

TETRAHEDRON

## Formation of 2,3-seco-Acids in the Biotransformation of the Diterpene Ribenone by *Gibberella fujikuroi*

Braulio M. Fraga, Pedro González, Melchor G. Hernández and Sergio Suárez

Instituto de Productos Naturales y Agrobiología, CSIC, PO Box 195, La Laguna, 38206-Tenerife, Canary Islands, Spain
<sup>†</sup> Instituto Universitario de Bio-Orgánica, Universidad de La Laguna, Tenerife, Spain

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Abstract: The biotransformation of ribenone (*ent*-3-oxo-13-*epi*-manoyl oxide) (4) by the fungus Gibberella fujikuroi led to compounds hydroxylated at C-1( $\alpha$ ), C-6( $\beta$ ), C-11( $\beta$ ) or C-12( $\beta$ ), in addition to the 2,3-seco diacids 15, 17 and 19. © 1999 Elsevier Science Ltd. All rights reserved.

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The microbiological transformation of diterpenoids with fungi has been one of the aims of our studies in recent years. The fungus *Gibberella fujikuroi* produces *ent*-13-*epi*-manoyl oxide (1), a labdane diterpene which seems to be a final metabolite of a biosynthetic branch.<sup>1</sup> In a previous work we have examined the biotransformation by this microorganism of two alcohols, *ent*-3 $\beta$ -hydroxy-13-*epi*-manoyl oxide (ribenol) (2) and *ent*-19-hydroxy-13-*epi*-manoyl oxide (3),<sup>2</sup> showing that the introduction of a new functional group in 1, such as a hydroxyl, may induce the fungus to transform these substrates. In relation to these works we have now studied the microbiological transformation of ribenone (4). Other authors have carried out the biotransformation of this diterpene with *Curvularia lunata*<sup>3</sup> and *Fusarium moniliformis*.<sup>4</sup> In another work we have also examined the biotransformation of two manoyl oxide derivatives of the normal series, jhanol and jhanidiol, by *G. fujikuroi*.<sup>5</sup>

The substrate utilized was the diterpene ribenone (4), which had been isolated for the first time from Solidago missouriensis.<sup>6</sup> It was later obtained from Sideritis canariensis<sup>7</sup> and Excoecaria agallocha,<sup>8</sup> and can



 $2 R = \alpha - OH_{1}$ 4 R = O





5  $R_1 = OH R_2 = H$ 5  $R_1 = OAC R_2 = H$ 7  $R_1 = H R_2 = OH$ 7  $R_1 = H R_2 = AC$ 

OR3 /





8  $R_1 = OH R_2 = H$ 8a  $R_1 = OAC R_2 = H$ 9  $R_1 = H R_2 = OH$ 9a  $R_1 = H R_2 = OAc$ 

10  $R_1 = \alpha - OH, H R_2 = R_3 = H$ 11  $R_1 = O R_2 = OH R_3 = H$ 11a  $R_1 = O R_2 = OAc R_3 = Ac$ 12  $R_1 = \beta - OH, H R_2 = R_3 = H$ 12a  $R_1 = \beta - OAc, H R_2 = H R_3 = Ac$ 

also be prepared by oxidation of ribenol (2).<sup>9</sup> The incubations with *G. fujikuroi* were carried out in the presence of AMO 1618, a compound that inhibits the formation of *ent*-kaur-16-ene by this fungus, without disturbing post-kaurene metabolism, which facilitates the isolation of the metabolites formed.<sup>10,11</sup> The fermentations were harvested after eight days, and the broth and mycelium extracts were combined. Chromatography of the extract of the incubation of 4 gave the metabolites 5-9, 11-13, 15, 17 and 19.

The structure of  $1\alpha$ -hydroxy-ribenone (5) was given to one of the metabolites obtained on the basis of the following considerations: The high resolution mass spectra did not show the molecular ion, but gave a fragment at m/z 305.2106 (C<sub>19</sub>H<sub>29</sub>O<sub>3</sub>) formed by loss of a methyl group. Its <sup>1</sup>H NMR spectrum was very similar to that of substrate 4, but with a geminal proton to a new alcohol group appearing as a double doublet at  $\delta$  3.95. The coupling constants (J = 7.9 and 4.7 Hz) indicated that the hydroxyl group must be equatorial and situated at C-1 or C-7. The C-1( $\alpha$ ) position was chosen considering the relative low value of the greater of these constants (7.9 Hz), compared with that observed in a normal chair (11-12 Hz), indicating that the

alcohol must be located at C-1( $\alpha$ ) in the cyclohexanone ring, which is a deformed chair. This assertion was then confirmed by the <sup>13</sup>C NMR spectroscopic data (table 1). On the other hand, double resonance experiments permitted us to assign the H-2( $\alpha$ ) and H-2( $\beta$ ) resonances at  $\delta$  2.32 and 2.98. Therefore, the structure of *ent*-1 $\beta$ -hydroxy-3-oxo-13-*epi*-manoyl oxide (5) was assigned to this substance.

The less polar compound isolated was 6, which in comparison with the substrate showed in the MS a molecular ion with two units fewer and the appearance of two new vinylic protons in the <sup>1</sup>H NMR spectrum at  $\delta$  5.86 and 7.14 (J = 10.2 Hz). The chemical shifts of these hydrogens indicated that this new double bond introduced into the molecule must be conjugated with the oxo group, which was confirmed in its IR and UV spectra. Thus, the structure of this substance was assigned as *ent*-3-oxo-1-en-13-*epi*-manoyl oxide (6). This compound must be an artifact which has been formed during the extraction procedure, because it was also produced when compound 5 was left, dissolved in CDCl<sub>3</sub>, in a NMR tube for several days. The substances 5 and 6 were the sole compounds previously obtained in the biotransformation of 4 with *F. moniliformis.*<sup>4</sup>

