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Biotransformation of 7α-hydroxy- and 7-oxo-*ent*-atis-16-ene derivatives by the fungus *Gibberella fujikuroi*

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ABSTRACT

The microbiological transformation of 7α,19-dihydroxy-*ent*-atis-16-ene by the fungus *Gibberella fujikuroi* gave 19-hydroxy-7-oxo-*ent*-atis-16-ene, 13(*R*),19-dihydroxy-7-oxo-*ent*-atis-16-ene, 7α,11β,19-trihydroxy-*ent*-atis-16-ene and 7α,16β,19-trihydroxy-*ent*-atis-16-ene, while the incubation of 19-hydroxy-7-oxo-*ent*-atis-16-ene afforded 13(*R*),19-dihydroxy-7-oxo-*ent*-atis-16-ene and 16β,17-dihydroxy-7-oxo-*ent*-atisan-19-al. The biotransformation of 7-oxo-*ent*-atis-16-en-19-oic acid gave 6β-hydroxy-7-oxo-*ent*-atis-16-en-19-oic acid, 6β,16β,17-trihydroxy-7-oxo-19-*nor*-*ent*-atis-4(18)-ene and 3β,7α-dihydroxy-6-oxo-*ent*-atis-16-en-19-oic acid.

1. Introduction

Although all the known natural gibberellins belong to the *ent*-kaur-16-ene series, the occurrence of gibberellin analogues of other tetracyclic diterpenes with *ent*-phyllocladene, *ent*-beyerene or *ent*-atisene skeleta is biosynthetically possible. The same applies for the kaurenolides and 6,7-*seco*-ring *ent*-kaur-16-enes, produced by the fungus *Gibberella fujikuroi* together with the gibberellins.

In a previous work we described the biotransformation of 7β-hydroxy-*ent*-atis-16-en-19-oic acid by this fungus, which led to the formation of atisagibberellins A_{12} and A_{14} (Hanson et al., 1979). However, oxygenated C-20 atisagibberellins or the corresponding C_{19}-gibberellins were not isolated. Later, Beale et al. (1983) obtained atisagibberellins, an atisenolide and a 6,7-*seco*-ring diacid by incubation of *ent*-atis-16-en-19-oic acid and its 13(*R*)-hydroxy-derivative with the mutant B1–41A of *G. fujikuroi*. We also carried out the preparation of atisenolides by incubation of 19-hydroxy-*ent*-atis-6,16-diene and *ent*-atis-6,16-dien-19-oic acid with this microorganism (Fraga et al., 1992).

Now, to complete our studies with this type of diterpenes we have examined the biotransformation of the 7α-hydroxy- and 7-oxo-*ent*-atis-16-ene derivatives **1**–**3** with the fungus *G. fujikuroi*. The aims were to prepare *seco*-ring B compounds, such as **5**, and compare the results with those previously obtained in the incubation of the corresponding *ent*-kaur-16-ene analogues, which led to the isolation of a 6,7-*seco*-acid, fujenoic acid (**6**), and other *ent*-kaur-16-ene derivatives (Fraga et al., 2005, 2007).

2. Results and discussion

The incubations were carried out in the presence of AMO 1618. This substance inhibits the production of *ent*-kaur-16-ene and consequently metabolites derived from it are not formed. However, the post-kaurene metabolism is not affected, it being possible to utilize the biosynthetic enzymes for the transformation of the artificial substrates (Dennis et al., 1965; Cross and Myers, 1969).

Substrate **1** was prepared by $LiAlH_4$ reduction of methyl 7-oxo-*ent*-atis-16-en-19-oate (**4**) (Fig. 1), which also afforded its 7-epimer (**7**) in very low yield. Compound **4** had been prepared, with a global yield 85%, by methylation, hydrolysis and oxidation of gummiferolic acid (**11**), a diterpenic angelate, isolated from *Margotia gummifera* in good yield (Pinar et al., 1978).

The incubation of 7α,19-dihydroxy-*ent*-atis-16-ene (**1**) with the fungus *G. fujikuroi* gave 19-hydroxy-7-oxo-*ent*-atis-16-ene (**2**), 13(*R*),19-dihydroxy-7-oxo-*ent*-atis-16-ene (**13**), 7α,11β,19-trihydroxy-*ent*-atis-16-ene (**14**) and 7α,16β,19-trihydroxy-*ent*-atisane (**15**) (Fig. 2).

The least polar metabolite isolated from this biotransformation was **2**, which showed in its HRMS the molecular ion at *m/z* 302.2253, corresponding to a molecular formula of $C_{20}H_{30}O_2$. Consequently, this metabolite had lost two hydrogens during the

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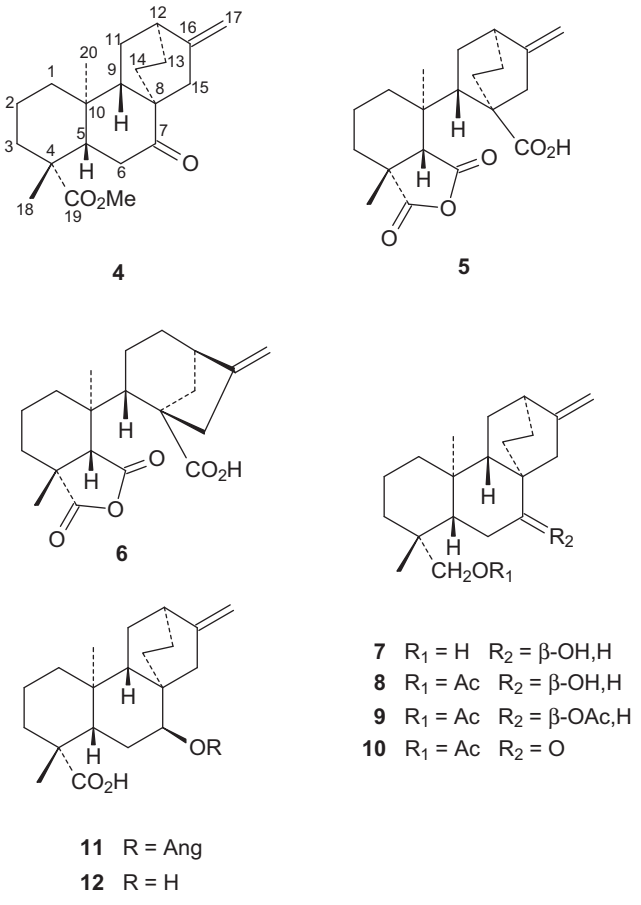

Fig. 1. Structures of compounds **4**–**12**.

incubation with respect to the substrate. The disappearances of the geminal proton to the secondary alcohol group and of the oxymethine in the ^{1}H and ^{13}C NMR spectra, respectively, and the presence in the latter of a new signal at δ 214.8, indicated that the secondary alcohol had been oxidized to a 7-oxo group. Thus, the structure of this substance was determined as 19-hydroxy-7-oxo-*ent*-atis-16-ene (**2**). Partial synthesis of this compound confirmed the assigned structure (see below).

The structure **13** was assigned to another metabolite obtained from this fermentation. The HRMS spectrum showed the molecular ion at 318.2185 ($C_{20}H_{30}O_3$). As in the metabolite **2**, the geminal proton to the 7α-hydroxyl group in the ^{1}H NMR spectrum, and the corresponding carbon in the ^{13}C NMR spectrum, were not observed. Alternatively, the presence in the molecule of a geminal hydrogen to a new secondary hydroxyl group at δ_H 4.04 (*dt*, *J* = 9.6 and 3.6 Hz), the corresponding oxymethine at δ_C 67.7, and the signal of a new oxo group at δ_C 214.4, were observed in both spectra. These two functions were located at C-13(*R*) and C-7 by assignment of the ^{13}C NMR spectrum using 2D NMR data (Table 1). Thus, the HMBC and NOESY experiments showed correlations of H-11, H-12 and H-14 with C-13, and of H-13 with H-12 and H-14, respectively. Double resonance experiments confirmed the assignment of the 13-hydroxyl group: irradiation of H-12 modifies the H-13 signal from a double triplet into a clean double doublet, whilst irradiation of H-14 collapses H-13 to a doublet. The stereochemistry of the 13-OH cannot be determined using the coupling constant of its geminal proton, because both hydrogens in the 13(*R*) or 13(*S*) configuration, have the same *J*-coupling. This is due to the symmetrical bridge that joins C-8 and C-12, which is perpendicular to the paper plane in the atisane skeleton. This fact also does not allow naming the stereochemistry of this alcohol as α or β. Consequently, the 13(*R*)-configuration assigned to this center was given considering the γ-gauche effect, −8.4 ppm, on the carbon resonance of C-11, which is produced by the presence of the 13-hydroxyl group. Thus, C-11 appears at δ_C 20.4 in **13** and 28.8 in **2**. These facts indicated that the structure of this metabolite was 13(*R*),19-dihydroxy-7-oxo-*ent*-atis-16-ene (**13**), which is formed via the 13(*R*)-hydroxylation of **2** (see also below). A compound of this type, 13(*R*)-angeloxy-*ent*-atis-16-en-19-oic acid, had been isolated from *Helianthus decapetalus* (Beale et al., 1983).

