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The biotransformation of *ent*-kaur-16-en-19-oic acid by *Rhizopus stolonifer*

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Abstract

Microbial transformation of *ent*-kaur-16-en-19-oic acid was carried out with *R. stolonifer*. After seven days of incubation, two metabolites, *ent*-7α-hydroxy-kaur-16-en-19-oic acid and *ent*-12β-hydroxy-kaur-9(11),16-dien-19-oic acid, were isolated as a result of hydroxylation and hydroxylation/dehydrogenation, respectively. Incubation for 15 days also afforded *ent*-16β,17-dihydroxy-kauran-19-oic acid. The metabolites were identified by spectroscopic methods. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Rhizopus stolonifer; Microbial transformation; *Ent*-kaur-16-en-19-oic acid

1. Introduction

Microorganisms are able to transform a huge variety of organic compounds, such as hydrocarbons, terpenoids, steroids, alkaloids, antibiotics and amino-acids (Kieslich, 1976). Many compounds of therapeutic and/or industrial interest are obtained by microbial transformation (Turner, 1994). *Ent*-kaur-16-en-19-oic acid (**1**), a diterpene that occurs in some species of the Brazilian flora of *Xylopia* (Annonaceae) (Takahashi, Boaventura, Bayma & Oliveira, 1995) and *Wedelia* (Asteraceae) (Yang et al., 1986), is known to have plant growth stimulating activity (Eall, Yeung & Pharis, 1996). Derivatives of **1** possess other biological activities, such as antimicrobial (Davino, Giesbrecht & Roque, 1989), antitumoral (Kupchan, Court, Dailey, Gilmore & Bryan, 1972), trypanosomicidal (Costa, Albuquerque & Vichnewski, 1996) and anti-HIV (Wu et al., 1996). As part of a program of diterpenoid transformation by microorganisms, we carried out the transformation of *ent*-kaur-16-en-19-oic acid (**1**), using *R. stolonifer*.

2. Results and discussion

Incubation of **1** with *R. stolonifer* for seven days yielded compounds **2** and **3**, the former being the major product (4.7%). The IR spectrum of **2** showed typical bands for carbon–carbon double bond stretching (1650 cm^{-1}), carboxyl (1700 cm^{-1}) and hydroxyl (3480 cm^{-1}) groups. ^{1}H NMR and ^{13}C NMR spectra of **2** showed signals corresponding to the same functional group at C-19 and to the C-16 double bond in comparison with starting material. New signals at δ_H 3.63 and δ_C 77.12 indicated that a hydroxylation had occurred. Assignments of the ^{13}C NMR signals for compound **2** indicated that the hydroxyl group was introduced at C-7. This was confirmed by the downfield shifts of C-6 and C-8 signals and the upfield shifts of C-5, C-9 and C-15 due to the γ-gauche effect. The axial hydroxylation at C-7 was identified by the γ-gauche effect (Hasan, Healey & Waterman, 1982; Hutchison, Lewer & Macmillan, 1984). Thus, γ-gauche interactions observed between the hydroxy substituent

