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The microbiological transformation of 7α-hydroxy-*ent*-kaur-16-ene derivatives by *Gibberella fujikuroi*

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Abstract

The biotransformation of 7α-hydroxy-*ent*-kaur-16-ene (*epi*-candol A) by the fungus *Gibberella fujikuroi* gave 7α,16α,17-trihydroxy-*ent*-kaur-16-ene and a *seco*-ring B derivative, fujenoic acid, whilst the incubation of candicandiol (7α,18-dihydroxy-*ent*-kaur-16-ene) and canditriol (7α,15α,18-trihydroxy-*ent*-kaur-16-ene) afforded 7α,18,19-trihydroxy-*ent*-kaur-16-ene and 7α,11β,15α,18-tetrahydroxy-*ent*-kaur-16-ene, respectively. The presence of a 7α-hydroxyl group in *epi*-candol A avoids its biotransformation along the biosynthetic pathway of g\ibberellins, and directs it to the *seco*-ring B acids route. The 15α-hydroxyl group in canditriol inhibits oxidation at C-19 and direct hydroxylation at C-11(β). The formation of fujenoic acid, from 7α-hydroxy-*ent*-kaur-16-ene, probably occurs via 7α-hydroxykaurenoic acid and 7-oxokaurenoic acid, with subsequent hydroxylation at the C-6(β) position.

Keywords: *Gibberella fujikuroi* MP-C; Diterpenes; *ent*-Kaur-16-ene derivatives; *epi*-Candol A; Candicandiol; Canditriol

1. Introduction

During the last years, we have studied the microbiological transformation of diterpenes with different skeletons by the fungus *Gibberella fujikuroi* MP-C (Fraga et al., 2003a). The aim of these works has been to know the specificity in the substrate of the enzymes involved in the biosynthetic processes of this fungus, and the characteristics of the active sites involved in them. Two of the themes studied were the biotransformations of 7β-hydroxy- (Fraga et al., 1978, 1980), and 7-oxo-*ent*-kaurene derivatives (Fraga et al., 2005), considering the importance of the 7β-hydroxyl group in the biosynthesis of gibberellins and *seco*-ring B compounds (MacMillan, 1997). To complete these studies it was necessary to investigate the incubation of the corresponding 7α-hydroxy derivatives with this fungus. Thus, we describe here the results obtained in the microbiological transformations of 7α-hydroxy-*ent*-kaur-16-ene (*epi*-candol A) (**1**), 7α,18-dihydroxy-*ent*-kaur-16-ene (candicandiol) (**5**) and 7α,15α,18-trihydroxy-*ent*-kaur-16-ene (canditriol) (**9**).

2. Results and discussion

Candicandiol (**5**) and canditriol (**9**) had been isolated from a variety of *Sideritis candicans* (Bretón et al., 1969; Rodríguez et al., 1970; Piozzi et al., 1971) and *S. infernalis* (Fernández et al., 1986), respectively. Canditriol (**9**) had also been obtained in the biotransformation of candicandiol by *Mucor plumbeus* (Fraga et al., 2003b). 7α-Hydroxy-*ent*-kaur-16-ene (*epi*-candol A) (**1**) had been isolated from the liverwort *Jungermania truncata* (Buchanan et al., 1996) and synthesized from *ent*-kaur-16-ene, via a 15-hydroxymethylene derivative to functionalize ring B (Node et al., 1986). We have now prepared **1** by sodium

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borohydride reduction of 7-oxo-*ent*-kaur-16-ene (**3**), in the same way than Fujita et al. (1979). This 7-oxo-derivative had been obtained by oxidation of candol A (**4**), and partially synthesized from epicandicandiol (**11**) (Fujita et al., 1979; Fraga et al., 2005). The diterpene candol A had been isolated from *S. candicans* (González et al., 1973).

The biotransformations with *G. fujikuroi* were carried out, during six days, in the presence of AMO 1618, a quaternary ammonium compound that inhibits the formation of *ent*-kaurene without perturbing the post-kaurene metabolism (Dennis et al., 1965; Cross and Myers, 1969), thus facilitating the isolation of the substances formed.

The incubation of 7α-hydroxy-*ent*-kaur-16-ene (*epi*-candol A) (**1**) with this fungus gave 7α,16α,17-trihydroxy-*ent*-kaur-16-ene (**13**) and fujenoic acid (**17**). The latter is in the fungus in the triacid form **20** (Rojas et al., 2004; Fraga et al., 2005) and the anhydride structure **17** is probably formed during the isolation. The metabolite **13** was obtained as the triacetate (**14**) and the 7α,17-diacetate (**15**), by acetylation and chromatography of the fractions containing it.

The diacetate (**15**) did not show the molecular ion in the high resolution MS, the peak of higher mass being observed at *m*/*z* 346.2508, which is formed by loss of acetic acid. Thus, the molecular formula of the corresponding alcohol formed in the feeding must be $C_{20}H_{34}O_3$. This indicated that during the incubation two oxygen and two hydrogen atoms were introduced in the molecule. The ^{1}H NMR spectrum of **15**, in comparison with that of the acetylated substrate **2**, showed the presence of an AB system as a pair of doublets at δ 4.15 and 4.22, with a coupling constant of 11.4 Hz, and the disappearance of the hydrogens of the exocyclic double bond. A 2D NMR study (COSY, NOESY, HMQC and HMBC) permitted us to give for the diacetate the structure **15**, and **13** to the corresponding alcohol formed in the feeding. Thus, in the HMBC spectrum correlations were observed of H-15 with C-17 and of H-17 with C-13, C-15 and C-16. Moreover, in the NOESY spectrum a correlation was observed between H-15 and H-17. Compound **13** can be originated by the epoxidation of the double bond and opening of the oxirane ring in aqueous medium.

