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THE MICROBIOLOGICAL TRANSFORMATION OF SOME ISOATISENE DITERPENOIDS INTO ISOATISAGIBBERELLINS AND ISOATISENOLIDES BY *GIBBERELLA FUJIKUROI*

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Key Word Index—*Gibberella fujikuroi*; gibberellins; kaurenolides; *ent*-atis-15-ene; isoatisagibberellins; isoatisenolides; gummiferolic acid.

Abstract—The microbiological transformations of *ent*-7α-hydroxy-atis-15-en-19-oic acid into isoatisagibberellins A_{12} and A_{15}, and of *ent*-19-hydroxy-atis-6,15-diene into 7β-hydroxyisoatisenolide and 7β,18-dihydroxyisoatisenolide have been demonstrated using *Gibberella fujikuroi*. The substrates incubated were chemically obtained from gummiferolic acid.

INTRODUCTION

The gibberellins are plant hormones that are biosynthetically derived from *ent*-kaur-16-ene (**1**). Although some diterpenes structurally related to **1** are known, such as *ent*-kaur-15-ene (isokaurene), *ent*-phyllocladene (13β-kaurene), *ent*-beyer-15-ene, *ent*-atis-16-ene, *ent*-atis-15-ene (isoatisene), and *ent*-trachylobane, the gibberellins which have been isolated so far belong to the *ent*-kaur-16-ene series [1]. However, nothing is known about gibberellin biosynthesis, which might preclude the natural occurrence of analogues of gibberellins with other skeletons, that show different arrangements of rings C and D. We have shown that the fungus *Gibberella fujikuroi* can metabolize substrates with the atisane [2], trachylobane [3, 4], beyerene [5, 6], and isokaurane [7] skeletons to give atisa-, trachyloba-, beyer- and iso-gibberellins. Other authors have also published the results of the incubation of trachylobane and atisane derivatives by a mutant of this fungus [8, 9]. To complete these studies, we describe in this work the microbiological transformation by 'wild-type' *Giibberella fujikuroi* of the synthetic isoatisene derivatives, *ent*-7α-hydroxyatis-15-en-19-oic acid (**2**) and *ent*-19-hydroxyatis-6-,15-diene (**6**), to give isoatisagibberellins and isoatisenolides, respectively. Both compounds, **2** and **6**, were synthetically prepared from gummiferolic acid (**3**), an atisane diterpene isolated from *Margotia gummifera* [10].

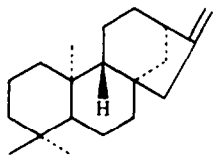
RESULTS AND DISCUSSION

The fermentations were carried out in the presence of AMO 1618, an inhibitor of the biosynthesis of *ent*-kaur-16-ene (**1**), in order to suppress the formation of the metabolites derived from this diterpene, and to facilitate the study of the product formed [11, 12]. The incubations were harvested after six days of growth, and the broth and mycelium extracts were mixed, and separated into acid and neutral fractions. The acid fraction was treated with diazomethane and purified by chromatography of its methyl esters.

The microbiological transformation of *ent*-7α-hydroxyatis-15-en-19-oic acid (**2**) gave isoatisagibberellin A_{12} (**8**) and isoatisagibberellin A_{15} (**12**), in the acid fraction. No metabolites were isolated from the neutral fraction. The high resolution mass spectrum of isoatisagibberellin A_{12} dimethyl ester (**10**) was in accordance with the molecular formula $C_{22}H_{32}O_4$, and its ^{1}H NMR spectrum contained three C-methyl signals, two singlets at δ0.78 and 1.08 for C-20 and C-18 methyls, respectively, and one doublet at 1.73, assigned to the C-17 methyl. The characteristic gibberellin double doublets of H-5 and H-6 appeared at 2.04 and 3.25 respectively, with values similar to those observed for the dimethyl ester of atisagibberellin A_{12} [2]. The two methoxyls appeared at 3.66 and 3.74 and the vinylic proton as a broad singlet at 5.77. The mass spectrum was very similar to those of gibberellin A_{12} dimethyl ester [13] and atisagibberellin A_{12} dimethyl ester [2], with the molecular ion at *m/z* 360 and fragments at *m/z* 328, 300 and 285.