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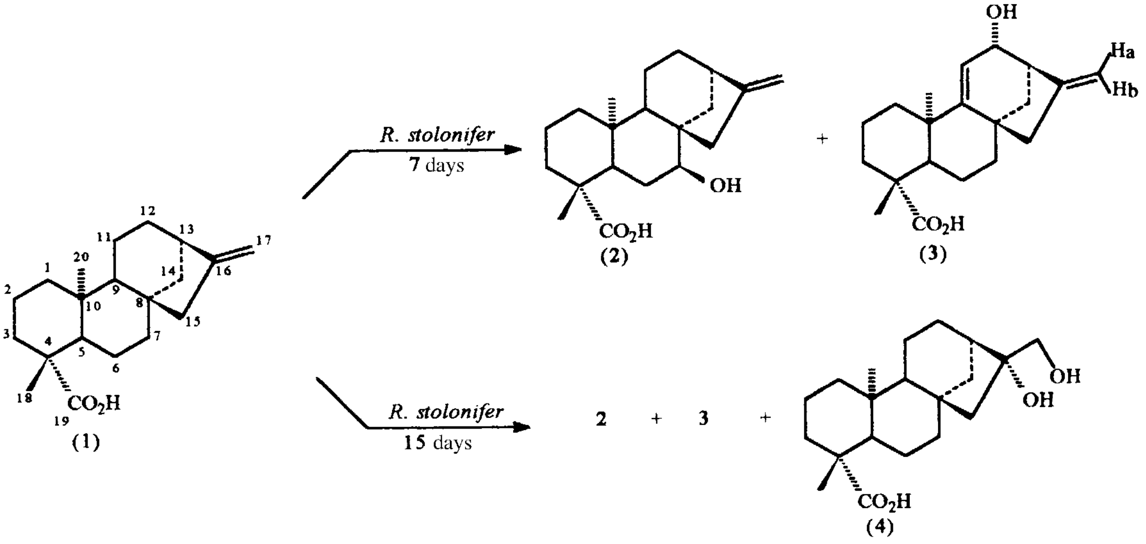

and C-5, C-9 and C-15 confirmed a 7β-hydroxylation. For a 7α-hydroxylation, we should observe the γ-gauche effect at C-14 and C-15.

The minor product isolated from this incubation (**3**) presented a M$^+$ 316 peak indicating that both hydroxylation and dehydrogenation had occurred. Compound **3** was identified by its characteristic ^{1}H NMR signals at δ 5.05 and 4.91 (one hydrogen each, broad singlet, H-17a and H-17b), δ 3.95 (J = 3.5 Hz, one hydrogen, broad triplet, H-12β) and an olefinic hydrogen doublet signal at δ 5.36 (J = 3.8 Hz). This signal is due to an endocyclic double bond between C-9 and C-11 by comparison of spectral data with published values (Ahmed, Jakupovic & Castro, 1991; Gao, Wang & Marby, 1987). These assignments were confirmed by ^{1}H-^{1}H COSY experiments which showed correlations of H-11 with H-12 and H-13 (δ 2.84), as well of H-13 with H-12 and H-14β (δ 1.44). The ^{13}C NMR spectrum showed a new signal at δ 72.6, assigned to C-12, now hydroxylated. The presence of a the hydroxyl group at C-12 is reflected in a downfield displacement of the C-13 signal (δ 46.1). The assignments of the remaining carbons were made by comparison of the ^{13}C NMR spectrum of **3** with data for **1**. After all carbons were assigned, they were correlated to the hydrogen signals by HMQC experiments. NOESY experiments defined the α-configuration of the hydroxyl group at C-12 as a correlation was found between H-12 and one of the hydrogens at C-17. This is only possible when the hydroxyl group occupies the α-position. Besides, no correlation between H-12 and H-14α was found, as expected for a hydroxyl group in a α-position. Data for compound **3** are in accordance with the literature (Ahmed et al., 1991).

Incubation of **1** with *R. stolonifer,* under the same conditions, for a longer period (15 days), led to the formation of a third metabolite (**4**). The spectrometric data of **4** suggested dihydroxylation of the original 16–17 double bond of substrate **1**. Thus, its ^{1}H NMR spectrum showed the absence of the unsaturated exocyclic methylene signals, but a new AB system at δ 4.10 and 4.20 (2H, J = 10.8 Hz) has appeared. Two additional oxygen atoms (mass spectrum m/z 336 [M]$^+$, chemical ionization) and two new oxygenated

Table 1
^{13}NMR assignments for *ent*-kaur-16-en-19-oic acid (**1**) and compounds **2**, **3** and **4** (CDCl$_3$, δ)^{ab}