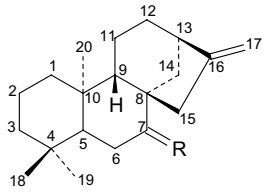
The second compound isolated from this biotransformation was identified as fujenoic acid (**17**) by comparison with a sample, obtained previously in the feeding of 7-oxo-*ent*-kaur-16-ene (**3**) (Fraga et al., 2005). This assignment was confirmed by studying the ^{1}H NMR spectra of its methyl ester (**18**). As stated above, the natural compound formed in the incubation must be the triacid **20**.

Since, **20** is an endogenous metabolite of the fungus, and was isolated in low yield as the anhydride **17**, it was necessary to confirm that this metabolite was formed in the biotransformation from **1**, because the possibility exists that the amount of the inhibitor AMO-1618 used in the incubation was insufficient. Thus, we made a new feeding with the corresponding deuterated derivative at C-18, [18-$^2H_1$]-7α-hydroxy-*ent*-kaur-16-ene (**1d**), obtaining the expected deuterated metabolites [18-$^2H_1$]-7α,16α,17-trihydroxy-*ent*-kaur-16-ene (**13d**), [18-$^2H_1$]-fujenoic acid (**17d**) and the [18-$^2H_1$]-triacid (**20d**). Compound **13d** was isolated as the diacetate (**15d**), by acetylation and chromatography of the fractions containing it, while the metabolite **20d** was obtained as its trimethyl ester (**21d**), by methylation of the corresponding fractions with CH_2N_2. The substrate **1d** was prepared in the following way: partial hydrolysis of candicandiol diacetate (**6**) afforded the 7α-monoacetate (**7**), which was treated with Ph_3P/CCl_4 to give the chloro derivative **8**. Reaction of this with tri-*n*-butyltin deuteride formed the deuterated derivative **2d**, which was hydrolysed with methanolic KOH to give **1d**.

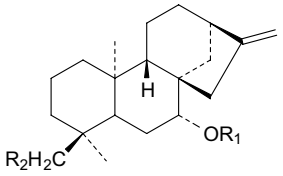
Table 1
^{13}C NMR data of **1**, **2**, **7**, **15**, **22**, **23** and **25** in $CDCl_3$

Carbon	1	2	7	15	22	23	25
1	40.2	40.1	39.7	40.0	39.4	40.1	39.8
2	18.5	18.4	17.8	18.4	17.9	18.4	17.5
3	41.8	41.7	34.9	41.7	30.6	31.5	35.6
4	33.1	33.1	37.5	33.2	42.1	40.3	36.4
5	53.4	52.8	45.6	52.6	49.5	47.4	45.0
6	29.4	25.8	25.4	26.0	30.1	26.5	26.2
7	75.2	77.1	77.1	76.9	75.4	77.0	72.7
8	49.8	48.2	48.2	49.0	50.3	48.6	50.1
9	55.4	55.2	55.2	55.8	55.6	55.7	60.7
10	39.4	39.2	39.1	38.2	40.1	39.4	39.7
11	17.9	17.8	17.9	17.7	18.3	18.0	68.1
12	33.6	33.3	33.4	26.3	33.7	32.1	39.2
13	43.1	42.9	42.9	44.9	43.5	43.2	39.9
14	30.5	31.8	31.9	29.5	30.5	33.7	30.6
15	43.3	43.2	43.2	48.0	43.4	43.3	81.2
16	155.1	154.8	154.7	79.3	155.0	154.8	156.8
17	103.4	103.4	103.1	66.5	103.8	104.2	109.2
18	33.5	33.3	71.4	33.3	73.4	69.4	72.0
19	21.6	21.5	17.5	21.5	64.9	65.1	17.6
20	17.7	17.6	18.1	17.9	17.9	18.4	17.7

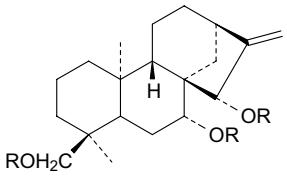
The incubation of candicandiol (**5**) with *G. fujikuroi* gave 7α,18,19-trihydroxy-*ent*-kaur-16-ene (**22**), which showed in its MS spectrum the molecular ion at *m*/*z* 320.2362, corresponding to a molecular formula of $C_{20}H_{32}O_3$. The new oxygen atom, introduced in the molecule of the substrate during the incubation, must be in the form of a new primary hydroxyl group, because in the ^{1}H NMR spectrum appears a new AB system as a pair of doublets at δ 3.72 and 3.90 ($J = 11$ Hz). Thus, the new hydroxyl group must be situated at C-19 or C-20. The first of these positions was chosen on the basis of biogenetic considerations, because it is well known that one of the principal steps in the biosynthesis of the gibberellins is the hydroxylation of *ent*-kaur-16-ene at C-19. We confirmed this location by assignment of its ^{13}C NMR spectrum and that of its triacetate **23** (Table 1). In the HMBC spectrum of the latter the following correlations were observed: H-5 with C-4, C-6, C-7, C-10, C-18 and C-19; H-9 with C-8, C-10, C-11 and C-12; H-15 with C-7, C-8, C-9 and C-14; H-18 and H-19 with C-3, C-4 and C-5; H-20 with C-1, C-5, C-9 and C-10.