The structure of the second gibberellin analogue obtained in the methylated acid fraction was determined as isoatisagibberellin A_{15} methyl ester, on the basis of the following considerations: the molecular formula was established as $C_{21}H_{28}O_4$ by high resolution mass spectrometry. Its ^{1}H NMR spectrum showed signals of an angular methyl (δ1.11, *s*), of a methyl on a double bond (1.74, *d*, J = 1.5 Hz), of a methoxyl (3.74, *s*) and of the H-5 and H-6 hydrogens (2.29 and 2.44) *d*, J = 11 Hz). The methylene protons at C-20 also appear in this spectrum as a doublet at δ4.11 (J = 12 Hz) and a double doublet at 4.57 (J = 12 and 1.5 Hz). The second coupling constant of this last hydrogen probably originates from a long-range coupling with the H-1α. The mass spectrum contains ions similar to those obtained for GA_{15} methyl ester at *m/z* 344, 326, 312 and 284 [13]. A third isoatisagibberellin was a minor compound in one of the fractions that contained isoatisagibberellin A_{15}, and it was tentatively identified as isoatisagibberellin A_{24} on the evidence of the high and low resolution mass spectrum of this fraction.

It has been previously noted [2, 9] that *ent*-atis-16-ene



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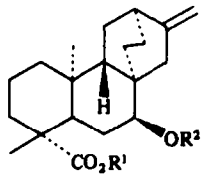
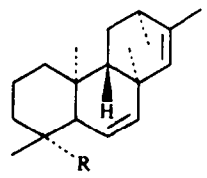
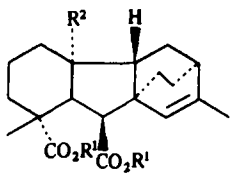


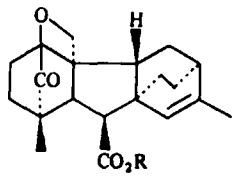
3 R^1 = H, R^2 = Ang
4 R^1 = Me, R^2 = Ang
5 R^1 = Me, R^2 = H



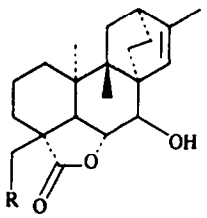
6 R = CH_2OH
7 R = CO_2Me



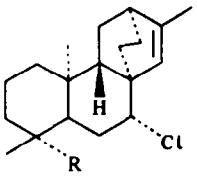
8 R^1 = H, R^2 = Me
9 R^1 = H, R^2 = CHO
10 R^1 = R^2 = Me
11 R^1 = Me, R^2 = CHO



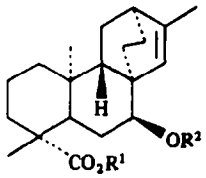
12 R = H
13 R = Me



14 R = H
15 R = OH



16 R = CO_2Me
17 R = CH_2OH



2 R^1 = R^2 = H
18 R^1 = H, R^2 = Ang
19 R^1 = Me, R^2 = H

diterpenes were only metabolized for a few steps along the gibberellin biosynthetic pathway, and atisagibberellins oxidized at C-20 were not obtained. In contrast we have now obtained isoatisagibberellins with oxygen at this carbon, indicating that a small variation in the atisene D ring, can affect the substrate specificity of the enzyme(s) responsible for these oxidations. On the other hand, no C_{19} analogues of gibberellin were isolated in the present work from the feedings of isoatisene derivatives, as also occurred in the incubation of *ent*-atis-16-ene diterpenes [2, 9].

The incubation of *ent*-19-hydroxyatis-6,15-diene (**6**) gave 7β-hydroxyisoatisenolide (**14**) and 7β-18-dihydroxyisotisenolide (**15**) in the neutral fraction. The acid fraction did not contain gibberellin metabolites. This result is as expected because the Δ^6-double bond in **6**, leads the metabolism to the kaurenolide biosynthetic pathway [14]. The less polar of these compounds, the 7β-hydroxyisoatisenolide (**14**), showed in its ^{1}H NMR spectrum signals of the three methyl groups, of the olefinic proton at C-15 and of the resonances originating from the hydrogens at C-6 and C-7 (δ4.74 and 4.11, $J_{5,6}$ = 7 Hz, $J_{6,7}$ = 4 Hz). These last signals are similar to those observed in the kaurenolides [15]. The more polar hydroxy-lactone showed a ^{1}H NMR spectrum similar to that of **14**, where the signal of one of the angular methyl groups has been substituted by a broad singlet at δ3.71 (2H) of a hydroxymethyl group. By comparison with the spectrum of 7β,18-dihydroxykaurenolide, this alcoholic function was assigned to C-18, and the structure established as 7β,18-dihydroxyisoatisenolide (**15**).

The substrates incubated were synthesized in the following way. Hydrolysis of the methyl ester of gummiferolic acid (**4**) gave the alcohol **5** [10], which was treated with *p*-toluenesulphonic acid in benzene to give the isomerized compound **19**. Reaction of **19** with thionyl chloride in pyridine gave the dehydrated product **7**, contaminated with the chlorine derivative **16**. Treatment of this mixture with lithium aluminium hydride afforded the alcohols **6** and **17**, which were separated by silica gel chromatography.

The hydroxy acid **2** was obtained by isomerization of gummiferolic acid (**3**), and then by hydrolysis of the product obtained (**18**).