Carbon	Compounds				**4** Etse et al., 1987	
	1	**2**	**3**	**4**	16α-	16β-
1	40.7	40.4	40.5	41.3	42.1	41.8
2	19.1	19.1	20.1	20.1	19.1	19.0
3	37.7	37.8	38.2	39.0	38.1	38.0
4	43.2	43.2	43.4	44.1	43.9	43.7
5	57.0	49.1	48.4	57.2	56.9	56.8
6	21.8	29.7	18.3	23.2	22.2	21.5
7	41.3	77.1	46.7	43.0	40.7	40.6
8	44.2	48.3	44.7	45.2	44.7	43.4
9	55.1	47.2	160.6	56.5	55.8	56.0
10	39.7	39.4	39.0	40.3	39.5	39.4
11	18.4	17.9	117.3	19.2	18.6	18.9
12	33.1	33.5	72.6	27.0	26.2	26.5
13	43.8	43.7	46.1	46.1	45.4	40.5
14	39.7	38.7	40.3	38.0	37.3	37.9
15	48.9	45.3	29.1	54.1	53.2	52.3
16	155.8	155.1	152.3	81.9	81.9	79.8
17	103.0	103.7	108.5	66.7	66.4	69.7
18	28.9	28.7	28.2	29.6	28.7	28.6
19	184.9	160.0	183.2	180.5	180.1	180.3
20	15.6	15.5	23.5	16.2	16.0	15.1

[a] Assignments based on DEPT 135 for **2**, **3** and **4**.

[b] Assignments based on ^{1}H–^{1}H COSY, NOESY and HMQC for **3**.

carbons (^{13}C NMR, δ 81.9, C-16 and δ 66.7, C-17) were also detected; all data confirmed the 16,17-dihydroxylation. The stereochemistry at C-16 was defined by comparison with the literature that relates the chemical shifts reported for the α- and β-16,17-dihydroxy-kauranoic acids (δ 81.9 for the 16α and δ 79.8 for 16β isomers) (Etse, Gray & Waterman, 1987; Herz & Kulanthaviel, 1984). The chemical shift found for compound **4** (δ 81.88) suggests the α-configuration for the 16-hydroxyl group (Table 1). Additionally, the comparison of the melting point of **4** with reported data is in accordance with this assignment (Herz & Kulanthaviel, 1984).

The results of the incubations indicate that the products obtained in the microbial transformation of **1** by *R. stolonifer* were formed by the hydroxylation in the B, C and D rings (compounds **2**, **3** and **4**, respectively), only one product of hydroxylation/dehydrogenation (**3**) being obtained. Incubation for a longer period of time increases the number of metabolites but decrease their yield. Therefore, it is important to monitor the incubations in order to improve the yield of the desired products. It is interesting to note the role of *ent*-7α-hydroxy-kaur-16-en-19-oic acid (**2**) as an intermediate in gibberellin biosynthesis and as a plant growth hormone (Brücher & Blechschmidt, 1991) as well as the anti-HIV activity of **4** (Kupchan et al., 1972).

This is the first report on the transformation of **1** to **3** and **4** by *R. stolonifer*. The transformation of **1** to **2** by this fungus was previously described (Ghisalberti, Jeffreries, Sefton & Sheppard, 1977).

3. Experimental

3.1. General

Melting points were determined on a Metler FPS apparatus and are uncorrected. IR spectra were recorded on a Shimadzu/IR-408 spectrophotometer. Crystalline samples were measured in KBr discs (1.0 mg%). ^{1}H NMR spectra were measured in $CDCl_3$ at 200 MHz (Bruker Advance DPX 200) and 400 MHz (Bruker Advance DRX 400), with TMS as internal standard. ^{13}C NMR spectra were determined at 50 and 100 MHz, in $CDCl_3$ soln. Assignments of ^{13}C chemical shifts were made with the aid of distortionless enhancement by polarisation transfer (DEPT) using a flip angle of 135°. MS were determined on an Autospec model VG spectrometer. 2D-NMR experiments (NOESY, COSY and HMQC) were performed to identify compound **3**.