Another metabolite isolated (**14**) had the molecular formula $C_{20}H_{32}O_3$ (*m/z* 320.2325), which indicated that a new hydroxyl group had been introduced into the molecule of the substrate **1**. The ^{13}C NMR spectrum of **14**, in comparison with that of **1**, showed the presence of a new secondary carbon bearing a hydroxyl group at δ_C 69.9, and the corresponding proton resonance at δ_H 3.70, which is overlapped with a H-19. The relative low value of resonance was indicative of a oxymethine situated between carbons bearing hydrogen atoms, such as C-2, C-11 or C-13. On the other hand, the form of resonance and coupling of the geminal proton to the new hydroxyl group at δ_H 3.45 (*t*, *J* = 3.5 Hz), in C_6D_6, and the high value of the C-9 resonance at δ_C 63.9, permitted us to assign it to C-11(β). This assertion was confirmed in the HMBC experiment with a cross-peak between H-9 and C-11. Other correlations observed in this spectrum were: H-20 with C-9 and C-1; H-18 with C-3, C-5 and C-19; H-5 with C-4, C-10 and C-19; H-6 with C-7; H-15 with C-9 and C-16; and H-17 with C-12 and C-15. Thus, the structure of this product was determined as 7α,11β,19-trihydroxy-*ent*-atis-16-ene (**14**).

To the fourth compound isolated from this biotransformation the structure of 7α,16β,19-trihydroxy-*ent*-atis-16-ene (**15**) was assigned, on the basis of the following considerations: Its ^{1}H and ^{13}C NMR spectra showed the disappearance of the exocyclic double bond signals and the presence in the molecule of a new methyl group at δ_H 1.30 and δ_C 30.4. These values indicated that this methyl should be geminal to a hydroxyl group, which was confirmed in the HMBC experiment. The MS spectrum also explains this fact showing the peak of higher mass at 304.2444 ($C_{20}H_{32}O_2$), which is formed from the molecular ion by loss of water. The β-stereochemistry of the alcohol at C-16 was assigned considering that the hydration of the exocyclic double bond in the *ent*-atis-16-ene derivatives occurs by the less hindered β-face. The natural compounds of this type normally have the 16β-configuration (Schmitz et al., 1983; Piacenza et al., 1985; Hao and Nie, 1998; Tavares et al., 2007). This fact was confirmed in the NOESY spectrum where correlations of H-17 with H-13 and H-15α, and of H-7 with H-15β were observed, which indicated an α-stereochemistry for the C-17 methyl group.

We also carried out the biotransformation of the two 7-oxo-*ent*-atis-16-ene derivatives **2** and **3**. The former had been obtained in the previous feeding, but as this compound had been isolated in low yield, we synthesized it as follows: The diol **7**, obtained by $LiAlH_4$ reduction of gummiferolic acid (**11**) (Pinar et al., 1978), was partially acetylated affording the 19-monoacetate **8**, which was oxidized to the corresponding 7-oxo derivative **10**. Alkaline hydrolysis of this compound gave 19-hydroxy-7-oxo-*ent*-atis-16-ene (**2**). The global yield from **11** was 74%.

The microbiological transformation of **2** afforded the metabolites **13** and **16** (Fig. 2). The less polar of these compounds was identified as 13(*R*),19-dihydroxy-7-oxo-*ent*-atis-16-ene (**13**), which was identical with a substance obtained in the incubation of **1**.

The second metabolite was obtained in very low yield and its structure determined as 16β,17-dihydroxy-7-oxo-*ent*-atisan-19-al (**16**). In the HRMS the peak of higher mass appears at *m/z* 319.1910 ($C_{19}H_{27}O_4$), which is formed from the molecular ion by loss of a methyl group. Thus, the molecular formula of this metabolite was $C_{20}H_{30}O_4$. The ^{1}H and ^{13}C NMR spectra showed

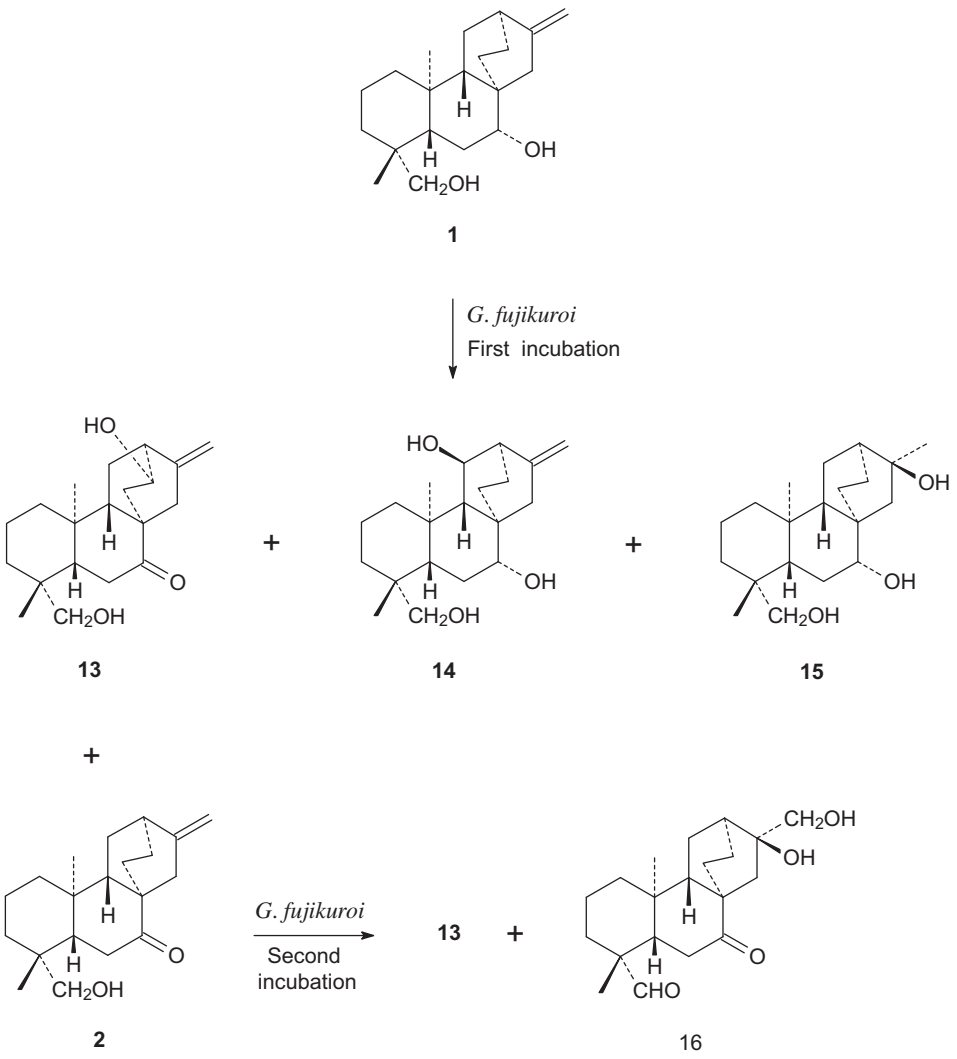

Fig. 2. Biotransformation of **1** and **2**.

Table 1
^{13}C NMR data of compounds **1**, **2**, **7**, **8**, **10** and **13**–**16**.