Another metabolite obtained in this feeding with G. fujikuroi was 11 $\beta$ -hydroxy-ribenone (7). In the high resolution MS the molecular ion appears at m/z 320.2355 indicating that the molecular formula of this substance was C<sub>20</sub>H<sub>32</sub>O<sub>3</sub>. The new oxygen introduced during the fermentation must be a part of a secondary alcoholic group, because its <sup>1</sup>H NMR spectrum showed a proton geminal to a new hydroxyl group at  $\delta$  4.18 as a triple doublet (J = 9.6 and 4.5 Hz). This coupling permitted us to assign the alcohol group at C-6( $\beta$ ) or C-11( $\beta$ ). The latter position was chosen considering the assignment of the <sup>13</sup>C NMR spectra of this alcohol (7) and its acetate (7a) (table 1). Therefore, the structure of this metabolite was determined as *ent*-11 $\alpha$ -hydroxy-3-oxo-13-*epi*-manoyl oxide (7). This product was identical with one previously isolated from the resinous wood of *E. agallocha*.<sup>8</sup>

The following metabolites obtained in this feeding were 8 and 9. Both have a molecular formula  $C_{20}H_{32}O_3$ . The <sup>1</sup>H NMR spectrum of 8 was similar to that of its isomer 7, showing now the resonance of the proton geminal to the hydroxyl group at  $\delta$  3.83 as a triple doublet with coupling constants of 11.3 and 4.4 Hz. These couplings are characteristic of an axial hydrogen geminal to an alcoholic group at C-6( $\beta$ ) or C-11( $\beta$ ). The first position was assigned considering the <sup>13</sup>C NMR data (table 1). Thus, the structure of *ent*-6 $\alpha$ -hydroxy-3-oxo-13-*epi*-manoyl oxide (8) was given to this substance. Its epimer at C-6 had been obtained in the biotransformation of 4 by C. *lanata*.<sup>3</sup>

The other isomer 9 showed in its <sup>1</sup>H NMR spectrum the hydrogen geminal to the hydroxyl group at  $\delta$  4.08 as a triplet (J = 3.1 Hz). This coupling constant is typical of an equatorial proton geminal to a hydroxyl group located at C-1( $\beta$ ), C-7( $\beta$ ) or C-12( $\beta$ ). The last position was chosen, considering that the resonance of the two H-15 were affected by the presence of this hydroxyl group. Then, the position of the 12 $\beta$ -hydroxyl was

definitively assigned considering its <sup>13</sup>C NMR spectrum. This fact was chemically confirmed by sodium borohydride reduction of this compound, which led to varodiol (10), which had been isolated from *Sideritis* varoi.<sup>12</sup> Consequently, its structure was determined as *ent*-12 $\alpha$ -hydroxy-3-oxo-13-*epi*-manoyl oxide (9).

To another metabolite obtained in this biotransformation was assigned the structure of *ent*-11 $\alpha$ , 12 $\alpha$ dihydroxy-3-oxo-13-*epi*-manoyl oxide (11). Its high resolution MS showed the molecular ion at 336.2313 in accordance with the molecular formula C<sub>20</sub>H<sub>32</sub>O<sub>4</sub>, indicating that two oxygen atoms have been introduced into the molecule during the fermentation. In its <sup>1</sup>H NMR spectrum two protons geminal to hydroxyl groups appear at  $\delta$  4.05 (br s) and 4.20 (m). Double resonance experiments showed that these hydrogens were at contiguous carbons, and situated between a methine ( $\delta$  1.76, d, J = 10.3 Hz) and a tetrasubstituted carbon. In this spectrum the hydrogens of the hydroxyl groups also appeared, as two doublets at  $\delta$  2.13 (J = 6.5 Hz) and 2.24 (J = 7.8 Hz) coupled with the H-12 and H-11, respectively, which indicated the existence of hydrogen bonds. All these data pointed to C-6( $\beta$ ), C-7( $\beta$ ) or C-11( $\beta$ ), C-12( $\beta$ ) for the location of these two hydroxyl groups. Since the resonance of the two H-15 were different from those observed in the substrate we assigned these alcohols at the aforementioned positions of ring C, which was confirmed by assignment of the <sup>13</sup>C NMR spectra of 11 and 11a (table 2).

Compound 12 has the molecular formula  $C_{20}H_{34}O_3$ . Its <sup>1</sup>H NMR spectrum showed resonances of protons geminal to two alcoholic groups at  $\delta$  3.42 (t, J = 2.8 Hz) and 4.08 (t, J = 3.5 Hz), which indicated, considering its molecular formula, that the 3-oxo group of ribenone (4) had been reduced into an alcohol group during the fermentation and that a new hydroxyl had also been introduced into its molecule. The chemical shift and couplings of the second geminal hydrogen were similar to those observed in the product 9 and were consequently assigned to C-12( $\beta$ ). The former hydrogen was located at C-3( $\beta$ ). Thus, the structure of this metabolite was determined as *ent*-3 $\alpha$ , 12 $\alpha$ -dihydroxy-13*-epi*-manoyl oxide (12) and then confirmed by assignment of its <sup>13</sup>C NMR spectrum (table 2). Compound 12 is the 3-epimer of varodiol (10). <sup>12</sup> An analogous reduction of the 3-oxo group of ribenone (4) has also been observed in its incubation with *C. lunata*.<sup>3</sup> Interestingly, the microbiological reduction with both fungi, *G. fujikuroi* and *C. lunata*, produced the  $\beta$ -alcohol, while the borohydride reduction (see above) afforded the  $\alpha$ -alcohol.

A series of 2,3-seco-acids were also formed in this biotransformation. Thus, the most simple of these compounds was 13. Its MS does not show the molecular ion, but at m/z 337.2013 a fragment ion is seen formed by loss of a methyl group. Thus, its molecular formula was determined as C<sub>20</sub>H<sub>32</sub>O<sub>5</sub>. In its <sup>1</sup>H NMR spectrum the characteristic H-2 signals of the substrate 4 did not appear, which indicated that the structure of ring A of the molecule had been altered during the incubation. This compound was converted into dimethyl ester 14 by methylation with diazomethane. These facts, the presence in the <sup>1</sup>H NMR spectrum of 13 of five

