc-petrol (1:4). Mixt. A afforded a compound, tentatively assigned the structure of *ent*-7,12-diacetoxy-19-hydroxygibberell-20-oic acid 20,19-lactone (15) (3 mg) as a gum. (Found: M 418.235. C₂₄H₃₄O₈ requires M 418.235.) ¹H NMR (200 MHz): δ 0.99 (3H, s, H-18), 1.13 (3H, d, *J* = 6.6 Hz, H-17), 2.04 and 2.07 (3H, each s, OAc), 3.97 (1H, dd, *J* = 11.6 and 6.6 Hz) and 4.25 (1H, dd, *J* = 11.6 and 3 Hz) (each H-7), 4.10 (1H, d, *J* = 11.8 Hz) and 4.33 (1H, dd, *J* = 11.8 and 2.1 Hz) (each H-19), 5.67 (1H, d, *J* = 7.4 Hz, H-12). MS *m/z*: 418 [M]⁺, 376, 375, 358, 298, 269, 225. Further elution gave *ent*-7-acetoxy-13,19-dihydroxygibberell-20-oic acid 20,19-lactone (5 mg) (17) as a gum. (Found: M 376.224 C₂₂H₃₂O₈ requires M 376.225.) IR $\nu_{\max}$ cm⁻¹: 3380, 1730. ¹H NMR (200 MHz): δ 0.97 (3H, s, H-18), 1.05 (3H, d, *J* = 6.6 Hz, H-17), 2.08 (3H, s, OAc), 3.92 and 4.15 (each 1H, d, *J* = 12.5 Hz, H-19), 3.96 (1H, dd, *J* = 11.8 and 5.6 Hz) and 4.36 (1H, dd, *J* = 11.8 and 1.6 Hz) (each H-7). MS *m/z*: 376 [M]⁺, 358, 331, 298, 286, 241, 219.

Chromatography of fr. B gave *ent*-7,16-diacetoxy-19-hydroxygibberell-20-oic acid 20,19-lactone (18) as a gum (2 mg). ¹H NMR (200 MHz): δ 0.97 (3H, s, H-18), 1.66 (3H, s, H-17), 1.95 and 2.07 (each 3H, s, OAc), 3.92 (1H, dd, *J* = 11.6 and 7.0 Hz) and 4.17 (1H, dd, *J* = 11.6 and 2.9 Hz) (each H-7), 4.10 (1H, dd, *J* = 11.5 and 1.6 Hz) and 4.33 (1H, dd, *J* = 11.5 and 2.0 Hz) (each H-19). MS *m/z*: 418 [M]⁺, 376, 358, 298, 239. Further elution gave *ent*-7-acetoxy-16,19-dihydroxygibberell-20-oic acid 20,19-lactone (19) (12 mg) as a gum. (Found: [M]⁺ 376.224 C₂₂H₃₂O₈ requires M 376.225.) IR $\nu_{\max}$ cm⁻¹: 3625, 1730. ¹H NMR (200 MHz): δ 0.97 (3H, s, H-18), 1.43 (3H, s, H-17), 2.08 (3H, s, OAc), 3.97 (1H, dd, *J* = 11.7 and 6.0 Hz) and 4.18 (1H, dd, *J* = 11.7 and 2.9 Hz) (each H-7), 4.09 (1H, dd, *J* = 11.7 and 1.7 Hz) and 4.34 (1H, dd, *J* = 11.7 and 2.0 Hz) (each H-19). MS *m/z*: 376, 358, 333, 316, 298, 258, 199.

Allylic oxidations with selenium dioxide. (a) *ent*-7,19-Dihydroxygibberell-16-en-20-oic acid 20,19-lactone (10) (30 mg) in dioxan (0.5 ml) was treated with SeO₂ (12 mg)

and 30% H₂O₂ (0.5 ml) at room temp. for 5 hr. H₂O was added and the product was recovered in EtOAc and chromatographed on silica gel. Elution with EtOAc-petrol (4:1) gave *ent*-7,15 β ,19-trihydroxygibberell-16-en-20-oic acid 20,19-lactone (11) (17 mg) identical (IR, ¹H NMR) to the material described above. (b) *ent*-19-Hydroxygibberell-16-en-7,20-dioic acid 20,19-lactone 7-methyl ester (12) (90 mg) in dioxan (5 ml) was treated with SeO₂ (25 mg) and 30% H₂O₂ (2 ml) for 1 hr at room temp. A saturated soln of Na₂SO₃ was added and the product was recovered in CHCl₃ and chromatographed on silica gel. Elution with EtOAc-petrol (1:4) gave *ent*-15 β ,19-trihydroxygibberell-16-en-7,20-dioic acid 20,19-lactone 7-methyl ester (20 mg) identical (IR, ¹H NMR) to the material described above. Further elution with EtOAc-petrol (1:3) gave *ent*-17,19-dihydroxy-15 β ,16 β -epoxygibberell-7,20-dioic acid 20,19-lactone 7-methyl ester (21) (30 mg) as a gum. (Found: M 376.189. C₂₁H₃₀O₈ requires M 376.188.) IR $\nu_{\max}$ cm⁻¹: 3477, 1728. ¹H NMR δ 0.81 (3H, s, H-18), 2.09 (1H, d, *J* = 11.2 Hz, H-5), 2.49 (1H, d, *J* = 11.2 Hz, H-6), 3.23 (1H, s, H-15), 3.74 (3H, s, OMe), 3.76 and 4.04 (each 1H, d, *J* = 12.7 Hz, H-17), 4.10 (1H, dd, *J* = 11.9 and 1.3 Hz) and 4.24 (1H, dd, *J* = 11.9 and 2.1 Hz, each H-19).

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