Position	1	2	7	8	10	13	14	15	16
1	39.0	39.0	39.3	39.2	38.9	39.0	39.3	38.9	38.3
2	17.7	17.6	17.2	17.7	17.6	17.6	17.6	17.6	17.9
3	35.6	35.8	35.9	36.5	36.6	35.8	35.6	35.6	34.8
4	37.7	38.1	37.6[a]	36.2	37.4	37.5	37.2	37.5	42.8
5	53.7	53.8	47.6[b]	47.6[a]	53.7	53.2	53.5	53.7	53.7
6	28.0	37.0	27.0[c]	26.9[b]	36.9	37.5	28.2	28.2	36.2
7	78.9	214.8	73.6	73.6	214.4	214.4	78.3	79.0	n.o.
8	38.1	47.4	37.7[a]	37.6	47.5	47.9	38.2[a]	39.3	47.8
9	52.0	52.8	47.7[b]	47.7[a]	52.8	52.5	63.9	50.4	50.1
10	38.1	37.4	37.8[a]	37.6	36.7	38.0	40.0[a]	37.8	37.7
11	28.5	28.4	27.0[c]	27.0[b]	28.4	20.4	69.9	23.1[a]	23.2[a]
12	36.2	35.8	36.3	36.3	35.8	44.6	44.4	37.6	31.3
13	26.3	26.0	27.8[c]	27.7[b]	26.0	67.7	23.7	22.9[a]	22.6[a]
14	20.9	27.6	28.3[c]	28.3[b]	27.6	37.3	19.5	19.4	26.8
15	43.8	40.0	41.6	41.6	40.0	39.3	43.3	52.7	44.1
16	151.8	150.2	152.0	151.9	150.0	146.7	145.7	71.6	73.5
17	105.0	106.3	105.0	105.0	106.3	109.1	110.8	30.4	68.2
18	26.9	26.4	26.7	27.1	26.8	26.4	26.9	26.8	23.8
19	65.4	65.3	65.9	67.4	66.9	65.2	65.6	65.6	204.7
20	14.4	14.0	13.9	13.9	13.9	14.8	15.8	14.4	13.1

[a,b,c]These values can be interchanged.
n.o., Not observed.

Table 2
^{13}C NMR data of compounds **3**, **17**, **19** and **21**.

Position	3	17	19	21
1	39.1	39.9	39.5	32.9
2	18.8	18.3	22.4	25.4
3	37.6	39.5	36.9	70.3
4	43.7	45.1	145.3	46.6[a]
5	53.9	58.8	58.4	58.4
6	38.2	73.9	70.7	219.3
7	214.9	214.0	214.9	82. 2
8	47.6	46.6	45.9	47.6[a]
9	51.9	52.0	49.3	49.6
10	38.0	39.0	39.8	46.9[a]
11	28.3	25.8	22.8	28.1[b]
12	35.8	35.7	31.4	35.8
13	26.1	28.5	23.7	26.3[b]
14	27.6	27.8	26.8	21.1
15	39.9	40.0	44.5	44.6
16	150.0	148.9	73.4	149.7
17	106.3	107.0	68.4	106.4
18	28.3	31.6	109.3	22.4
19	182.4	179.8	–	175.3
20	12.0	13.7	13.5	12.9

[a,b]These values can be interchanged.

resonances at δ_H 9.78 and δ_C 204.7, respectively. These signals were due to an unstable aldehyde group, which is formed by oxidation of the C-19 alcohol. In both spectra the disappearance of the signals originated by the exocyclic double bond was observed, being substituted by those due to a hydroxymethylene group, δ_H 3.50 and 3.60 (each *d*, *J* = 11.0 Hz) and δ_C 68.2. This primary alcohol was geminal to a hydroxyl group situated at C-16 (δ_C 73.5). In the HMBC experiment correlations of H-15 with C-17 and of H-20 with C-1, C-5 and C-9 could be observed. The small amount isolated, and the instability of this metabolite, did not permit us to run a NOESY spectrum to determine the configuration at C-16. However, comparison of the ^{13}C NMR data of C-16 and other surrounding carbons (C-12, C-13, C-14, C-15 and C-17) (Tables 1 and 2) of **16** with the corresponding of **19**, where a NOESY spectrum could be obtained, indicated that both metabolites had the same stereochemistry at C-16. Moreover, the formation of the 16β,17-diol, in these compounds, must occur in the same way (see below).

The third substrate, 7-oxo-*ent*-atis-16-en-19-oic acid (**3**), was prepared by hydrolysis of gummiferolic acid (**11**) and subsequent oxidation of the obtained 7β-alcohol (**12**), with a global yield of 82%. The biotransformation of **3** gave 6β-hydroxy-7-oxo-*ent*-atis-16-en-19-oic acid (**17**), 6β,16β,17-trihydroxy-7-oxo-19-*nor*-*ent*-atis-4(18)-ene (**19**) and 3β,7α-dihydroxy-6-oxo-*ent*-atis-16-en-19-oic acid (**21**) (Fig. 3).

The least polar of these metabolites was **17**. Its molecular formula ($C_{20}H_{28}O_4$) indicated that a new oxygen group had been

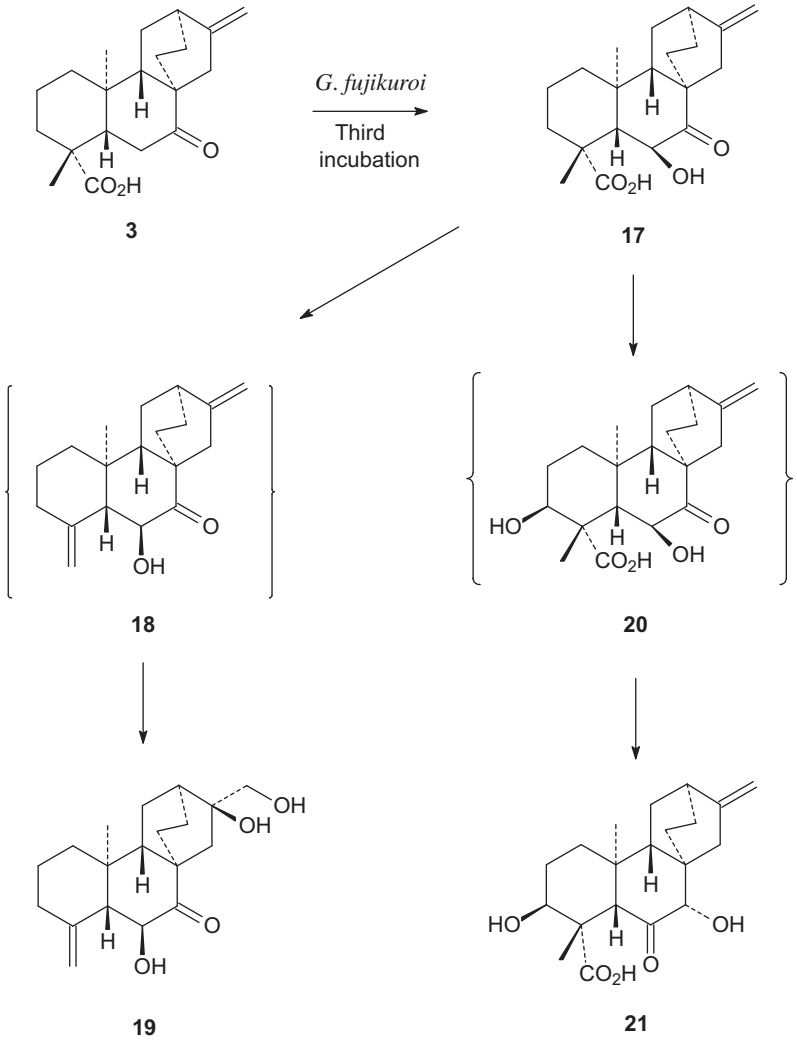

Fig. 3. Biotransformation of **3**.