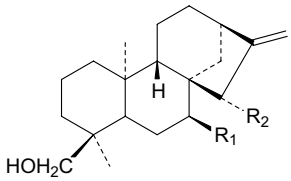
1 R = α–OH,H
1d R = α–OH,H, [18-$^2H_1$]
2 R = α–OAc,H
2d R = α–OAc,H, [18-$^2H_1$]
3 R = O
4 R = β–OH,H



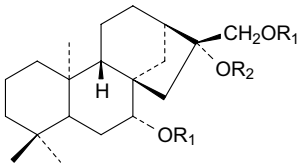
5 R_1 = H R_2 = OH
6 R_1 = Ac R_2 = OAc
7 R_1 = Ac R_2 = OH
8 R_1 = Ac R_2 = Cl



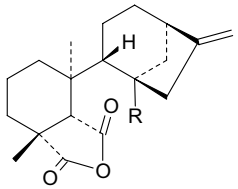
9 R = H
10 R = Ac



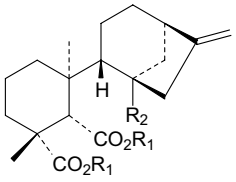
11 R_1 = OH R_2 = H
12 R_1 = H R_2 = OH



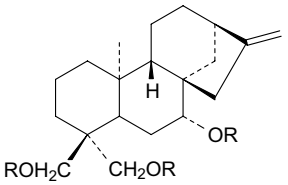
13 R_1 = R_2 = H
13d R_1 = R_2 = H, [18-$^2H_1$]
14 R_1 = R_2 = Ac
15 R_1 = Ac R_2 = H
15d R_1 = Ac R_2 = H, [18-$^2H_1$]



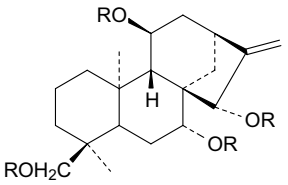
16 R = CHO
17 R = CO_2H
17d R = CO_2H, [18-$^2H_1$]
18 R = CO_2Me
18d R = CO_2Me, [18-$^2H_1$]



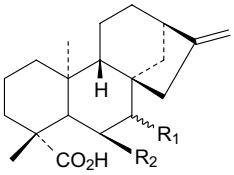
19 R_1 = H R_2 = CHO
20 R_1 = H R_2 = CO_2H
20d R_1 = H R_2 = CO_2H, [18-$^2H_1$]
21d R_1 = Me R_2 = CO_2Me, [18-$^2H_1$]



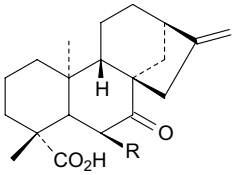
22 R = H
23 R = Ac



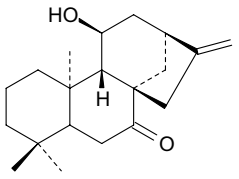
24 R = H
25 R = Ac



26 R_1 = α–OH, R_2 = H
27 R_1 = α–OH, R_2 = OH
28 R_1 = β–OH, R_2 = H
29 R_1 = β–OH, R_2 = OH



30 R = H
31 R = OH



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The biotransformation of canditriol (**9**) afforded 7α,11β,15α,18-tetrahydroxy-*ent*-kaur-16-ene (**24**). The latter was obtained as its tetraacetate **25**, by acetylation and chromatography of the fractions containing it. Its molecular formula, $C_{28}H_{40}O_8$, indicated that a new hydroxyl group had been introduced in the molecule of canditriol (**9**) during its incubation. The ^{13}C NMR spectrum of **25**, in comparison with that of canditriol triacetate (**10**), showed the presence of a new secondary carbon bearing an acetoxyl group at δ 68.1. Its relative low value of resonance indicated that it is a carbon situated between carbons bearing hydrogen atoms, such as C-2, C-6 or C-11, but not C-12 which resonates at about δ 74.0. On the other hand, in the ^1H NMR spectrum a geminal proton to a new acetoxyl group appeared as a doublet at δ 5.11 ($J = 4.7$ Hz), which permitted us to assign it to C-11, because the chemical shift and the coupling constant of this proton are typical of a hydrogen geminal to a

β-acetoxyl group at this carbon (Fraga et al., 1986). Finally, the structure **25** was confirmed by assignment of the ^{13}C NMR spectrum (Table 1). Moreover, this compound **25** was identical with the corresponding tetraacetate of a product which had been isolated from the incubation of candidiol (**12**) with *G. fujikuroi* (Fraga et al., 1992). Therefore, the structure of the metabolite formed in the incubation was determined as 7α,11β,15α,18-tetrahydroxy-*ent*-kaur-16-ene (**24**).