С	4	5	5a	7	7 <b>a</b>	8	8a	9
1	38.1	78.0	78.8	40.3	40.1	38.1	37.9	37.7
2	33.7	45.1	41.5	33.9	33.8	32.7	32.5	33.6
3	217.4	214.6	214.3	217.8	217.7	218.8	217.6	217.3
4	47.2	47.1	46.7	47.7	47.7	47.1	47.3	47.2
5	54.5	50.9	50.4	54.8	54.4	58.8	55.5	54.9
6	20.7	20.5	20.5	20.8	20.6	68.2	71.2	20.8
7	42.0	41.7	41.7	42.3	42.4	52.3	47.3	41.8
8	75.5	75.5	75.2	76.8	77.1	74.8	74.4	76.2
9	57.5	58.2	57.5	62.6	59.2	56.8	56.7	48.9
10	36.3	42.5	41.0	37.6	37.6	36.8	36.9	35.9
11	16.3	18.1	17.6	65.7	68.1	16.5	16.5	23.9
12	34.7	34.8	34.7	45.3	39.2	34.8	34.7	68.9
13	73.6	73.5	73.4	74.2	74.2	73.7	73.7	75.7
14	147.2	147.1	147.2	147.6	146.6	147.0	146.7	146.9
15	109.7	109.9	109. <b>8</b>	110.0	110.9	110.0	110.0	110.7
16	32.5	32.6	32.5	32.3	31.4	32.6	32.5	26.9 <sup>a</sup>
17	23.2	23.3	23.1	24.6	25.1	24.0	23.8	23.5
18	26.6	27.5	28.2	27.0	26.8	31.8	31.2	26.6 <sup>a</sup>
19	20.8	20.0	19.8	20.8	20.7	19.3	19.5	20.7
20	15.4	11.1	12.0	16.1	16.3	16.9	16.6	15,5

Table 2. <sup>13</sup>C NMR spectroscopic data of compounds 9a, 11-14 and 17

С	9a	11	11a	12	12a	13	14	17
1	37.9	39.6	39.8	32.1	32.9	40.4	41.3	41.5
2	33.6	33.9	33.6	25.2	22.7	177.7	171.6	175.6
3	217.2	219.6	219.6	76.0	78.0	186.8	179.6	189.1
4	47.3	47.6	47.5	36.2	35.9	45.4	46.2	39.4
5	54.5	54.8	53.9	49.0 <sup>a</sup>	49.9 <sup>a</sup>	48.2	47.9	56.6
6	20.7	20.9	20.6	19.6	19.4	20.6	21.9	74.2
7	41.6	42.3	41.6	42.7	42.4	42.4	41.7	45.7
8	76.2	<b>n</b> .o.	76.6	76.1	76.1	75.7 <sup>a</sup>	75.6	75.8
9	49.4	56.1	53.0	49.5 <sup>a</sup>	50.1 <sup>a</sup>	50.3	50.7	52.0
10	35.7	37.6	37.2	37.6	36.8	41.1	41.6	44.0
11	21.8	67.0	68.6	23.6	21.5	16.6	16.7	16.2
12	71.2	72.6	71.4	69.0	71.4	34,4	34.6	34.3
13	75.0	<b>n</b> .o.	75.8	n.o	74.8	73.3 <sup>a</sup>	73.2	74.1
14	146.3	146.2	144.8	147.1	146.5	147.4	147.5	147.1
15	111.1	111.6	112.1	110.6	111.0	109.7	109.6	110.0
16	27.1	25.9	27.2 <sup>a</sup>	26.9	27.1	32.3 <sup>b</sup>	32.4	32.4
17	23.3	25.5	24.5	24.3	23.9	23,9	23.9	24.8
18	26.6	26.9	26.4 <sup>a</sup>	28.2	27.8	32.3 <sup>b</sup>	27.5	25.9
19	20.8	20.9	20.8	21.7	21.3	23.9	23.0	20.5
20	15.3	15.9	16.5	15.6	15.4	20.8	19.6	20.2

<sup>a,b</sup> These values can be interchanged.

n.o. Not observed.

methyl groups and the assignment of its <sup>13</sup>C NMR data (table 2) indicated to us that the two acid groups must be at C-2 and C-3, as a consequence of the cleavage of ring A during the fermentation. Thus, the structure of this metabolite was determined as *ent-2,3-seco-13-epi*-manoyl oxide 2,3-dioic acid (13). This structure was confirmed by a 2D NMR study (COSY, HMQC and HMBC) of the dimethyl ester 14.

Another three seco-acids 15, 17 and 19 were isolated from the incubation, which were hydroxylated at the same positions, C-1( $\alpha$ ), C-6( $\beta$ ) and C-11 ( $\beta$ ), respectively, as the metabolites also derived from ribenone described above. Compound 15 was obtained in the monomethyl ester form 16 by methylation of the fractions



containing it. Its structure was determined on the basis of the following considerations: Its high resolution MS was in accordance with the formula  $C_{21}H_{34}O_6$ . The <sup>1</sup>H NMR spectrum showed resonances of five methyls, a methoxy group, a vinylic group and a proton geminal to a alcoholic group. This last signal appears as a sharp singlet at  $\delta$  4.54, which indicated that the hydroxyl group must be situated at C-1. Its  $\alpha$ -stereochemistry [1(R)-OH] was given considering that this compound can be biosynthetically formed from 5, which possesses a 1 $\alpha$ -OH group.

The arguments used above in the assignment of the hydroxyl groups at C-6( $\beta$ ) and C-11( $\beta$ ) in compounds 8 and 7 were the same ones utilized to locate them in substances 17 and 19, respectively, and consequently we will not repeat them. On the other hand, the formation of the single methyl esters 16 and 18, in the reaction of 15 and 17 with diazomethane, should be explained considering an association by hydrogen bridge between the 3-oxo group and the 1 $\alpha$ -OH or the 6 $\beta$ -OH, respectively. In an analogous manner, the formation of 20 occurs by the association of the 11-OH with the 2-oxo group.

Several consequences may be deduced from the results obtained in the biotransformation of 4:

1) This incubation confirms that although *ent*-13-*epi*-manoyl oxide (1) may be a final metabolite in G. *fujikuroi*, the introduction of a new oxygenated function in its molecule, as occurs in 2, and also in 3 and 4, may lead the fungus to transform these compounds. This may be due to the increase in polarity with respect to 1, which facilitates its transport across membranes.