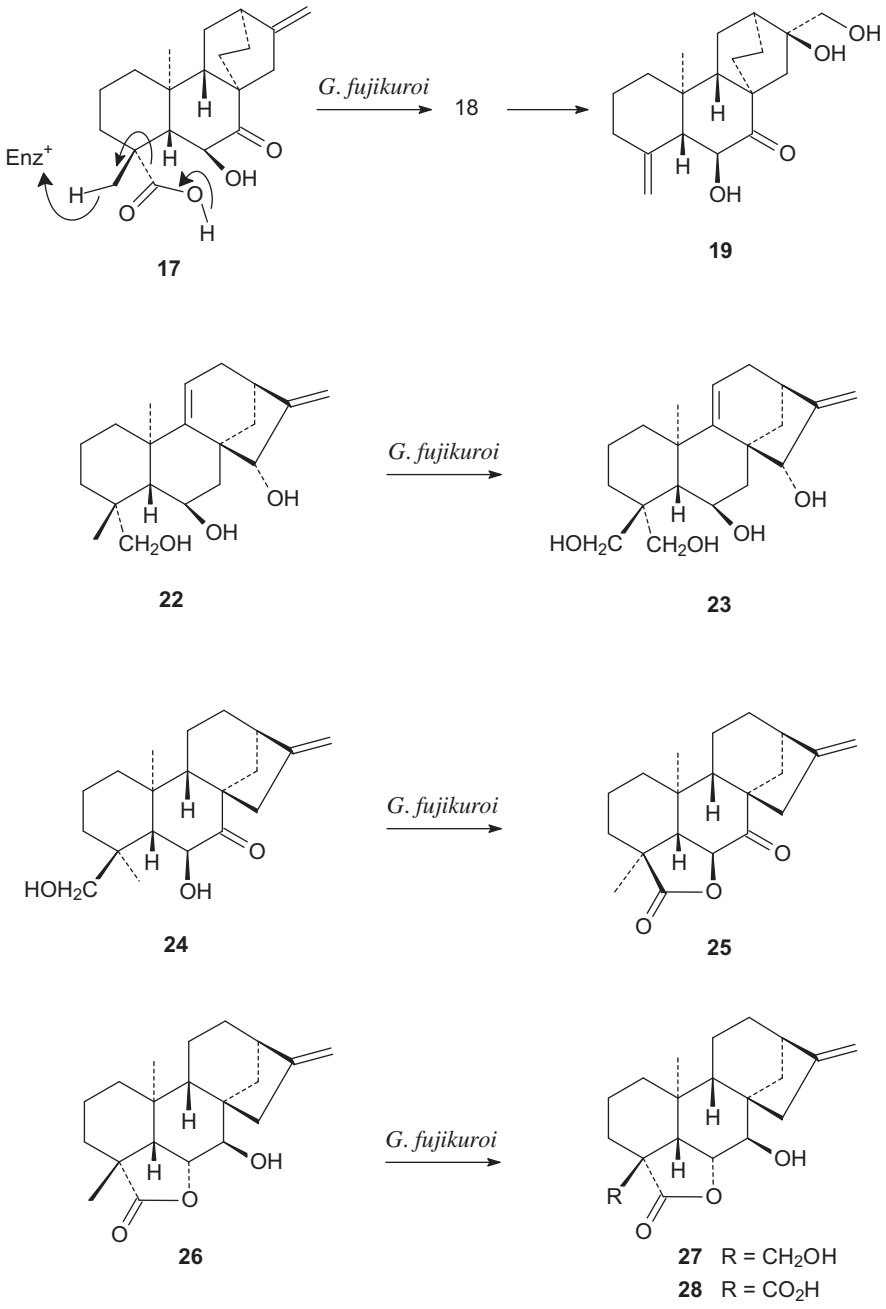

Fig. 4. Formation of **19**, **23**, **25**, **27** and **28**.

introduced into the molecule during the fermentation. This must be a part of a secondary hydroxyl group, because a geminal hydrogen to this alcohol appeared at δ_H 4.95 (*d*, *J* = 12.6 Hz) in the ^{1}H NMR spectrum, and the corresponding oxymethine at δ_C 73.9 in the ^{13}C NMR one. The form of resonance and the coupling constant in the proton spectrum indicated that the axial geminal proton was situated at C-6(α). This position was confirmed by the unambiguous assignment of the ^{13}C NMR spectrum using 2D NMR data (Table 2). The HMBC experiment showed correlations of H-6 with C-5 and C-7. Consequently, the structure of this metabolite was determined as 6β-hydroxy-7-oxo-*ent*-atis-16-en-19-oic acid (**17**).

The structure 6β,16β,17-trihydroxy-7-oxo-19-*nor*-*ent*-atis-4(18)-ene (**19**) was assigned to the second metabolite of this feeding on the following basis: In its HRMS, the peak of higher mass appeared at *m/z* 289.1818 ($C_{18}H_{25}O_3$), which is formed from the molecular ion by loss of a hydroxymethylene group. The presence of the 6β-hydroxyl was deduced from the NMR spectra. Thus, the resonance of its geminal proton at δ_H 4.43 (*d*, *J* = 12.1 Hz) and of the corresponding oxymethine at δ_C 70.7 were displayed. Other functions observed in these spectra were the hydroxymethylene group at δ_H 3.53 and 3.61 (each 1H, *J* = 11.0 Hz) and δ_C 68.4, respectively, and the signals corresponding to the 4,18-double bond at δ_H 4.97, 5.07 and δ_C 145.3, 109.3, respectively. In the HMBC spectrum the two H-18 showed correlations with C-3 and C-5. Other observed cross-peaks were H-6 with C-5, and H-17 with C-16. The α-stereochemistry of the C-17 hydroxymethylene group was determined observing the NOESY spectrum, which showed a correlation of H-17 with H-13. This configuration at C-16 is the one usually observed in natural compounds (Satti et al., 1987; Lal et al., 1990; Gustafson et al., 1991). The precursor of this type of products must be a 16β,17-epoxide, which is formed via the less hindered β-face. Opening of the oxirane ring and neutralization by a hydroxyl

group, probably of water origin, led to the corresponding 16β,17-diol. Compound **19** can be formed via the intermediate **18** by enzymatic hydrogen abstraction from the C-18 methyl, migration of the two electrons of the 4,19-bond and concomitant decarboxylation of the C-19 acid (Fig. 4). *ent*-Atisane derivatives with 16,17-dihydroxyl groups have been shown to possess interesting biological properties (Kurata et al., 2008; Tachibana et al., 2008).

The last compound isolated **21** showed in its HRMS that two oxygen atoms had been introduced in the molecule during the feeding (*m/z* 348.1928, $C_{20}H_{28}O_5$), which are part of two secondary hydroxyl groups. Thus, two geminal protons were observed in the ^{1}H NMR spectrum, the first at δ_H 4.18 (*t*, *J* = 2.5 Hz) being assigned at C-3(α). It showed in the ^{13}C NMR spectrum gauche effects with C-1 and C-5, while in the HSQC experiment a cross-peak was observed with the carbon at δ_C 70.3. In the HMBC experiment a correlation of H-3 with C-2 and C-5 was displayed. The second geminal hydrogen appeared at δ_H 4.19 (*d*, *J* = 1.4 Hz) and the corresponding carbon at δ_C 82.2, which is more typical of an oxymethine at C-7 than at C-6. The resonance of H-5 at δ_H 3.21 observed in the ketol **21**, in comparison with 1.36 in **17**, and correlations of H-7 with C-6 and C-15 in the HMBC experiment also confirmed this assertion. This means that the oxo group at δ_C 219.3 must be at C-6 and the hydroxyl group at C-7, which must be a consequence of the previous formation of **20** and subsequent isomerization to another ketol **21** (Fig. 3). Other correlations observed in the HMBC experiment were: H-5 with C-3, C-4, C-6, C-9, C-18, C-19 and C-20; H-15 with C-9 and C-17; H-9 with C-1, C-5, C-8, C-10, C-14 and C-15; H-18 with C-3, C-4, C-5 and C-19; and H-20 with C-1, C-5 and C-9. The α-equatorial stereochemistry was assigned to the C-7 alcohol considering the *J*-coupling through a carbonyl of 1.4 Hz, observed between the axial H-5 and the axial H-7 (Bhacca and Williams, 1964). These facts were confirmed in the NOESY experiment where cross-peaks of H-7 with H-5, H-9 and H-15, and of H-5 with H-7, H-9 and H-18, were displayed. Consequently, the structure of this compound was determined as 3β,7α-dihydroxy-6-oxo-*ent*-atis-16-en-19-oic acid (**21**). The observed isomerization perhaps occurs after the 3β-hydroxylation, via the intermediate **20**. The occurrence in plants of isomeric α-ketols in the beyerane class of diterpenes has been reported (Connolly and Harding, 1972; Ansell et al., 1993).