The results of these microbiological biotransformations led us to the following conclusions:

1. The presence of the 7α-hydroxyl group in *epi*-candol A (**1**) avoids its biotransformation along the biosynthetic pathway of gibberellins, and directs it to the *seco*-ring B acids route. We must point out that its 7-epimer, candol A (7β-hydroxy-*ent*-kaur-16-ene) (**4**), was transformed into gibberellins A_3, A_4, A_7 and fujenal (**16**) by incubation with *G. fujikuroi* (Fraga et al., 1980).
2. In this fungus 6β,7β-dihydroxy-*ent*-kaur-16-en-19-oic acid (**29**) is the precursor of the diacid-aldehyde **19** and the triacid **20**, the yield of the latter being lower in comparison with that of the former (Cross et al., 1970; Rojas et al., 2004). Now, in the biotransformation of 7α-hydroxy-*ent*-kaur-16-ene (**1**), the formation of the triacid **20** (isolated as its anhydride **17**) and not of the diacid-aldehyde **19**, indicates that an intermediate of the type 6β,7α-dihydroxy (**27**), necessary for the formation of **19**, was not produced in this feeding. In consequence, the formation of the triacid (**20**) in the incubation of **1** must occur via an alternative way, such as an 7-oxo-group (Bearder et al., 1975; Fraga et al., 2005).
3. In the biotransformation of **1**–**20**, an oxidation of C-19 must first occur to afford 7α-hydroxykaurenoic acid (**26**), followed by the formation of the 7-oxo derivative **30**, and then hydroxylation at C-6(β) to give **31**. We have postulated the presence of a 6β-hydroxy-7-oxo intermediate in the formation of **20** (Fraga et al., 2005). If the substrate **1** were first oxidized to 7-oxo-*ent*-kaur-16-ene (**3**), before the oxidation at C-19, the 11β-hydroxy derivative **32** should be formed in this incubation (Fraga et al., 2005).
4. The enzyme responsible for the oxidation of the 7α-OH of **26** to the 7-oxo-derivative **30** is probably the same one that acts in the hydroxylation of the 7β-position of *ent*-kaur-16-en-19-oic acid in the biosynthesis of gibberellins and *seco*-ring B compounds. This step is produced with retention of configuration (Castellaro et al., 1990) and occurs only in the presence of an acid group at C-19 or an equivalent polar group, such as a 3α,18-diol (Fraga et al., 1981).
5. Studies carried out with a genetically transformed strain of *G. fujikuroi* have shown that the transformation of 7β-hydroxykaurenoic acid (**28**) into *seco*-ring B derivatives is due to the P-450-1 monooxygenase (GA_{14} synthase) (Rojas et al., 2004). This enzyme is probably also responsible for the formation of fujenoic triacid (**20**) from 7α-hydroxykaurenoic acid (**26**) via the corresponding 7-oxo derivative **30**. These diterpenes, **26** and **30**, have not been isolated from this fungus.
6. The C-19 oxidation, which is characteristic of the biosynthesis of gibberellins, has been observed now, at the hydroxyl level, in the feeding of candicandiol (**5**), whilst in the incubation of epicandicandiol (**11**) it had occurred at the acid level (Fraga et al., 1978). Thus, the first diterpene (**5**) has been worse metabolized than the second (**11**) by this fungus. This can be explained considering the presence of a 7α-OH group in **5** and of a 7β-OH in **11**. This last hydroxyl position occurs in 7β-hydroxy-*ent*-kaur-17-en-19-oic acid, a natural metabolite of the fungus and precursor of the gibberellins.
7. In the feeding of candicandiol (**5**) the 6β-hydroxylation, necessary for the ring cleavage and formation of the aldehyde **19** (Cross et al., 1970), or of its anhydride form **16**, was not produced. This was probably due to the fact that the 7α-hydroxyl was not oxidized to a 7-oxo group, probably because oxidation of C-7 requires an acid group at C-19 (see conclusions 3 and 4).
8. The 15α-hydroxyl group in canditriol (**9**) inhibited the oxidation at C-19, and direct hydroxylation at C-11(β), which confirmed our previous results obtained in the microbiological transformation of candidiol (15α,18-dihydroxy-*ent*-kaur-16-ene) (**12**) (Fraga et al., 1980) and other 15α-hydroxy-derivatives (Fraga et al., 1988, 1991, 1992).

3. Experimental

3.1. General procedures

Mps were determined with a Reichert Thermovar apparatus and are uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ soln at 500.13 and 125.77 MHz, respectively, with a Bruker AMX2-500 spectrometer. Chemical shifts are given in ppm (δ). Mass spectra were taken at 70 eV (probe) in a Micromass Autospec spectrometer. Dry column chromatographies were made on silica gel Merck 0.02–0.063 mm. The fungal strain was *Gibberella fujikuroi* IMI 58289. The substances were crystallised from petrol-EtOAc except where otherwise indicated.

3.2. Incubation procedure

Gibberella fujikuroi MP-C (*Fusarium fujikuroi*) IMI 58289 inhibited with 5×10^{-5} M AMO 1618, was grown in shake culture at 25 °C for 2 days in 65–75 conical flasks (250 ml), each containing sterile medium (50 ml) (Hanson et al., 1972). 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 diluted 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.