2) The oxidation at C-19, which occurs at alcohol, aldehyde and acid level, after the formation of *ent*-kaur-16-ene in the biosynthetic pathway of gibberellins and kaurenolides, is not produced in the incubation of 4, or in the biotransformations of ribenol (2) and the 19-hydroxy-derivative 3. On the other hand, the hydroxylations at C-11( $\beta$ ) and C-12( $\beta$ ) produced in this feeding were also observed in the fermentation of 2, whilst those at C-12( $\beta$ ) and C-(7 $\alpha$ ) were produced in the incubation of 3.<sup>2</sup> The non-oxidation of C-19 and these hydroxylations observed may be induced by the presence of the oxygen atom of ring C in these *ent*-13-*epi*-manoyl-oxide derivatives. We have shown that an oxygen atom situated on the  $\alpha$ -face of ring D in *ent*-kaurane products, for example a 15 $\alpha$ , 16 $\alpha$ -epoxy,<sup>13</sup> a 15 $\alpha$ - or a 16 $\alpha$ -hydroxy derivative,<sup>14,15</sup> inhibits oxidation at C-19 and directs the hydroxylation at C-11( $\beta$ ) and C-7( $\alpha$ ). Thus, the substrates in both cases, in these *ent*-kaurane and *ent*-13-*epi*-manoyl oxide derivatives, possess an oxygen atom in a similar spatial region.

3) The formation of the 2,3-seco-acids is very interesting from the biosynthetic point of view, in relation to the specificity in the substrate of the enzymes involved in the metabolism of G. *fujikuroi*, and of the characteristics of the receptor involved in this process. In this microorganism, seco-ring B compounds such as fujenal (21) (or the corresponding diacid 22) and the triacid 23, are mainly formed from 7 $\beta$ -hydroxy-kaur-16-en-19-oic acid (24), via the 6 $\beta$ ,7 $\beta$ -dihydroxy derivative 25,<sup>16-18</sup> and, in a lesser proportion, from 7 $\beta$ -hydroxy-kaur-16-en-19-oic acid (24), via the 6 $\alpha$ ,7 $\beta$ -diol 26.<sup>18,19</sup> On the other hand, it has been shown that *ent*-7-oxokaur-16-en-19-oic

acid (27) was completely metabolized to the triacid 23 by a G. *fujikuroi* mutant.<sup>20</sup> Thus, the formation of the 2,3-*seco*-acid 13 from 4 must occur in a similar way to the triacid 23 from 27. This 7-oxo derivative 27 does not appear to be a natural metabolite of the fungus.

4) We have determined the low energy conformations of ribenone (4) and of *ent*-7-oxo-kaurenoic acid (27) using computational methods. Then, we have compared both structures, superimposing carbons 1, 2 and 3 of 4 with 5k, 6k and 7k of 27 [k of kaurene] observing the superimposing and equivalence of the other carbon atoms of ring A and B of these molecules, respectively, such as 4 (8k), 5 (9k) and 10 (10k), and also of other carbon joined to these rings, such as 6 (11k), 9 (1k), 18 (15k), 19 (14k) and 20 (20k) (Figure 1). These facts seem to indicate the same type of receptor, and consequently that the same enzyme(s) is (are) probably involved in the formation of the *seco*-acids 13 and 23.

5) If the same enzyme(s) is (are) involved in the cleavage of ring A of 4 and ring B of 27, it is clear that the presence of an acid group at C-19 in the latter is unnecessary for the cleavage of ring B to take place.

6) The mechanism of formation operating in the biotransformation of 27 into 23 has not been studied, but its analogy with the biotransformation reported herein of ribenone (4) into 13 can afford some insight. It is interesting that in both cases aldehyde intermediates were not obtained, which seems to indicate the absence of precursors of the type  $6\beta$ ,  $7\beta$ - (25) or  $6\alpha$ ,  $7\beta$ -diol (26) in the former process, or of a  $2\beta$ ,  $3\beta$ -diol in the latter. However, this is not conclusive proof, because a rapid oxidation of aldehydes to acids should be produced, although fujenal 21 is a relatively stable and abundant compound of the fungus. Therefore, intermediates of the type 6-hydroxy-7-oxo- or 2-hydroxy-3-oxo- as precursor of 23 or 13, respectively, seem more probable.

We must also consider that now in the incubation of 4 a reduction of the 3-oxo group to the 3 $\beta$ -alcohol occurs in the formation of 12, but, curiously, a *seco*-acid with a 12-hydroxyl group was not isolated. In any case, a bioreduction of the 7-oxo of 27 to give the gibberellin precursor 24, as a first step in the formation of the 6 $\beta$ ,7 $\beta$ -diol 25, does not occur, because gibberellins were not obtained in the biotransformation of 27.<sup>20</sup>

We think that the formation of 2,3-seco-acids in this feeding of 4 with G. fujikuroi reopens a interesting issue pending resolution, the mechanism of the quantitative formation of the 6,7-seco-acid 23 in the incubation of 7-oxo-ent-kaur-16-en-19-oic acid (27) with this fungus,<sup>20</sup> and its relation, if any, with the natural formation of fujenal (21) and the triacid 23.<sup>16,17</sup> The study of the formation of these seco-ring compounds is very interesting for two main reasons: a) In this fungus a relationship has been suggested between the production of gibberellins and that of the seco-acids, because from 24 a similar enzyme may form gibberellin A<sub>12</sub> aldehyde or the 6 $\beta$ ,7 $\beta$ -dihydroxy-derivative 25,<sup>21</sup> and also the ring-cleavage of the latter.<sup>19</sup> b) There is another important group of natural ent-kaurene derivatives with a seco-ring B skeleton, such as enmein (29), which possess antitumoral, antibacterial, insect antifeedant and plant growth inhibitory properties. More than 50 diterpenes of this type have been obtained from plants of the genera *Isodon* and *Rabdosia* (Labiatae).<sup>22,23</sup>



Figure 1. Comparison of the structures of ribenone (4) and 7-oxo-*ent*-kaur-16-en-19-oic acid (27) superimposing the C-1, C-2 and C-3 of the former over C-5, C-6 and C-7 of the latter. The numbering of 4 has been indicated. The hydrogen atoms have been omitted for reasons of clarity.