3. Conclusions

Several conclusions can be deduced from the results obtained in the microbiological transformations of the *ent*-atis-16-ene derivatives **1**–**3**:

1. We had indicated that the oxidation by this fungus of a 7α-hydroxyl to a 7-oxo group, in the *ent*-kaur-16-ene series, probably required the presence in the molecule of a 19-acid group (Fraga et al., 2007). However, the biotransformation of **1** indicated that a 19-hydroxyl group is sufficient to carry out this 7-oxidation.
2. A 7α-hydroxy- or a 7-oxo group in *ent*-atis-16-ene derivatives hinders the oxidation of C-19, which is characteristic of the biosynthesis of gibberellins, kaurenolides and *seco*-ring B compounds. However, this does not occur in the biotransformation by *G. fujikuroi* of analogous substrates of the *ent*-kaur-16-ene series, which are efficiently transformed into *seco*-ring B derivatives (Fraga et al., 2005, 2007).
3. The formation of a 6β-hydroxy-7-oxo intermediate in the biotransformation by *G. fujikuroi* of 7α-hydroxy or 7-oxo-*ent*-kaur-16-ene derivatives (Fraga et al., 2005) into 6,7-*seco*-ring compounds, such as fujenoic acid (**6**) or the corresponding triacid, has now been confirmed in the *ent*-atis-16-ene series with the biotransformation of **3**.
4. In the incubation of 7-oxo-*ent*-atis-16-en-19-oic acid (**3**) a 6β-hydroxylation took place, but the reaction did not progress to form a 6,7-*seco*-ring compound **5**, or the corresponding triacid, as occurs in the feeding of 7-oxo-*ent*-kaur-16-en-19-oic acid, which gave fujenoic acid (**6**) in very good yield (Fraga et al., 2005). This difference in behaviour, and the difficulty in the C-19 oxidation (see conclusion 2), can be attributed to steric hindrance produced by the C/D rings in the *ent*-atis-16-ene diterpenes. We must remember here that atisagibberellins functionalized at C-20 could not be obtained by biotransformation, probably for the same reason (Hanson et al., 1979; Beale et al., 1983).
5. The oxidative decarboxylation produced in the incubation of **3** to form the metabolite **19**, probably via **18**, must be highlighted. This is the first time that this reaction has been observed in a biotransformation by *G. fujikuroi*. Its biosynthesis implies an enzymatic abstraction of H-18 (see above, Fig. 4). Previously, we had obtained with this fungus the C-18 functionalized metabolites **23** and **25** in the biotransformation of **22** (unpublished work) and **24** (Fraga et al., 2005), respectively (Fig. 4). Now, the same H-18 abstraction led to a decarboxylation due to the presence of the C-19 acid group.
6. The metabolites **17**, **22** and **24** precursors of **19**, **23** and **25**, respectively, possess a 6β-hydroxyl group, which suggests that its presence in the molecule is a prerequisite to carry out the oxidation of C-18 by this fungus. We must also indicate that these levels of oxidation have also been observed in another two metabolites of *G. fujikuroi*, such as 7β,18-dihydroxykaurenolide (**27**) and the corresponding 18-acid (**28**) (Fig. 4), which are also functionalized at C-6 but with the α-stereochemistry (Cross et al., 1963; Hanson and Sarah, 1979).
7. The major dihydroxykaurenolides produced by the fungus *G. fujikuroi* are 7β,18- and 3β,7β-dihydroxykaurenolide, which are formed from 7β-hydroxy-kaurenolide (**26**) (Cross et al., 1968; Rojas et al., 2001). This functionalization, at C-18 and C-3(β), is the same now observed in the *ent*-atisane derivatives **19** and **21**, respectively, formed from the ketol **17** (Fig. 3), which could indicate that the same enzyme is working in both processes. This hypothesis is also in accordance with the results obtained from studies on the gibberellin P450-1 monooxygenase of this fungus, which catalyses the oxidation of C-7, C-6, C-3 and C-18 in the biosynthetic pathway of kaurenolides and *seco*-ring B acids, in addition to the GA_{14} synthesis (Rojas et al., 2001, 2004). If the same enzyme acts on the functionalization of C-3 and C-18, we also can conclude that the 3β-hydroxylation to form **21** is produced before, and not after, the ketol isomerization (Fig. 3).

4. Experimental

4.1. General procedures

Mps were determined with a Reichert Thermovar apparatus and are uncorrected. IR spectra were recorded in a Perkin Elmer 1600 FT. ^{1}H NMR spectra were recorded in $CDCl_3$ solution at 500.13 MHz with a Bruker AMX-500 spectrometer, and the ^{13}C NMR at 125.03 MHz in a Bruker AMX-500, except those of **1** and **15**, which were recorded at 50 MHz in a Bruker AC-200. Mass spectra were taken at 70 eV (probe) in a Shimadzu Q2000, and high resolution mass spectra in a Micromass Autospec spectrometer. Optical rotations have been measured at 25 °C in a Perkin Elmer 343 Plus. HPLC was performed using a Beckman System Gold 125P. Purification by HPLC was achieved using a silica gel column (Ultrasphere Si 5 μm, 10 × 250 mm). Dry column chromatography was made on silica gel Merck 0.040–0.063 mm. The substances were crystallized from petrol–EtOAc except where otherwise indi-

cated. Molecular mechanics calculations were carried out with the program Hyperchem 7.0 (Hypercube).

4.2. Microorganism

The fungal strain was *G. fujikuroi* MP-C (*Fusarium fujikuroi*) IMI 58289 and was a gift from Prof. J.R. Hanson, Department of Chemistry (University of Sussex, UK).

4.3. Incubation procedure

The fungus *G. fujikuroi*, inhibited with 5×10^{-5} M AMO 1618, was grown on shake culture at 25 °C for two days in 54–80 conical flasks (250 ml), each containing 50 ml of sterile medium comprising (per dm^3) glucose (80 g), NH_4NO_3 (0.48 g), KH_2PO_4 (5 g), $MgSO_4$ (1 g), and trace elements solution (2 ml). The trace elements solution contained (per 100 ml) $Co(NO_3)_2$ (0.01 g), $CuSO_4$ (0.015 g), $ZnSO_4$ (0.16 g), $MnSO_4$ (0.01 g), $(NH_4)_6Mo_7O_{24}$ (0.01 g). The substrate dissolved in EtOH (11–16 ml) and Tween 80 (three drops) was evenly distributed between the flasks and the incubation allowed to continue for a further 6 days. The broth was filtered and the culture filtrate extracted with EtOAc. The mycelium was treated with liquid nitrogen, crushed in a mortar and extracted with EtOAc. Both extracts were combined and separated into 'acidic' and 'neutral' fractions with aqueous $NaHCO_3$. The acidic fraction was methylated with CH_2N_2.

4.4. Preparation of the substrate 7α,19-dihydroxy-ent-atis-16-ene (**1**)

Methyl 7-oxo-*ent*-atis-16-en-19-oate (**4**) (330 mg) in dry THF (50 ml) was treated with $LiAlH_4$ (165 mg) at reflux for 3 h. The excess of reagent was destroyed with drops of EtOAc. The reaction mixture was poured over aqueous HCl (3%) (120 ml) and extracted with EtOAc in the usual way. The solution was concentrated in vacuo and chromatographed on SiO_2, eluting with a petrol–EtOAc gradient to afford **7** (11 mg) (Pinar et al., 1978) and **1** (280 mg).

4.4.1. 7β,19-Dihydroxy-ent-atis-16-ene (**7**)

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.90 (1H, *td*, *J* = 13.6 and 4.5 Hz, H-1β), 0.96 (3H, *s*, H-20), 0.97 (3H, *s*, H-18), 1.02 (2H, *m*, H-3β and H-11), 1.40 (2H, *m*, H-2 and H-11), 1.77 (2H, *m*, H-3α and H-6), 1.94 (1H, *dt*, *J* = 16.7 and 2.1 Hz, H-15), 2.24 (1H, *m*, H-12), 2.42 (1H, *ddd*, *J* = 16.7, 5.8 and 2.6 Hz, H-15), 3.46 (1H, *t*, *J* = 2.9 Hz, H-7), 3.49 and 3.75 (each 1H, *d*, *J* = 10.9 Hz, H-19), 4.62 (1H, *dd*, *J* = 4.2 and 2.1 Hz, H-17), 4.75 (1H, *dd*, *J* = 4.6 and 2.3 Hz, H-17); EIMS *m/z* (rel. int.): 304 $[M]^+$ (5), 286 (39), 273 (11), 268 (4), 255 (100), 243 (4), 199 (8), 173 (10), 164 (11); HRMS: $[M]^+$ at *m/z* 304.2401. $C_{20}H_{32}O_2$ requires 304.2402.