3.3. 7α-Hydroxy-ent-kaur-16-ene (epi-candol A) (**1**)

Found: C, 83.24; H, 11.33. Calc. for $C_{20}H_{32}O$: C, 83.272; H, 11.18%; $[M]^+$ at *m/z* 288.2447. $C_{20}H_{32}O$ requires 288.2379; ^1H NMR (500 MHz): δ 0.69 (1H, *td*, *J* = 13.1 and 3 Hz, H-1β), 0.79 (3H, *s*, H-19), 0.83 (1H, *dd*, *J* = 12 and 1.5 Hz, H-5), 0.84 (3H, *s*, H-18), 1.00 (1H, *m*, H-9), 1.01 (3H, *s*, H-20), 1.09 (1H, *td*, *J* = 13.6 and 4.4 Hz, H-3β), 1.36 (1H, *q*, *J* = 12 Hz, H-6α), 1.40 (2H, *m*, H-2 and H-3), 1.48 and 1.68 (each 1H, *m*, H-12), 1.77 (2H, *m*, H-1α and H-6β), 1.90 (1H, *dd*, *J* = 16.5 and 1.4 Hz, H-15β), 2.64 (1H, *dt*, *J* = 16.5 and 3 Hz, H-15α), 2.66 (1H, br *s*, H-13), 3.44 (1H, *dd*, *J* = 12 and 4.3 Hz, H-7), 4.73 and 4.80 (each 1H, br *s*, H-17); EIMS *m/z* (rel. int.): 288 $[M]^+$ (100), 270 (39), 255 (17), 245 (14), 227 (5), 199 (6), 190 (22), 164 (36), 149 (19). *Acetate* (**2**). $[M-C_2H_2O]^+$ at *m/z* 288.2476. $C_{20}H_{32}O$ requires 288.2453; ^1H NMR (500 MHz): δ 0.71 (1H, *td*, *J* = 13.5 and 3.8 Hz, H-1β), 0.79 (3H, *s*, H-19), 0.85 (3H, *s*, H-18), 0.92 (1H, *d*, *J* = 12.0 Hz, H-5), 1.04 (3H, *s*, H-20), 1.07 (1H, br *d*, *J* = 6.2 Hz, H-9), 1.11 (1H, *td*, *J* = 13.8 and 4.8 Hz, H-3β), 1.39 (1H, *q*, *J* = 12.0 Hz, H-6α), 1.48 (1H, *m*, H-14), 1.93 (1H, br *d*, *J* = 17 Hz, H-15β), 2.03 (3H, *s*), 2.27 (1H, *dt*, *J* = 17 and 2.4 Hz, H-15α), 2.64 (1H, br *s*, H-13), 4.71 (1H, *dd*, *J* = 12.0 and 4.5 Hz, H-7), 4.74 and 4.80 (each 1H, br *s*, H-17); EIMS *m/z* (rel. int.): 288 $[M-C_2H_2O]^+$ (1), 270 (100), 255 (37), 241 (7), 227 (10), 199 (12), 190 (55), 185 (15), 173 (10), 160 (11), 145 (13).

3.4. Incubation of 7α-hydroxy-ent-kaur-16-ene (**1**)

The biotransformation of **1** (220 mg) gave in the neutral fraction starting material (60 mg) and 7α,16α,17-trihydroxy-*ent*-kaur-16-ene (**13**), which was obtained as the triacetate (**14**) (1 mg) and the 7,17-diacetate (**15**) (6 mg), by acetylation and chromatography of the fractions containing it. Fujenoic acid (**17**) (3 mg) was obtained in the acid fraction.

3.4.1. 7α,16α,17-Triacetoxy-ent-kaurane (**14**)

$[M-C_2H_2O]^+$ at *m/z* 388.2605. $C_{24}H_{36}O_4$ requires 388.2614; ^1H NMR (500 MHz): δ 0.72 (1H, *td*, *J* = 13 and 3.5 Hz, H-1β), 0.78 (3H, *s*, H-19), 0.85 (3H, *s*, H-18), 1.02 (3H, *s*, H-20), 1.66 (1H, br *d*, *J* = 12 Hz, H-15), 1.73 (1H, *dt*, *J* = 13 and 2 Hz, H-1α), 1.82 (1H, *dd*, *J* = 12 and 4.2 Hz, H-6β), 1.96, 2.02 and 2.04 (each 3H, *s*), 2.12 (1H, br *d*, *J* = 15 Hz, H-14), 2.53 (1H, br *s*, H-13), 4.45 and 4.82 (each 1H, *d*, *J* = 11 Hz, H-17), 4.63 (1H, *dd*, *J* = 11.3 and 4.2 Hz, H-7). EIMS *m/z* (rel. int.): 388 $[M-C_2H_2O]^+$ (11), 346 (5), 328 (75), 313 (14), 286 (100), 268 (95), 253 (67), 240 (13), 225 (25), 211 (12), 197 (20), 183 (27).

3.4.2. 7α,17-Diacetoxy-16α-hydroxy-ent-kaurane (15)