EXPERIMENTAL

Incubation experiments. Gibberella fujikuroi (ACC 917) inhibited with 5×10^{-5} M AMO 1618, was grown in shake culture at 25° for 2 days in 65–75 conical flasks (250 ml) each containing sterile medium (50 ml) [16]. The substrate (see below) in EtOH (13–15 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 $NaHCO_3$. The acidic fraction was methylated with CH_2N_2.

The incubation of *ent*-7α-hydroxy-atis-15-en-19-oic acid (**2**) (285 mg) gave, after methylation of the acid fraction and chromatography on silica gel (0.2–0.05) eluting with petrol–EtOAc (5%), isoatisagibberellin A_{12} dimethyl ester (**10**) (4 mg), isoatisagibberellin A_{15} methyl (**13**) (8 mg) and traces of a compound tentatively identified as isoatisagibberellin A_{24} dimethyl ester (**11**).

The incubation of *ent*-19-hydroxy-atis-6,15-diene (**6**) (135 mg) gave 7β-hydroxyisoatisenolide (**14**) (3 mg) and 7β,18-dihydroxyisoatisenolide (**15**) (4 mg). These two compounds were separated by chromatography on silica gel, eluting with mixtures of petrol–EtOAc (95:5 and 80:20, respectively).

Isoatisagibberellin A_{12} dimethyl ester (**10**). $[M]^+$ at *m/z* 360.2311, $C_{22}H_{32}O_4$ requires 360.2299; ^{1}H NMR (80 MHz): δ0.78 and 1.08 (each 3H, *s*), 1.73 (3H, *d*, *J* = 1.6 Hz, H-17), 2.04 and 3.25 (each 1H, *d*, *J* = 11 Hz, H-5 and H-6), 3.66 and 3.74 (each 3H, *s*), 5.79 (1H, *br s*, H-15); EIMS *m/z* (re. int.): 360 $[M]^+$ (11), 328 (27), 300 (73), 285 (27), 272 (27), 241 (56), 219 (11).

Isoatisagibberellin A_{15} methyl ester (**13**). $[M]^+$ at *m/z* 344.1976, $C_{21}H_{28}O_4$ requires 344.1986; ^{1}H NMR (200 MHz): δ1.11 (3H, *s*, H-18), 1.74 (3H, *d*, *J* = 1.5 Hz, H-17), 2.29 and 2.44 (each 1H, *d*, *J* = 11 Hz, H-5 and H-6), 3.74 (3H, *s*), 4.11 (1H, *d*, *J* = 12 Hz, H-20), 4.5 (1H, *dd*, *J* = 12 and 1.5 Hz, H-20), 5.83 (1H, *br s*, H-15); EIMS *m/z* (rel. int.): 344 $[M]^+$ (4), 326 (6), 312 (28), 284 (63), 256 (9), 239 (8).

Isoatisagibberellin A_{24} dimethyl ester (**11**). This product was tentatively identified as a minor compound in one of the fractions that contained **13**. Compound **11**, $[M]^+$, at *m/z* 374.2089, $C_{22}H_{30}O_5$ requires 374.2090 EIMS *m/z* (rel. int.) 374 $[M]^+$ (1), 342 (0.5), 286 (21).

ent-6β,7α-*Dihydroxyatis-15-en-19-oic acid* 19,6β-*lactone* (7β-*hydroxyisoatisenolide*) (**14**). $[M]^+$ at *m/z* 316.2008, $C_{20}H_{28}O_3$ requires 316.1992; ^{1}H NMR (200 MHz): δ0.96 and 1.30 (each 3H, *s*), 1.78 (3H, *d*, *J* = 1.7 Hz, H-17), 4.11 (1H, *d*, *J* = 4 Hz, H-7) 4.74 (1H, *dd*, *J* = 7 and 4 Hz, H-6), 6.40 (1H, *br s*, H-15); EIMS *m/z* (rel. int.): 316 $[M]^+$ (4), 301 (2), 298 (1), 288 (14).

ent-6β,7α,18-*Trihydroxyatis-15-en-19-oic acid* 19,6β-*lactone* (7β,18-*dihydroxyisoatisenolide*) (**15**). $[M]^+$ at *m/z* 332.1981, $C_{20}H_{28}O_4$ requires 332.1986; ^{1}H NMR (200 MHz): δ 1.00 (3H, *s*, H-20), 1.77 (3H, *d*, *J* = 1.6 Hz, H-17), 3.71 (2H, *br s*, H-18), 4.10 (1H, *d*, *J* = 5 Hz, H-7), 4.86 (1H, *dd*, *J* = 7 and 5 Hz, H-6), 6.38 (1H, *br s*, H-15); EIMS *m/z* (rel. int.): 332 $[M]^+$ (8), 304 (26), 289 (10), 274 (12), 271 (19), 269 (12), 250 (14).