4.4.2. 7α,19-Dihydroxy-ent-atis-16-ene (**1**)

Colourless crystal, m.p. 79–80 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.77 (1H, *td*, *J* = 13.2 and 4.8 Hz, H-1β), 0.92 (1H, *td*, *J* = 13.7 and 4.2 Hz, H-3β), 0.94 (3H, *s*, H-20), 0.96 (3H, *s*, H-18), 1.00 (1H, *d*, *J* = 11.4 Hz, H-5), 1.11 (1H, *dd*, *J* = 11.4 and 6.7 Hz, H-9), 1.77 (2H, *m*, H-3α and H-6), 1.81 and 2.59 (each 1H, *br d*, *J* = 16.8 Hz, H-15), 2.23 (1H, *br s*, H-12), 3.25 (1H, *dd*, *J* = 11.5 and 4.3 Hz, H-7), 3.46 and 3.69 (each 1H, *d*, *J* = 11.0 Hz, H-19), 4.58 and 4.73 (each 1H, *br s*, H-17); EIMS *m/z* (rel. int.): 304 $[M]^+$ (84), 286 (25), 273 (11), 261 (100), 256 (82), 255 (61), 253 (16), 243 (13), 241 (26), 199 (14), 187 (12), 185 (11), 173 (13), 164 (36), 159 (17), 147 (21); $[M]^+$ at *m/z* 304.2396. $C_{20}H_{32}O_2$ requires 304.2402.

4.5. Incubation of 7α,19-dihydroxy-ent-atis-16-ene (**1**)

The substrate **1** (270 mg) in EtOH (16 ml) was distributed between 80 conical flasks. Its biotransformation gave in the neutral fraction 19-hydroxy-7-oxo-*ent*-atis-16-ene (**2**) (1 mg), starting material (160 mg), 13(*R*),19-dihydroxy-7-oxo-*ent*-atis-16-ene (**13**) (9 mg), 7α,11β,19-trihydroxy-*ent*-atis-16-ene (**14**) (3.5 mg) and 7α,16β,19-trihydroxy-*ent*-atisane (**15**) (12 mg). Transformed products were not obtained in the acid fraction.

4.5.1. 19-Hydroxy-7-oxo-ent-atis-16-ene (**2**)

Colourless crystal, m.p. 119–120 °C; $[\alpha]_D$: −7.6° (*c*, 0.58); IR (CCl_4) ν_{max} 3640, 2932, 1702 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.92 (1H, *td*, *J* = 13.2 and 3.6 Hz, H-1β), 0.96 (3H, *s*, H-18), 0.99 (1H, *td*, *J* = 13.7 and 3.7 Hz, H-3β), 1.14 (3H, *s*, H-20), 1.30 (1H, *m*, H-14), 1.39 (1H, *dd*, *J* = 14.6 and 2.7 Hz, H-5), 1.45 (1H, *m*, H-2), 1.48 (2H, *m*, H-9 and H-11), 1.57 (1H, *ddt*, *J* = 13.7 and 3.3 Hz, H-2α), 1.65 (3H, *m*, H-11 and 2H-13), 1.68 (1H, *m*, H-1α), 1.80 (1H, *br d*, *J* = 13.7 Hz, H-3α), 1.96 (1H, *m*, H-14), 2.10 (1H, *br d*, *J* = 17.2 Hz, H-15), 2.31 (1H, *br s*, H-12), 2.45 (1H, *dd*, *J* = 15.5 and 2.7 Hz, H-6β), 2.55 (1H, *dd*, *J* = 15.5 and 14.6 Hz, H-6α), 2.65 (1H, *br d*, *J* = 17.2 Hz, H-15), 3.54 and 3.74 (each 1H, *d*, *J* = 10.8 Hz, H-19), 4.67 and 4.79 (each 1H, *br s*, H-17); EIMS *m/z* (rel. int.): 302 $[M]^+$ (100), 284 (2), 271 (9), 189 (4), 175 (5), 162 (56), 147 (13); HRMS: $[M]^+$ at *m/z* 302.2253. $C_{20}H_{30}O_2$ requires 302.2246.

4.5.2. 13(R),19-Dihydroxy-7-oxo-ent-atis-16-ene (**13**)

A gum; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.93 (1H, *td*, *J* = 13.2 and 3.9 Hz, H-1β), 0.96 (3H, *s*, H-18), 0.99 (1H, *td*, *J* = 13.8 and 3.8 Hz, H-3β), 1.20 (3H, *s*, H-20), 1.40 (1H, *dd*, *J* = 13.5 and 4.2 Hz, H-5), 1.46 (3H, *m*, H-2β, H-9 and H-11), 1.58 (1H, *ddt*, *J* = 13.8 and 3.4 Hz, H-2α), 1.74 (1H, *br d*, *J* = 13.2 Hz, H-1α), 1.77 (1H, *dd*, *J* = 9.6 and 1.2 Hz, H-14), 1.80 (2H, *m*, H-3α and H-14), 2.03 (2H, *m*, H-11 and H-15), 2.34 (1H, *br s*, H-12), 2.48 (1H, *dd*, *J* = 15.9 and 4.2 Hz, H-6β), 2.52 (1H, *br d*, *J* = 17.1 Hz, H-15), 2.53 (1H, *dd*, *J* = 15.9 and 13.5 Hz, H-6α), 3.53 (1H, *dd*, *J* = 10.9 and 0.6 Hz, H-19), 3.75 (1H, *d*, *J* = 10.9 Hz, H-19), 4.04 (1H, *dt*, *J* = 9.6 and 3.6 Hz, H-13), 4.79 (1H, *dd*, *J* = 3.9 and 2.0 Hz, H-17), 4.91 (1H, *m*, H-17); EIMS *m/z* (rel. int.): 318 $[M]^+$ (100), 300 (15), 289 (11), 273 (40), 269 (17), 243 (9), 178 (29), 160 (14), 145 (18), 134 (26); HRMS: $[M]^+$ at *m/z* 318.2185. $C_{20}H_{30}O_3$ requires 318.2195.

4.5.3. 7α,11β,19-Trihydroxy-ent-atis-16-ene (**14**)

A gum; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.86 (1H, *d*, *J* = 3.3 Hz, H-9), 0.96 (3H, *s*, H-20), 0.97 (1H, *td*, *J* = 13.6 and 3.6 Hz, H-3β), 0.99 (3H, *s*, H-18), 1.05 (1H, *dd*, *J* = 12.7 and 1.7 Hz, H-5), 1.08 (1H, *m*, H-1β), 1.35 (1H, *m*, H-14), 1.87 (1H, *br d*, *J* = 17.0, H-15β), 2.29 (1H, *td*, *J* = 3.8 and 1.9 Hz, H-12), 2.64 (1H, *dt*, *J* = 17.0 and 2.2 Hz, H-15α), 3.31 (1H, *dd*, *J* = 11.4 and 4.8 Hz, H-7), 3.49 and 3.72 (each 1H, *d*, *J* = 10.9 Hz, H-19), 3.70 (1H, overlapped with H-19, H-11), 4.90 and 4.93 (each 1H, *dd*, *J* = 4.0 and 2.1 Hz, H-17); ^{1}H NMR (500 MHz, C_6D_6): δ 0.62 (1H, *d*, *J* = 3.3 Hz, H-9), 0.68 (3H, *s*), 0.73 (1H, *dd*, *J* = 12.7 and 1.7 Hz, H-5), 0.89 (3H, *s*), 1.66 (1H, *br d*, *J* = 17.0 Hz, H-15β), 2.08 (1H, *br s*, H-12), 2.58 (1H, *dt*, *J* = 17.0 and 2.2 Hz, H-15α), 2.91 (1H, *dd*, *J* = 11.4 and 4.7 Hz, H-7), 3.18 and 3.39 (each 1H, *d*, *J* = 10.5 Hz, H-19), 3.45 (1H, *t*, *J* = 3.5 Hz, H-11), 4.86 and 4.90 (each 1H, *dd*, *J* = 4.3 and 2.4 Hz, H-17); EIMS *m/z* (rel. int.): 320 $[M]^+$ (28), 302 (25), 287 (33), 284 (35), 271 (42), 253 (37), 243 (14), 225 (9), 210 (8), 197 (10), 189 (11), 185 (13), 181 (17), 171 (17), 161 (11), 148 (36), 137 (31), 131 (39), 123 (97), 81 (100); HRMS: $[M]^+$ at *m/z* 320.2325. $C_{20}H_{32}O_3$ requires 320.2351.