## EXPERIMENTAL

Mps were determined with a Reichert Thermovar apparatus and are uncorrected. IR and UV spectra were recorded in a Bruker IFS 55 and a Jasco V-560 spectrophotometer, respectively. <sup>1</sup>H NMR spectra were recorded in CDCl<sub>3</sub> solutions at 200.1 and 500.1 MHz, with a Bruker AC-200 or a Bruker AMX2-500 spectrometer, respectively, and the <sup>13</sup>C NMR spectra were run at 50.3 MHz, with a Bruker AC-200. Low- and high-resolution mass spectra were taken at 70 eV (probe) in a Shimadzu QP-2000 and a Micromass Autospec spectrometer, respectively. Conformations of minimum energy and comparison of structures were determined by computational methods employing the *Hyperchem 5.1* program of Hypercube Inc. Dry column chromatographies were made on silica gel Merck (0.02-0.063 mm). The fungal strain was *Gibberella fujikuroi* (IMI 58289) from the International Mycological Institute, Englefield Green, Egham, Surrey, UK.

Incubation procedure and isolation of products. The fungus G. fujikuroi inhibited with  $5\times10^{-5}$  M AMO 1618 was grown on shake culture at 25°C for two days in 75 conical flasks (250 ml), each containing 50 ml of sterile medium.<sup>17</sup> The substrate 4 (220 mg) dissolved in EtOH (15 ml) was equally distributed among the flasks and the incubation allowed to continue for a further six days. The broth was filtered and extracted with EtOAc. The mycelium was treated with liquid nitrogen, crushed with a mortar and extracted with EtOAc. The two extracts were combined, dried and concentrated. Chromatography of the residue on silica gel, eluting with petrol-EtOAc mixtures, gave starting material (4) (88 mg), ent-1en-3-oxo-13-epi-manoyl oxide (6) (3 mg), ent-1\beta-hydroxy-3-oxo-13-epi-manoyl oxide (5) (8 mg), ent-11\alpha-hydroxy-3-oxo-13-epi-manoyl oxide (8) (9 mg), ent-12\alpha-hydroxy-3-oxo-13-epi-manoyl oxide (7) (4 mg), ent-6\alpha-hydroxy-2,3-seco-13-epi-manoyl oxide (8) (9 mg), ent-12\alpha-hydroxy-3-oxo-13-epi-manoyl oxide (10) (5 mg), ent-2,3-seco-13-epi-manoyl oxide-2,3-dioic acid (13) (6 mg), ent-1\beta-hydroxy-2,3-seco-13-epi-manoyl oxide-dioic acid (15) (2 mg), ent-11\alpha-hydroxy-2,3-seco-13-epi-manoyl oxide (12) (5 mg). Compound (15) was identified in 3-methyl ester form (16) by methylation with diazomethane of the fractions containing it.

ent-1 $\beta$ -Hydroxy-3-oxo-13-epi-manoyl oxide (5). White needles, m.p. 114-116°C (MeOH); [M-CH<sub>3</sub>]<sup>+</sup> at m/z 305.2106, C<sub>19</sub>H<sub>29</sub>O<sub>3</sub> requires 305.2116; I.R. (CHCl<sub>3</sub>)  $\upsilon_{max}$  3350, 1695, 1400, 1370, 1100, 905 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.78, 1.03, 1.07, 1.14 and 1.26 (each 3H, s), 2.32 (1H, dd, J = 14.7 and 4.7 Hz, H-2), 2.98 (1H, dd, J = 14.7 and 7.9 Hz, H-2), 3.95 (1H, dd, J = 7.9 and 4.7 Hz, H-1), 4.93 (1H, d, J = 11 Hz, H-15),

4.99 (1H, d, J = 17.9 Hz, H-15), 6.00 (1H, dd, J = 17.9 and 11 Hz, H-14); EIMS m/z (rel. int.) 305 [M-CH<sub>3</sub>]<sup>+</sup> (19), 287 (11), 278 (1), 269 (2), 264 (1), 256 (10), 213 (3). Acetate (5a). [M]<sup>+</sup> at m/z 362.2467, C<sub>22</sub>H<sub>24</sub>O<sub>4</sub> requires 362.2457; I.R. (film)  $\upsilon_{max}$  2930, 1730, 1710, 1450, 1410, 1235, 1090, 1070, 990 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.82, 1.05, 1.09, 1.13 and 1.25 (each 3H, s), 2.05 (3H, s), 2.71 (1H, dd, J = 15.6 and 3.1 Hz, H-2), 3.11 (1H, dd, J = 15.6 and 8.7 Hz, H-2), 4.94 (1H, d, J = 11.1 Hz, H-15), 4.98 (1H, d, J = 18.6 Hz, H-15), 5.01 (1H, dd, J = 8.6 and 3.1 Hz, H-1), 6.00 (1H, dd, J = 18.6 and 11.1 Hz, H-14); EIMS m/z (rel. int.) 362 [M]<sup>+</sup> (1), 347 (38), 302 (2), 287 (86), 259 (1), 232 (6).

*ent-3-Oxo-1-en-13-epi-manoyl oxide (6).* White needles (petroleum ether-EtOAc), m.p. 170-173°C; [M]<sup>+</sup> at m/z 302.2240, C<sub>20</sub>H<sub>30</sub>O<sub>2</sub> requires 302.2245; U.V. (EtOH)  $\lambda_{max}$  228 nm ( $\varepsilon = 6.42 \times 10^3$ ); I.R. (film)  $\upsilon_{max}$  2930, 1660, 1450, 1400, 1260, 1090, 1075, 910 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.98, 1.07, 1.15, 1.16 and 1.30 (each 3H, s), 4.96 (1H, d, J = 10 Hz, H-15), 5.01 (1H, d, J = 18 Hz, H-15), 5.86 (1H, d, J = 10.2 Hz, H-2), 6.03 (1H, dd, J = 18 and 11 Hz, H-14), 7.14 (1H, d, J = 10.2 Hz, H-1); EIMS m/z (rel. int.) 302 [M]<sup>+</sup> (2), 287 (100), 275 (3), 232 (5), 217 (5), 213 (9), 204 (20), 199 (5), 189 (13), 185 (6).