Isomerization of gummiferolic acid. A crude mixture enriched in gummiferolic acid (**3**) (1150 mg) in C_6H_6 (40 ml) was treated with *p*-TsOH (20 mg) for 2.5 hr. Usual work-up and chromatography on silica gel impregnated with $AgNO_3$ (15%) and elution with petrol–EtOAc (9:1) gave **18** (640 mg): ^{1}H NMR (60 MHz): δ0.94 and 1.16 (each 3H, *s*), 1.75 (3H, *d*, *J* = 1.5 Hz, H-17), 5.20 (1H, *br*, *s*, H-7), 5.68 (1H, *br s*, H-15); EIMS *m/z* (rel. int.): 400 $[M]^+$ (5), 300 (46), 285 (18), 272 (89), 254 (28), 239 (25), 226 (31), 211 (43), 197 (10), 185 (16), 183 (15), 171 (20), 157 (46).

Hydrolysis of **18**. The product **18** (620 mg) in 2.5 N methanolic KOH (20 ml) was treated under reflux for 10 hr. The soln was acidified, the product recovered with EtOAc in the usual way, and purified by dry CC eluting with petrol–EtOAc (20:3) to afford *ent*-7α-hydroxyatis-15-en-19-oic acid (**2**) (420 mg), mp 245–255°, $[M]^+$ at *m/z* 31.8.2179, $C_{20}H_{30}O_3$ requires 318.2192; ^{1}H NMR (80 MHz): δ0.91 and 1.24 (each 3H, *s*), 1.77 (3H, *d*, *J* = 1.6 Hz, H-17), 3.83 (1H, *t*, *J* = 2.7 Hz, H-7), 5.99 (1H, *br s*, H-15); EIMS *m/z* (rel. int.): 318 $[M]^+$ (38), 303 (21), 300 (13), 290 (10), 285 (6), 272 (14), 257 (16), 236 (27), 164 (59).

Isomerization of **5**. The crude compound **5** (900 mg) [10] was treated with *p*-TsOH as above for **3** to give **19** (450 mg), mp 186–191° (lit. [10] 190–192°), $[M]^+$ at *m/z* 332.2369, $C_{21}H_{32}O_3$ requires 332.2351; ^{1}H NMR (200 MHz): δ0.76 and 1.15 (each 3H, *s*), 1.75 (3H, *d*, *J* = 1.6 Hz, H-17), 3.63 (3H, *s*), 3.80 (1H, *t*, *J* = 3 Hz, H-7), 5.97 (1H, *br*, *s*, H-15); EIMS *m/z* (rel. int.): 332 $[M]^+$ (50), 317 (18), 314 (14), 304 (8), 299 (5), 289 (8), 286 (11), 273 (18), 255 (33), 250 (30), 164 (72).

Formation of **6**. The alcohol **19** (430 mg) was dissolved in dry pyridine (6 ml), and $SOCl_2$ (0.6 ml) was added dropwise to this soln at 0°, which was left for 3 hr, then diluted with H_2O and extracted in the usual way. The residue (260 mg) in dry THF (10 ml) was refluxed with $LiAlH_4$ (170 mg) for 2 hr. Excess of reagent was destroyed by adding EtOAc and then H_2O. The mixture was washed with 2% HCl and extracted with EtOAc. Chromatography of the residue, eluting with petrol–EtOAc (9:1), gave *ent*-19-hydroxyatis-6,15-diene (**6**) (140 mg), mp 104–106°, $[M]^+$ at *m/z* 286.2291, $C_{20}H_{30}O$ requires 286.2287; ^{1}H NMR (200 MHz): δ0.92 and 1.02 (each 3H, *s*), 1.74 (3H, *d*, *J* = 1.5 Hz, H-17), 3.58 and 3.66 (each 1H, *d*, *J* = 11 Hz, H-19), 5.61 (1H, *dd*, *J* = 10 and 3 Hz, H-6), 5.79 (1H, *dd*, *J* = 10 and 2 Hz, H-7), 5.81 (1H, *br s*, H-15); EIMS *m/z* (rel. int.): 286 $[M]^+$ (43), 258 (100), 227 (37), 225 (27), 211 (15), 197 (10). Further elution afforded *ent*-7β-chloro-19-hydroxyatis-15-ene (**17**), ^{1}H NMR (200 MHz): δ0.97 (6H, *s*), 1.76 (3H, *d*, *J* = 1.6 Hz, H-17), 3.50 and 3.69 (each 1H, *d*, *J* = 11 Hz, H-19), 4.34 (1H, *t*, *J* = 3 Hz, H-7), 6.04 (1H, *br s*, H-15). EIMS *m/z* (rel. int.): 322 $[M]^+$ (25), 291 (57), 255 (35), 206 (25), 195 (21).

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