4.5.4. 7α,16β,19-Trihydroxy-ent-atisane (**15**)

A gum; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.84 (1H, *td*, *J* = 12.8 and 4.0 Hz, H-1β), 0.95 (3H, *s*, H-20), 0.97 (3H, *s*, H-18), 1.02 (1H, *br d*, *J* = 10.8 Hz, H-5), 1.13 (1H, *br d*, *J* = 13.5 Hz, H-15), 1.21 (1H, *m*, H-9), 1.30 (3H, *s*, H-17), 1.66 (1H, *br d*, *J* = 12.8 Hz, H-1α), 1.77 (2H, *m*, H-3 and H-6), 1.92 (1H, *d*, *J* = 13.5 Hz, H-15), 2.03 (1H, *m*, H-13), 3.26 (1H, *dd*, *J* = 11.5 and 4.7 Hz, H-7), 3.47 and 3.72 (each

1H, *d*, *J* = 10.9 Hz, H-19); EIMS *m/z* (rel. int.): 304 $[M\text{-}H_2O]^+$ (45), 291 (8), 289 (13), 286 (9), 273 (63), 261 (27), 256 (48), 255 (44), 246 (9), 215 (10), 164 (11), 159 (12), 151 (11), 147 (13), 145 (12), 135 (10), 134 (13), 131 (14), 123 (100); HRMS: $[M-H_2O]^+$ at *m/z* 304.2444. $C_{20}H_{32}O_2$ requires 304.2402.

4.6. Preparation of the substrate 19-hydroxy-7-oxo-ent-atis-16-ene (**2**)

4.6.1. Partial acetylation of 7β,19-dihydroxy-ent-atis-16-ene (**7**)

The diol **7** (330 mg) in pyridine (2 ml) at 0 °C was treated with Ac_2O (2 ml) and stirred for 90 min. Usual work-up and chromatography afforded starting material (**7**) (138 mg), 19-acetoxy-7β-hydroxy-*ent*-atis-16-ene (**8**) (213 mg) and 7β,19-diacetoxy-*ent*-atis-16-ene (**9**) (traces).

*4.6.1.1. 19-Acetoxy-7β-hydroxy-ent-atis-16-ene (**8**).* Colourless crystal, m.p. 101–103 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.90 (1H, *td*, *J* = 13.6 and 3.8 Hz, H-1β), 0.95 (3H, *s*, H-18), 0.98 (3H, *s*, H-20), 1.06 (2H, *m*, H-3β and H-11), 1.40 (2H, *m*, H-2 and H-11), 1.94 (1H, *dt*, *J* = 16.7 and 2.2 Hz, H-15α), 2.05 (3H, *s*, OAc), 2.24 (1H, *m*, H-12), 2.42 (1H, *ddd*, *J* = 16.7, 5.7 and 2.6 Hz, H-15β), 3.47 (1H, *t*, *J* = 2.8 Hz, H-7), 3.92 and 4.22 (each 1H, *d*, *J* = 10.9 Hz, H-19), 4.62 and 4.75 (each 1H, *dd*, *J* = 4.4 and 2.2 Hz, H-17); EIMS *m/z* (rel. int.): 346 $[M]^+$ (23), 328 (39), 273 (26), 255 (92), 240 (10), 225 (16), 173 (19), 164 (41), 123 (77), 109 (60), 93 (92), 81 (100); HRMS: $[M]^+$ at *m/z* 346.2498. $C_{22}H_{34}O_3$ requires 346.2508.

4.6.2. Oxidation of **8**

Compound **8** (270 mg) in acetone (15 ml) was treated with Jones reagent in the usual way affording 19-acetoxy-7-oxo-*ent*-atis-16-ene (**10**) (264 mg): colourless crystal, m.p. 116–117 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.91 (1H, *td*, *J* = 13.2 and 3.8 Hz, H-1β), 0.95 (3H, *s*, H-18), 1.05 (1H, *td*, *J* = 13.8 and 4.0 Hz, H-3β), 1.15 (3H, *s*, H-20), 1.31 (1H, *m*, H-14), 1.39 (1H, *dd*, *J* = 13.9 and 3.6 Hz, H-5), 1.44 (1H, *m*, H-2), 1.48 (2H, *m*, H-9 and H-11), 1.57 (1H, *ddt*, *J* = 13.7 and 3.5 Hz, H-2α), 1.65 (3H, *m*, H-11 and 2H-13), 1.70 (2H, *m*, H-1α and H-3α), 1.96 (1H, *m*, H-14), 2.05 (3H, *s*, OAc), 2.10 (1H, *br d*, *J* = 17.2 Hz, H-15β), 2.31 (1H, *br s*, H-12), 2.46 (1H, *dd*, *J* = 15.4 and 3.6 Hz, H-6β), 2.52 (1H, *dd*, *J* = 15.4 and 13.9 Hz, H-6α), 2.65 (1H, *dt*, *J* = 17.2 and 2.6 Hz, H-15α), 3.94 and 4.23 (each 1H, *d*, *J* = 11.0 Hz, H-19), 4.66 and 4.78 (each 1H, *dd*, *J* = 4.2 and 2.1 Hz, H-17); EIMS *m/z* (rel. int.): 344 $[M]^+$ (81), 284 (18), 271 (6), 256 (4), 241 (3), 213 (5), 201 (8), 189 (13), 175 (24), 162 (24), 147 (46); HRMS: $[M]^+$ at *m/z* 344.2349. $C_{22}H_{32}O_3$ requires 344.2351.

4.6.3. Hydrolysis of 19-acetoxy-7-oxo-ent-atis-16-ene (**10**)

Compound **10** (263 mg) in MeOH (15 ml) was treated with Na_2CO_3 (315 mg) with stirring at room temperature for 2 h and then at 40 °C for 30 min. The solvent was evaporated off and the syrupy oil neutralized with HCl solution (3%). Extraction with EtOAc in the usual way afforded 19-hydroxy-7-oxo-*ent*-atis-16-ene (**2**) (215 mg).

4.7. Incubation of 19-hydroxy-7-oxo-ent-atis-16-ene (**2**)

The substrate **2** (213 mg) in EtOH (11 ml) was distributed between 54 flasks. Its microbiological transformation and usual work-up gave acid (79 mg) and neutral fractions (617 mg). Chromatography of the latter afforded starting material **2** (32 mg), 13(*R*),19-dihydroxy-7-oxo-*ent*-atis-16-ene (**13**) (13 mg) (identical with that obtained in the feeding of **1**) and 16β,17-dihydroxy-7-oxo-*ent*-atisan-19-al (**16**) (1 mg). Transformed products were not obtained in the acid fraction.

4.7.1. 16β,17-Dihydroxy-7-oxo-ent-atisan-19-al (**16**)

Colourless oil; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.01 (1H, *m*, H-1β), 1.02 (6H, *s*, H-18 and H-20), 1.11 (2H, *m*, H-3β and H-14), 1.26 (1H, *m*, H-11), 1.37 (1H, *dd*, *J* = 14.6 and 3.4 Hz, H-15), 1.59 (1H, *dd*, *J* = 14.8 and 3.0 Hz, H-5), 1.65 (1H, *m*, H-2), 1.74 (2H, *m*, H-1α and H-9), 1.77 (1H, *d*, *J* = 14.6 Hz, H-15), 1.93 (1H, *br s*, H-12), 1.97 (1H, *m*, H-14), 2.05 (1H, *m*, H-11), 2.16 (1H, *br d*, *J* = 13.6 Hz, H-3α), 2.65 (1H, *dd*, *J* = 15.3 and 3.0 Hz, H-6β), 2.83 (1H, *dd*, *J* = 15.3 and 14.8 Hz, H-6α), 3.50 and 3.60 (each 1H, *d*, *J* = 11.0 Hz, H-17), 9.78 (1H, *s*, H-19); EIMS *m/z* (rel. int.): 334 $[M]^+$ (1), 319 (100), 303 (14), 291 (10), 286 (11), 275 (13), 255 (5), 229 (6), 217 (6), 178 (8), 161 (14), 137 (26); HRMS: $[M-CH_3]^+$ at *m/z* 319.1910. $C_{19}H_{27}O_4$ requires 319.1909.