ent-11α-Hydroxy-3-oxo-13-epi-manoyl oxide (7). White needles, m.p 117-119°C (MeOH);  $[M]^+$  at m/z 320.2355, C<sub>20</sub>H<sub>32</sub>O<sub>3</sub> requires 320.2351; <sup>1</sup>H NMR (500 MHz) δ 1.01, 1.05, 1.12, 1.25 and 1.28 (each 3H, s), 1.38 (1H, d, J = 9.6 Hz, H-9), 2.50 (3H, complex signal, H-2 and H-12), 4.18 (1H, td, J = 9.6 and 4.5 Hz, H-11), 4.96 (1H, d, J = 11.1 Hz, H-15), 5.09 (1H, d, J = 17.9 Hz, H-15), 6.01 (1H, dd, J = 17.9 and 11.1 Hz, H-14); EIMS m/z (rel. int.) 320 [M]<sup>+</sup> (4), 305 (100), 293 (2), 287 (45), 275 (3), 269 (16), 257 (2), 235 (13), 217 (12). Acetate (7a). White needles, m.p 141-144°C; [M]<sup>+</sup> at m/z 362.2461, C<sub>22</sub>H<sub>34</sub>O<sub>4</sub> requires 362.2457; I.R. (CHCl<sub>3</sub>) υ<sub>max</sub> 2900, 1710, 1115, 1060, 910 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz) δ 0.94, 1.04, 1.12, 1.25 and 1.32 (each 3H, s), 1.65 (1H, d, J = 10.1 Hz, H-9), 2.05 (3H, s), 2.47 (3H, complex signal, H-2 and H-12), 5.00 (1H, d, J = 11.1 Hz, H-15), 5.25 (1H, d, J = 17.9 Hz, H-15), 5.31 (1H, ddd, J = 10.1, 7.2 and 4.8 Hz, H-11), 5.94 (1H, dd, J = 17.9 and 11.1 Hz, H-14); EIMS m/z (rel. int.) 362 [M]<sup>+</sup> (1), 347 (7), 302 (7), 287 (100), 275 (5).

*ent*-6 $\alpha$ -Hydroxy-3-oxo-13-*epi*-manoyl oxide (8). Colorless gum, [M]<sup>+</sup> at m/z 320.2349, C<sub>20</sub>H<sub>32</sub>O<sub>3</sub> requires 320.2351; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.63, 1.15, 1.25, 1.27 and 1.33 (each 3H, s), 1.55 (1H, d, J = 11.3 Hz, H-7), 1.77 (1H, d, J = 11.3 Hz, H-5), 2.08 (1H, dd, J = 11.3 and 4.4 Hz, H-7), 3.83 (1H, td, J = 11.3 and 4.4 Hz, H-6), 4.93 (1H, d, J = 11.0 Hz, H-15), 4.98 (1H, d, J = 17.9 Hz, H-15), 5.99 (1H, dd, J = 17.9 and 11.0 Hz, H-14); EIMS m/z (rel. int.) 320 [M]<sup>+</sup> (4), 305 (100), 287 (31), 269 (17), 250 (3), 222 (8), 217 (14), 204 (13), 199 (8), 189 (6), 177 (6). Acetate (8a). [M]<sup>+</sup> at m/z 362.2463, C<sub>22</sub>H<sub>34</sub>O<sub>4</sub> requires 362.2457; I.R. (film)  $\upsilon_{max}$  2930, 1700, 1460, 1380,1090, 915 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.68, 1.05, 1.14, 1.26 and 1.31 (each 3H, s), 205 (3H, s), 2.10 (1H, d, J = 11.3 Hz, H-5), 2.20 (1H, dd, J = 11.3 and 4.3 Hz, H-7), 2.37 and 2.74 (each 1H, m, H-2), 4.95 (3H, complex signal, H-6 and H-15), 5.98 (1H, dd, J = 18.3 and 11.1 Hz, H-14); EIMS m/z (rel. int.) 362 [M]<sup>+</sup> (1), 347 (33), 287 (100), 269 (28), 232 (9), 217 (20), 204 (30), 199 (11).

*ent*-12 $\alpha$ -Hydroxy-3-oxo-13-*epi*-manoyl oxide (9). Colorless oil; [M]<sup>+</sup> at m/z 320.2357, C<sub>20</sub>H<sub>32</sub>O<sub>3</sub> requires 320.2351; I.R. (CHCl<sub>3</sub>)  $\upsilon_{max}$  3400, 2900, 1690, 1075, 905 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.81, 0.99, 1.06, 1.17 and 1.23 (each 3H, s), 2.47 (2H, m, H-11), 4.08 (1H, t, J = 3.1 Hz, H-12), 4.95 (1H, d, J = 18.1 Hz, H-15), 4.97 (1H, d, J = 11.4 Hz, H-15), 6.05 (1H, dd, J = 18.1 and 11.4 Hz, H-14); EIMS m/z (rel. int.) 320 [M]<sup>+</sup> (2), 305 (3), 302 (2), 293 (1), 287 (2), 277 (16), 250 (7), 235 (2), 217 (2), 207 (73). Acetate (9a). [M]<sup>+</sup> at m/z 362.2461, C<sub>22</sub>H<sub>34</sub>O<sub>4</sub> requires 362.2457; I.R. (film)  $\upsilon_{max}$  2930, 1380, 1730, 1700, 1170, 1080, 920 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.83, 1.03, 1.11, 1.12 and 1.29 (each 3H, s), 2.13 (3H, s), 5.03 (1H, d, J = 11.4 Hz, H-15), 5.08 (1H, d, J = 18.2 Hz, H-15), 5.37 (1H, t, J = 3.1 Hz, H-12), 6.05 (1H, dd, J = 18.2 and 11.4 Hz, H-14); EIMS m/z (rel. int.) 362 [M]<sup>+</sup> (1), 347 (3), 335 (1), 302 (5), 292 (5), 287 (7), 232 (8), 206 (100).