$[M-HOAc]^+$ at *m/z* 346.2475. $C_{22}H_{34}O_3$ requires 346.2508; ^1H NMR (500 MHz): δ 0.69 (1H, *td*, *J* = 12.2 and 3.4 Hz, H-1β), 0.78 (3H, *s*, H-19), 0.85 (3H, *s*, H-18), 0.91 (1H, *dd*, *J* = 12.4 and 1.9 Hz, H-5), 1.03 (3H, *s*, H-20), 1.11 (1H, *td*, *J* = 13.3 and 4.3 Hz, H-3β), 1.45 and 1.63 (each 1H, br *d*, *J* = 14.8 Hz, H-15), 1.66 (1H, br *d*, *J* = 11.0 Hz, H-14), 1.73 (1H, br *d*, *J* = 12.2 Hz, H-1α), 1.80 (1H, *ddd*, *J* = 12.4, 4.3 and 1.9 Hz, H-6β), 2.04 (1H, br *s*, H-13), 2.07 and 2.08 (each 3H, *s*), 2.14 (1H, *dd*, *J* = 11.0 and 4.2 Hz, H-14), 4.15 and 4.22 (each 1H, *d*, *J* = 11.4 Hz, H-17), 4.68 (1H, *dd*, *J* = 11.9 and 4.3 Hz, H-7); EIMS *m/z* (rel. int.): 346 $[M-HOAc]^+$ (24), 331 (11), 328 (20), 313 (7), 286 (41), 273 (100), 271 (22), 268 (27), 255 (17), 253 (19), 231 (17), 230 (57), 229 (17), 215 (14), 208 (14), 189 (14), 173 (10), 161 (11).

3.5. Preparation of the substrate [18-$^2H_1$]-7α-hydroxy-ent-kaur-16-ene (**1d**)

3.5.1. Partial hydrolysis of **6**

Candicandiol diacetate (**6**) (Fernández et al., 1986) (970 mg) dissolved in MeOH (40 ml) was stirred with K_2CO_3 (300 mg) for 3 h at room temp. The solvent was removed under vacuum and the residue extracted with EtOAc. Usual work-up and chromatography, eluting with petrol-EtOAc (85:15), afforded starting material (28 mg), candicandiol 7α-monoacetate (**7**) (310 mg) and candicandiol (**5**) (425 mg).

3.5.2. Candicandiol 7α-monoacetate (**7**)

$[M]^+$ at *m/z* 346.2470. $C_{22}H_{34}O_3$ requires 346.2508; ^1H NMR (500 MHz): δ 0.73 (3H, *s*, H-19), 0.75 (1H, *td*, *J* = 12.8 and 3.3 Hz, H-1β), 1.08 (3H, *s*, H-20), 1.14 (1H, br *d*, *J* = 7.0 Hz, H-9),1.24 (1H, br *d*, *J* = 12.6 Hz, H-3β), 1.30 (1H, *dd*, *J* = 12.3 and 2.4 Hz, H-5), 1.41 (1H, *t*, *J* = 11.8 Hz, H-6α), 1.79 (1H, *dt*, *J* = 12.8 and 2.2 Hz, H-1α), 1.94 (1H, *dd*, *J* = 16.8 and 1.7 Hz, H-15α), 2.03 (3H, *s*, OAc), 2.30 (1H, *dt*, *J* = 16.8 and 2.8 Hz, H-15β), 2.65 (1H, br *s*, H-13), 3.03 and 3.39 (each 1H, *d*, *J* = 10.9 Hz, H-18), 4.69 (1H, *dd*, *J* = 11.8 and 4.3 Hz, H-7), 4.75 and 4.82 (each 1H, br *s*, H-17); EIMS *m/z* (rel. int.): 346 $[M]^+$ (1), 304 (1), 286 (45), 271 (6), 255 (38), 239 (10), 206 (6), 199 (4), 187 (8), 173 (7).

3.5.3. Chloration of candicandiol 7α-monoacetate (**7**)

The monoacetate **7** (310 mg) and Ph_3P (960 mg), dissolved in CCl_4 (15 ml), were heated under reflux for 12 h. The precipitate was separated by filtration and washed with Et_2O. The solvents were removed under vacuum and the residue chromatographed on silica, eluting with petrol-EtOAc (98:2), to afford 7α-acetoxy-18-chloro-ent-kaur-16-ene (**8**) (224 mg): $[M]^+$ at *m/z* 364.2123. $C_{22}H_{33}ClO_2$ requires 364.2169; ^1H NMR (500 MHz): δ 0.75 (1H, *td*, *J* = 12.8 and 3.8 Hz, H-1β), 0.88 (3H, *s*, H-19), 1.08 (3H, *s*, H-20), 1.14 (1H, br *d*, *J* = 6.9 Hz, H-9), 1.34 (1H, *t*, *J* = 12.8 Hz, H-3β overlapped with H-5), 1.41 (1H, *q*, *J* = 12.0 Hz, H-6α), 1.52 (2H, *m*, H-2β and H-6β), 1.79 (1H, *dt*, *J* = 12.8 and 3.4 Hz, H-1α), 1.95 (1H, *dd*, *J* = 16.9 and 1.7 Hz, H-15β), 2.03 (3H, *s*, OAc), 2.29 (1 H, *dt*, *J* = 16.9 and 2.7 Hz, H-15α), 2.65 (1H, br *s*,

H-13), 3.21 and 3.38 (each 1H, *d*, $J = 11.1$ Hz, H-18), 4.73 (1H, *dd*, $J = 11.6$ and 4.5 Hz, H-7), 4.75 and 4.82 (each 1H, br *s*, H-17); EIMS *m/z* (rel. int.) 364 $[M]^+$ (0.1), 322 (1), 304 (100), 289 (37), 275 (6), 269 (13), 255 (32), 224 (28), 213 (5), 199 (10), 185 (11).