4.8. Preparation of the substrate 7-oxo-ent-atis-16-en-19-oic acid (**3**)

Compound **12** (238 mg) in acetone (10 ml) was treated with Jones reagent as above affording 7-oxo-*ent*-atis-16-en-19-oic acid (**3**) (230 mg). Colourless crystal; m.p. 175–176 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.94 (1H, *td*, *J* = 13.4 and 4.1 Hz, H-1β), 1.05 (1H, *td*, *J* = 13.6 and 4.1 Hz, H-3β), 1.09 (3H, *s*, H-20), 1.24 (3H, *s*, H-18), 1.35 (1H, *ddd*, *J* = 14.9, 6.5 and 1.5 Hz, H-14), 1.48 (1H, *dd*, *J* = 14.6 and 2.8 Hz, H-5), 1.49 (3H, *m*, H-2β, H-9 and H-11), 1.65 (3H, *m*, H-11 and 2H-13), 1.72 (1H, *br d*, *J* = 13.4 Hz, H-1α), 1.87 (1H, *ddt*, *J* = 13.9 and 3.6 Hz, H-2α), 2.05 (1H, *m*, H-14), 2.11 (1H, *br d*, *J* = 17.2 Hz, H-15), 2.23 (1H, *br d*, *J* = 13.6 Hz, H-3α), 2.32 (1H, *br s*, H-12), 2.62 (1H, *dd*, *J* = 15.6 and 2.8 Hz, H-6β), 2.65 (1H, *dt*, *J* = 17.2 and 2.5 Hz, H-15), 3.07 (1H, *dd*, *J* = 15.6 and 14.6 Hz, H-6α), 4.68 and 4.79 (each 1H, *dd*, *J* = 4.0 and 2.0 Hz, H-17); EIMS *m/z* (rel. int.): 316 $[M]^+$ (100), 270 (16), 255 (11), 227 (3), 215 (8), 201 (7), 174 (11), 162 (69), 147 (42), 119 (24). HRMS: $[M]^+$ at *m/z* 316.2044. $C_{20}H_{28}O_3$ requires 316.2038.

4.9. Incubation of 7-oxo-ent-atis-16-en-19-oic acid (**3**)

The substrate **3** (225 mg) in EtOH (12 ml) was distributed between 58 conical flasks. Its biotransformation gave starting material (92 mg), 6β-hydroxy-7-oxo-*ent*-atis-16-en-19-oic acid (**17**) (8 mg), 6β,16β,17-trihydroxy-7-oxo-19-*nor*-*ent*-atis-4(18)-ene (**19**) (1 mg) and 3β,7α-dihydroxy-6-oxo-*ent*-atis-16-en-19-oic acid (**21**) (11 mg), in the neutral fraction. Transformed products were not obtained in the acid fraction.

4.9.1. 6β-Hydroxy-7-oxo-ent-atis-16-en-19-oic acid (**17**)

Colourless oil; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.92 (1H, *td*, *J* = 13.3 and 3.8 Hz, H-1β), 1.08 (1H, *td*, *J* = 13.7 and 4.1 Hz, H-3β), 1.19 (3H, *s*, H-20), 1.36 (1H, *d*, *J* = 12.6 Hz, H-5), 1.41 (1H, *m*, H-14), 1.49 (3H, *m*, H-2β, H-9 and H-11), 1.51 (3H, *s*, H-18), 1.68 (4H, *m*, H-1α, H-11, 2H-13), 1.84 (1H, *ddt*, *J* = 13.9 and 3.6 Hz, H-2α), 2.09 (1H, *m*, H-14), 2.24 (2H, *m*, H-3α and H-15), 2.37 (1H, *br s*, H-12), 2.69 (1H, *dt*, *J* = 17.3 and 2.5 Hz, H-15), 4.11 (1H, *br s*, OH), 4.72 and 4.83 (each 1H, *dd*, *J* = 4.2 and 2.1 Hz, H-17), 4.95 (1H, *d*, *J* = 12.6 Hz, H-6); EIMS *m/z* (rel. int.): 332 $[M]^+$ (6), 314 (28), 286 (41), 271 (17), 258 (13), 243 (16), 231 (20), 215 (18), 189 (12), 181 (11), 153 (100); HRMS: $[M]^+$ at *m/z* 332.1992. $C_{20}H_{28}O_4$ requires 332.1988.

4.9.2. 6β,16β,17-Trihydroxy-7-oxo-19-nor-ent-atis-4(18)-ene (**19**)

Colourless oil; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.10 (3H, *s*, H-20), 1.15 (1H, *td*, *J* = 13.2 and 4.7 Hz, H-1β), 1.21 (1H, *m*, H-14), 1.38 (1H, *ddd*, *J* = 13.3, 7.8 and 2.2 Hz, H-11α), 1.51 (1H, *dd*, *J* = 14.7 and 3.5 Hz, H-15), 1.60 (4H, *m*, 2H-2 and 2H-13), 1.72 (1H, *br d*, *J* = 13.2 Hz, H-1α), 1.82 (1H, *d*, *J* = 14.7 Hz, H-15), 1.87 (1H, *ddd*, *J* = 10.9, 7.8 and 1.5 Hz, H-9), 1.96 (2H, *m*, H-3 and H-12), 2.02 (1H, *d*, *J* = 12.1 Hz, H-5), 2.08 (1H, *m*, H-14), 2.15 (1H, *m*, H-11β), 2.37 (1H, *br d*, *J* = 13.1 Hz, H-3), 3.53 and 3.61 (each 1H, *d*, *J* = 11.0 Hz, H-17), 4.43 (1H, *d*, *J* = 12.1 Hz, H-6), 4.97 (1H, *br s*, H-

18), 5.07 (1H, *dd*, *J* = 2.8 and 1.4 Hz, H-18); EIMS *m/z* (rel. int.): 289 $[M\text{-}CH_2OH]^+$ (100), 271 (9), 243 (4), 215 (2), 137 (16); HRMS: $[M-CH_2OH]^+$ at *m/z* 289.1818. $C_{18}H_{25}O_3$ requires 289.1804.

*4.9.3. 3β,7α-Dihydroxy-6-oxo-ent-atis-16-en-19-oic acid (**21**)*

A gum; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.95 (3H, *s*, H-20), 1.27 (1H, *m*, H-14), 1.34 (3H, *s*, H-18), 1.79 (1H, *dddd*, *J* = 13.5, 11.6, 2.9 and 2.9 Hz, H-11), 1.93 (1H, *ddd*, *J* = 11.6, 6.0 and 1.2 Hz, H-9), 2.18 (1H, *m*, H-2), 2.22 (1H, *br d*, *J* = 16.7 Hz, H-15β), 2.32 (1H, *br s*, H-12), 2.74 (1H, *dt*, *J* = 16.7 and 2.2 Hz, H-15α), 3.21 (1H, *d*, *J* = 1.4 Hz, H-5), 4.18 (1H, *t*, *J* = 2.5 Hz, H-3), 4.19 (1H, *d*, *J* = 1.4 Hz, H-7), 4.68 (1H, *dd*, *J* = 3.9 and 2.1 Hz, H-17) and 4.82 (1H, *dd*, *J* = 4.3 and 2.1 Hz, H-17); ^{1}H NMR (500 MHz, C_6D_6): δ 0.71 (3H, *s*, H-20), 0.87 (1H, *m*, H-14), 1.10 (3H, *s*, H-18), 1.77 (1H, *ddd*, *J* = 16.7, 5.6 and 2.6 Hz, H-15β), 2.01 (1H, *m*, H-12), 2.26 (1H, *m*, H-2α), 2.62 (1H, *dt*, *J* = 16.7 and 2.2 Hz, H-15α), 2.82 (1H, *d*, *J* = 1.4 Hz, H-5), 3.46 (1H, *d*, *J* = 1.4 Hz, H-7), 4.00 (1H, *t*, *J* = 2.5 Hz, H-3), 4.67 (1H, *dd*, *J* = 3.9 and 2.1 Hz, H-17), 4.82 (1H, *dd*, *J* = 4.3 and 2.1 Hz, H-17); EIMS *m/z* (rel. int.): 348 $[M]^+$ (41), 320 (9), 315 (6), 312 (6), 302 (62), 284 (23), 269 (20), 257 (12), 227 (11), 213 (14), 197 (32), 181 (23), 169 (23), 151 (85); HRMS: $[M]^+$ at *m/z* 348.1928. $C_{20}H_{28}O_5$ requires 348.1937.

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