Reduction of 9.- Compound 9 (10 mg) in MeOH (4 ml) was treated at room temperature and stirred with sodium borohydride (3 mg) for 90 min. The mixture was poured into water, acidified with dilute HCl and extracted with EtOAc. The solvent was evaporated giving 10 (9 mg), which was identical with varodiol.

*ent*-11 $\alpha$ ,12 $\alpha$ -Dihydroxy-3-oxo-13-*epi*-manoyl oxide (11). [M]<sup>+</sup> at m/z 336.2313, C<sub>20</sub>H<sub>32</sub>O<sub>4</sub> requires 336.2300; I.R. (film)  $\upsilon_{max}$  3600, 2930, 1740, 1700, 1130, 955 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  1.03, 1.05, 1.12,

1.29 and 1.32 (each 3H, s), 1.76 (1H, d, J = 10.3 Hz, H-9), 2.13 (1H, d, J = 6.5 Hz,-OH), 2.24 (1H, d, J = 7.8 Hz, OH), 2.49 (3H, complex signal, H-2 and H-1( $\alpha$ ), 4.05 (1H, br s, H-12), 4.20 (1H, m, H-11), 5.06 (1H, d, J = 11.2 Hz, H-15), 5.12 (1H, d, J = 18 Hz, H-15), 6.05 (1H, dd, J = 18 and 11.2 Hz, H-14); EIMS *m/z* (rel. int.) 336 [M]<sup>+</sup> (1), 321 (3), 318 (1), 309 (2), 293 (9), 285 (3), 279 (2), 275 (7), 266 (9), 248 (11), 235 (6), 207 (31). Diacetate (11a). [M]<sup>+</sup> at *m/z* 420.2511, C<sub>24</sub>H<sub>36</sub>O<sub>6</sub> requires 420.2511; I.R. (film)  $\nu_{max}$  2930, 1740, 1710, 1460, 1370, 1240, 1130, 1080, 1030, 1020, 980 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.85, 1.04, 1.13, 1.14 and 1.39 (each 3H, s), 2.00 and 2.16 (each 3H, s), 2.16 (1H, d, J = 11.6 Hz, H-9), 5.11 (1H, d, J = 11.4 Hz, H-15), 5.38 (1H, d, J = 18.2 Hz, H-15), 5.53 (1H, dd, J = 11.5 and 3 Hz, H-11), 5.63 (1H, d, J = 3.0 Hz, H-12), 5.97 (1H, dd, J = 18.2 and 11.4 Hz, H-14); EIMS *m/z* (rel. int.) 420 [M]<sup>+</sup> (7), 405 (7), 360 (7), 350 (41), 345 (8), 335 (32), 308 (13), 303 (13), 290 (100), 285 (53), 275 (24), 248 (97), 230 (18), 215 (10), 206 (24).

*ent*-3 $\alpha$ ,12 $\alpha$ -Dihydroxy-13-*epi*-manoyl oxide (12). White needles, m.p. 202-205°C (MeOH); [M]<sup>+</sup> at *m*/z 322.2534, C<sub>20</sub>H<sub>34</sub>O<sub>3</sub> requires 322.2507; I.R. (CHCl<sub>3</sub>)  $\upsilon_{max}$  3550, 3400, 2900, 1695, 1440, 1370, 1070, 1050, 905 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.77, 0.83, 0.96, 1.25 and 1.26 (each 3H, s), 3.42 (1H, t, J = 2.8 Hz, H-3), 4.08 (1H, t, J = 3.5 Hz, H-12), 4.98 (1H, d, J = 18.2, H-15), 4.99 (1H, d, J = 11.4 Hz, H-15), 6.08 (1H, dd, J = 18.2 and 11.4 Hz, H-14); EIMS *m*/z (rel. int.) 322 [M]<sup>+</sup> (1), 304 (9), 295 (1), 289 (2), 286 (16), 277 (6), 271 (16), 259 (6), 219 (3), 201 (3). Diacetate (12a). [M-CH<sub>3</sub>]<sup>+</sup> at *m*/z 391.2488, C<sub>23</sub>H<sub>35</sub>O<sub>5</sub> requires 391.2484; I.R. (film)  $\upsilon_{max}$  2920, 1730, 1450, 1370, 1120, 960 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.75, 0.86, 0.88, 1.13 and 1.27 (each 3H, s), 2.08, 2.15 (each 3H, s), 4.66 (1H, t, J = 2.8 Hz, H-3), 5.02 (1H, d, J = 11.4 Hz, H-15), 5.08 (1H, d, J = 18.2 Hz, H-15), 6.05 (1H, dd, J = 18.2 and 11.4 Hz, H-14); EIMS *m*/z (rel. int.) 391 [M-CH<sub>3</sub>]<sup>+</sup> (2), 364 (1), 346 (2), 331 (6), 322 (8), 286 (2), 279 (4), 276 (10), 271 (6), 250 (46), 216 (3), 201 (6).

*ent-2,3-seco-13-epi-Manoyl* oxide-2,3-dioic acid (13).  $[M-CH_3]^+$  at m/z 337.2013,  $C_{19}H_{29}O_5$  requires 337.2014; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.84, 1.14, 1.17, 1.23 and 1.28 (each 3H, s), 2.47 and 2.72 (each 1H, d, J = 19 Hz, H-1), 4.93 (1H, d, J = 11 Hz, H-15), 4.97 (1H, d, J = 17.9 Hz, H-15), 6.00 (1H, dd, J = 17.9 and 11 Hz, H-14); EIMS m/z (rel. int.) 337  $[M-CH_3]^+$  (36), 319 (100), 301 (19), 295 (5), 277 (11), 273 (13), 259 (17), 221 (6), 205 (7). Dimethyl ester (14).  $[M]^+$  at m/z 380.2578,  $C_{22}H_{36}O_5$  requires 380.2562; I.R. (film)  $\upsilon_{max}$  2940, 1730, 1460, 1360, 1150, 910 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.81, 1.13, 1.21, 1.24 and 1.25 (each 3H, s), 2.03 (1H, dd, J = 11.2 and 9.8 Hz, H-9), 2.41 (2H, s, H-1), 2.53 (1H, dd, J = 12.3 and 12 Hz, H-5), 3.62 and 3.67 (3H each, s), 4.92 (1H, d, J = 11 Hz, H-15), 4.96 (1H, d, J = 17.9 Hz, H-15), 5.99 (1H, dd, J = 17.9 and 11 Hz, H-14); EIMS m/z (rel. int.) 380 [M]<sup>+</sup> (1), 365 (100), 333 (47), 321 (9), 315 (7), 305 (7), 301 (7), 291 (35), 273 (22), 261 (14), 237 (5), 229 (11).