3.5.4. Reduction of the 18-chloro derivative **8**

7α-Acetoxy-18-chloro-*ent*-kaur-16-ene (**8**) (290 mg), dissolved in dry toluene (15 ml), was refluxed with tri-*n*-butyltin deuteride (600 μl) and 2,2′-azobisisobutyronitrile (traces) under nitrogen for 13 h. The solvent was evaporated, the residue dissolved in Et_2O and stirred with H_2O saturated with KF for 30 min. The solution was filtered, dried and the solvent removed under vacuum to give a residue, which was chromatographed on silica. Elution with petrol-EtOAc (98:2) afforded 7α-acetoxy-ent-[18-^{2}H]kaur-16-ene (**2d**) (245 mg): $[M]^+$ at *m/z* 331.2598. $C_{22}H_{33}DO_2$ requires 331.2622; ^{1}H NMR (500 MHz): δ 0.85 (2H, *s*, H-18); ^{13}C NMR (125 MHz): δ 33.1 [*t*, $J(^{13}C–^2H) = 19$ Hz]; EIMS *m/z* (rel. int.) 331 $[M]^+$ (0.1), 271 (100), 256 (30), 242 (8), 228 (5), 214 (3), 201 (7), 191 (32), 185 (7), 173 (3).

3.5.5. Hydrolysis of **2d**

The acetate **2d** (240 mg) was stirred in a 3% methanolic KOH soln (15 ml), at room temperature, for 16 h to afford 7α-hydroxy-*ent*-[18-^{2}H]kaur-16-ene (**1d**) (200 mg): $[M]^+$ at *m/z* 289.2519. $C_{20}H_{31}DO$ requires 289.2516; ^{1}H NMR (500 MHz): 0.85 (2H, *s*, H-18); ^{13}C NMR (125 MHz): δ 33.2 [*t*, $J(^{13}C–^2H) = 20$ Hz]; EIMS *m/z* (rel. int.) 289 $[M]^+$ (13), 271 (42), 256 (8), 246 (2), 199 (1), 191 (3), 164 (3), 149 (2), 124 (2), 105 (6), 91 (12).

3.6. Incubation of [18-$^2H_1$]-7α-hydroxy-ent-kaur-16-ene (**1d**)

The biotransformation of **1d** (200 mg) gave in the neutral fraction starting material (45 mg) and [18-$^2H_1$]-7α,16α,17-trihydroxy-*ent*-kaur-16-ene (**13d**), which was obtained as the 7,17-diacetate (**15d**) (5 mg), by acetylation and chromatography of the fractions containing it. [18-$^2H_1$]-Fujenoic acid (**17 d**) (7 mg) and the deuterated triacid (**20d**) were obtained in the acid fraction. The latter was isolated as its trimethyl ester (**21d**) (11 mg) by methylation with CH_2N_2 and purification by chromatography.

3.6.1. [18-$^2H_1$]-7α,17-Diacetoxy-16α-hydroxy-ent-kaurane (**15d**)

$[M–HOAc]^+$ at *m/z* 347.2572. $C_{22}H_{33}DO_3$ requires 347.2571; ^{1}H NMR (500 MHz): δ 0.84 (2H, *s*, H-18); EIMS *m/z* (rel. int.): 347 $[M–HOAc]^+$ (16), 332 (6), 329 (11), 314 (3), 287 (13), 274 (100), 269 (11), 256 (9), 254 (7), 248 (11), 231 (37), 216 (6), 200 (9), 159 (38), 157 (26), 149 (12).

3.6.2. [18-$^2H_1$]-Fujenoic acid (**17d**)

$[M]^+$ at *m/z* 347.1837. $C_{20}H_{25}DO_5$ requires 347.1843; ^{1}H NMR (500 MHz): δ 1.34 (2H, *s*, H-18); EIMS *m/z* (rel. int.): 347 $[M]^+$ (1), 329 (33), 301 (10), 257 (24), 255 (14), 242 (16), 182 (4), 166 (25), 165 (21), 154 (18), 148 (17), 147 (19), 137 (11), 119 (30), 110 (100). *Methyl ester* (**18d**): $[M]^+$ at *m/z* 361.1999. $C_{21}H_{27}DO_5$ requires 361.2000. ^{1}H NMR (500 MHz): δ 0.92 (3H, *s*, H-20), 1.18 (1H, *m*, H-3β), 1.33 (2H, *s*, H-18), 1.72 (1H, *dd*, $J = 12.4$ and 2.1 Hz, H-14), 1.81 (1H, *m*, H-1α), 1.89 (1H, *m*, H-11), 1.96 (1H, *m*, H-14), 2.30 (1H, *m*, H-3α), 2.38 (1H, *dt*, $J = 17.6$ and 2.7 Hz, H-15β), 2.52 (1H, *s*, H-5), 2.61 (1H, *dd*, $J = 17.6$ and 1.8 Hz, H-15α), 2.68 (1H, *d*, $J = 7.4$ Hz, H-9), 2.77 (1H, br *s*, H-13), 3.64 (3H, *s*, OMe), 4.78 and 4.87 (each 1H, br *s*, H-17); EIMS *m/z* (rel. int.): 361 $[M]^+$ (2), 329 (21), 301 (31), 273 (5), 167 (10), 149 (9).

3.6.3. [18-$^2H_1$]-Triacid (**20d**)

Obtained as its trimethyl ester (**21d**) by methylation with CH_2N_2 of the fractions containing it: $[M]^+$ at *m/z* 407.2410. $C_{23}H_{33}DO_6$ requires 407.2418; ^{1}H NMR (500 MHz): δ 1.25 (2H, *s*, H-18); EIMS *m/z* (rel. int.): 407 $[M]^+$ (2), 389 (1), 376 (6), 343 (2), 315 (3), 284 (3), 256 (5), 247 (1), 228 (66), 196 (100), 168 (62).

3.7. Incubation of candicandiol (**5**)

The fermentation of **5** (400 mg) gave in the neutral fraction starting material (310 mg) and 7α,18,19-trihydroxy-*ent*-kaur-16-ene (**22**) (18 mg), which was characterized as its triacetate **23**, by acetylation and chromatography of the fractions containing it.

3.7.1. 7α,18,19-Trihydroxy-ent-kaur-16-ene (**22**)

Mp 188–190 °C (from EtOAc), $[M]^+$ at *m/z* 320.2362. $C_{20}H_{32}O_3$ requires 320.2351; ^{1}H NMR (500 MHz): δ 0.74 (1H, *dt*, $J = 13.0$ and 4.2 Hz, H-1β), 1.00 (3H, *s*, H-20), 1.04 (1H, *d*, $J = 7.2$ Hz, H-9), 1.06 (1H, *d*, $J = 12.5$ Hz, H-5), 1.45 (1H, *q*, $J = 12.5$ Hz, H-6α), 1.52 (1H, *m*, H-2), 1.82 (1H, *dt*, $J = 13$ and 2 Hz, H-1α), 1.90 (3H, m, H-6β, H-11 and H-15), 2.64 (1H, br *d*, $J = 17.1$ Hz, H-15), 2.66 (1H, br *s*, H-13), 3.40 and 3.81 (each 1H, *d*, $J = 10.6$ Hz, H-18), 3.41 (1H, *dd*, $J = 12.5$ and 4 Hz, H-7), 3.72 and 3.90 (each 1H, *d*, $J = 11$ Hz, H-19), 4.74 and 4.81 (each 1H, br *s*, H-17); EIMS *m/z* (rel. int.): 320 $[M]^+$ (8), 302 (2), 288 (4), 284 (3), 270 (3), 256 (4), 253 (5), 213 (5), 185 (8), 164 (8), 149 (46).

3.7.2. Triacetate (**23**)

$[M–HOAc]^+$ at *m/z* 386.2469. $C_{24}H_{34}O_4$ requires 386.2457; ^{1}H NMR (500 MHz): δ 0.80 (1H, *td*, $J = 12.9$ and 3.9 Hz, H-1β), 1.10 (3H, *s*, H-20), 1.14 (1H, *d*, $J = 6.9$ Hz, H-9), 1.85 (1H, *dt*, $J = 12.9$ and 3.1 Hz, H-1α), 1.96 (1H, *d*, $J = 17.0$ Hz, H-15), 2.03 (6H, *s*), 2.09 (3H, *s*), 2.30 (1H, *dt*, $J = 17.0$ and 2.7 Hz, H-15), 2.67 (1H, br *s*, H-13), 3.87 and 4.03 (each 1H, *d*, $J = 11$ Hz, H-18), 4.00 and 4.26 (each 1H, *d*, $J = 11$ Hz, H-19) 4.63 (1H, *dd*, $J = 11.0$ and 3.6 Hz, H-7), 4.76 and 4.83 (each 1H, br *s*, H-17); EIMS *m/z* (rel. int.): 386 $[M–HOAc]^+$ (9), 344 (1), 326 (29), 284 (4), 266 (66),

253 (29), 251 (23), 237 (8), 223 (11), 211 (9), 197 (12), 185 (10).

3.8. Incubation of canditriol (**9**)

The biotransformation of **9** (210 mg) afforded in the neutral fraction starting material (114 mg) and 7α,11β,15α,18-tetrahydroxy-*ent*-kaur-16-ene (**24**). The latter was obtained as its tetraacetate **25** (14 mg), by acetylation and chromatography of the fractions containing it.

3.8.1. 7α,11β,15α,18-Tetrahydroxy-ent-kaur- 16-ene (**24**)

This compound was obtained as its tetraacetate (**25**) by acetylation of several fractions containing it, $[M]^+$ at *m/z* 504.2750. $C_{28}H_{40}O_8$ requires 504.2723; ^1H NMR (500 MHz): δ 0.78 (3H, *s*, H-19), 1.01 (3H, *s*, H-20), 1.57 (1H, *s*, H-9), 1.69 (1H, *d*, $J = 12$ Hz, H-14), 1.95, 1.97, 2.02 and 2.09 (each 3H, *s*), 2.14 (1H, *dd*, $J = 12$ and 4.9 Hz, H-14), 2.78 (1H, br *s*, H-13), 3.59 and 3.79 (each 1H, *d*, $J = 11.4$ Hz, H-18), 4.84 (1H, *dd*, $J = 11$ and 5.3 Hz, H-7), 4.95 and 5.08 (each 1H, br *s*), 5.11 (1H, *d*, $J = 4.7$ Hz, H-11), 5.90 (1H, br *s*, H-15); EIMS *m/z* (rel. int.): 504 $[M]^+$ (1), 444 (15), 402 (43), 384 (17), 369 (6), 360 (11), 342 (100), 324 (34), 309 (19), 300 (11), 282 (81), 264 (40), 251 (62).

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