*ent*-1 $\beta$ -Hydroxy-2,3-*seco*-13-*epi*-manoyl oxide-2,3-dioic acid 3-methyl ester (16). [M]<sup>+</sup> at m/z 382.2373, C<sub>21</sub>H<sub>34</sub>O<sub>6</sub> requires 382.2355; I.R. (film)  $\upsilon_{max}$  3400, 2930, 1740, 1460, 1380, 1290, 1150, 860 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  1.04, 1.15, 1.23, 1.29 and 1.33 (each 3H, s), 3.77 (3H, s), 4.54 (1H, s, H-1), 4.95 (1H, d, J = 11 Hz, H-15), 4.98 (1H, d, J = 18 Hz, H-15), 6.99 (1H, dd, J = 18 and 11 Hz, H-14); EIMS m/z (rel. int.) 382 [M]<sup>+</sup> (1), 367 (1), 349 (62), 337 (2), 317 (14), 307 (4), 299 (2), 279 (16), 271 (6), 219 (6), 217 (2).

*ent*-6α-Hydroxy-2,3-*seco*-13-*epi*-manoyl oxide-2,3-dioic acid (17).  $[M-H_2O]^+$  at *m/z* 350.2126, C<sub>20</sub>H<sub>30</sub>O<sub>5</sub> requires 350.2093; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.95, 1.16, 1.21, 1.34 and 1.44 (each 3H, s), 2.40 (1H, dd, J = 11.2 and 4.0 Hz, H-7), 2.47 (1H, d, J = 11.6 Hz, H-5), 2.48 and 2.58 (1H each, d, J = 15.9 Hz, H-1), 4.25 (1H, ddd, J = 11.7, 11.6 and 4 Hz, H-6), 4.96 (1H, d, J = 11 Hz, H-15), 5.00 (1H, d, J = 17.9 Hz, H-15), 6.01 (1H, dd, J = 17.9 and 11 Hz, H-14); EIMS *m/z* (rel. int.) 350 [M-H<sub>2</sub>O]<sup>+</sup> (1), 335 (100), 332 (1), 317 (22), 299 (2), 289 (3), 283 (6), 275 (31), 271 (5), 263 (6), 257 (4), 219 (17), 205 (10), 203 (4), 197 (10), 193 (12), 179 (8). **2-Methyl ester (18)**. [M]<sup>+</sup> at *m/z* 382.2374, C<sub>21</sub>H<sub>34</sub>O<sub>6</sub> requires 382.2355; I.R. (film)  $\upsilon_{max}$  3600, 2920, 1770, 1730, 1460, 1290, 1150, 910 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz)  $\delta$  0.93, 1.15, 1.20, 1.33 and 1.43 (each 3H, s), 2.39 (1H, dd, J = 11.7 and 4 Hz, H-7), 2.42 (1H, d, J = 11.7 Hz, H-5), 2.43 and 2.52 (1H each, d, J = 15.9 Hz, H-1), 4.23 (1H, ddd, J = 11.7 and 4 Hz, H-6), 4.95 (1H, d, J = 11.1 Hz, H-15), 4.99 (1H, d, J = 18 Hz, H-15), 6.00 (1H, dd, J = 18 and 11.1 Hz, H-14); EIMS *m/z* (rel. int.) 382 [M]<sup>+</sup> (1), 364 (2), 349 (100), 317 (23), 279 (14), 275 (3), 257 (2), 219 (5), 211 (11), 205 (6), 197 (10).

ent-11α-Hydroxy-2,3-seco-13-epi-manoyl oxide-2,3-dioic acid (19).  $[M-H_2O-CH_3]^+$  at m/z335.1863, C<sub>19</sub>H<sub>27</sub>O<sub>3</sub> requires 335.1858; <sup>1</sup>H NMR (500 MHz) δ 1.14, 1.19, 1.25, 1.26 and 1.44 (each 3H, s), 2.13 (1H, dd, J = 13 and 6.6 Hz, H-12), 2.16 (1H, d, J = 10.8 Hz, H-9), 2.34 (1H, t, J = 13 Hz, H-12), 2.48 and 2.75 (each 1H, d, J = 14.3 Hz, H-1), 4.77 (1H, ddd, J = 13, 10.7 and 6.6 Hz, H-11), 5.01 (1H, d, J = 10.8 Hz, H-15), 5.16 (1H, d, J = 17.4 Hz, H-15), 6.01 (1H, dd, J = 17.4 and 10.8 Hz, H-14); EIMS m/z (rel. int.) 335  $[M-H_2O-CH_3]^+$  (83), 317 (4), 299 (1), 289 (23), 275 (6), 271 (6), 267 (5), 221 (37), 219 (9), 203 (2), 201 (5), 179 (10). 2-Methyl ester (20).  $[M]^+$  at m/z 382.2362, C<sub>21</sub>H<sub>34</sub>O<sub>6</sub> requires 382.2355; I.R. (film) υ<sub>max</sub> 3400, 2930, 2850, 1730, 1250, 1080, 1020, 860 cm<sup>-1</sup>, <sup>1</sup>H NMR (500 MHz) δ 1.12, 1.17, 1.20, 1.35 and 1.43 (each 3H, s), 2.13 and 2.50 (each 1H, d, J = 14.3 Hz, H-1), 3.71 (3H, s), 4.74 (1H, ddd, J = 13, 10.7 and 6.7 Hz, H-11), 5.02 (1H, d, J = 10.8 Hz, H-15), 5.15 (1H, d, J = 17.5 Hz, H-15), 6.02 (1H, dd, J = 17.5 and 10.8 Hz, H-14); EIMS m/z (rel. int.) 382  $[M]^+$  (1), 364 (1), 349 (11), 307 (24), 289 (4), 279 (60), 261 (9).

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