3.2. Isolation of *1*

The *ent*-kaur-16-en-19-oic acid (**1**) was isolated from a hexane extract of green fruits of *Xylopia frutescens* by silica gel column chromatography as described previously (Costa et al., 1996) and from an EtOH extract of aerial parts of *Wedelia paludosa* by silica gel column chromatography.

3.3. Microorganism, media and culture conditions

Rhizopus stolonifer (CCT2002) was obtained from Coleção de Culturas Tropicais (Fundação André Tosello, Brazil). PDA Medium containing potato infusion (200 g), dextrose (20 g), agar (15 g) at pH 6.5 was used for storage of *R. stolonifer*. In the transformation experiments a medium composed of dextrose (20 g), peptone (5 g), yeast extr. (3 g), KH_2PO_4 (5 g) at pH 5.6 in H_2O (1 l) was used. The microorganism was grown by a two-stage fermentation procedure. Erlenmeyer flasks (500 ml) containing 100 ml of medium were inoculated with a suspension of *R. stolonifer*. Incubations were maintained at 28°C in a rotary shaker, operating at 150 rpm for 48 h. An aliquot (3.0 ml) was used to inoculate similar flasks that were incubated under the same conditions. After 24 h of incubation, a solution of substrate **1** in dimethylformamide (DMF) was added to a final concentration of 0.1 g/l and incubation was continued for seven days. In the second experiment, incubation was maintained for 15 days.

3.4. Extraction, purification and analysis of microbial transformation products

After seven days incubation, cultures were filtered and the mycelium was extracted with EtOAc (3 × 200 ml). The extracts were combined and dried over anhydrous sodium sulphate, the solvent being removed under reduced pressure, in a rotary evaporator, giving 101 mg of residue. After flash column chromatography, 23.7 mg (4.7%) of *ent*-7α-hydroxy-kaur-16-en-19-oic acid (**2**) and 8.9 mg (1.8%) of *ent*-12β-hydroxy-kaur-9(11),16-dien-19-oic acid (**3**) were isolated. Proceeding as described above, 22.0 mg (4.4%) of **2**, 6.8 mg (1.4%) of **3** and 20.1 mg (4.0%) of *ent*-16β,17-dihydroxy-kauran-19-oic acid (**4**) were isolated from 195.0 mg of residue obtained from the 15-day culture.

3.5. Ent-kaur-16-en-19-oic acid (*1*)

White solid. M.p. 175.9–176.8°C (lit. Etse et al. (1987) [171–173°]). IR ν_{max} cm^{-1}: 3000–2800 (OH), 1700 (CO_2H), 1650 ($C{=}CH_2$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.94 and 1.23 (3H each, s, Me), 2.62 (1H, m, H-13), 4.72 and 4.78 (2H, s, 2H-17). ^{13}C NMR: Table 1.

3.6. *Ent-7α-hydroxy-kaur-16-en-19-oic acid (**2**)*

White solid. M.p. 238.6–241.3°C (lit. Mitra, Das, Kirtaniya, Patra and Mitra (1980) [239–243°]). IR ν_{max} cm^{-1}: 3480 (OH), 1700 (COOH). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.96 and 1.24 (3H each, s, Me), 2.59 (1H, m, H-13), 3.63 (1H, s, H-7), 4.80 (1H, s, H-17b), 4.82 (1H, s, H-17a). ^{13}C NMR: Table 1. EIMS 70 eV, *m/z* (rel. int.): 318 [M]$^+$ (8), 300 [M-H_2O]$^+$ (100), 285 [300-Me]$^+$ (15), 239 [285-HCOOH]$^+$ (9).

3.7. *ent-12β-Hydroxy-kaur-9(11),16-dien-19-oic acid (**3**)*

White solid. M.p. 159.8–161.6°C (lit. Gao et al. (1987) [178–180°]). IR ν_{max} cm^{-1}: 3420 (OH), 1700 (CO_2H), 1650 and 880 (C=CH_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.04 and 1.16 (3H each, s, Me), 3.95 (1H, t, J = 3.5 Hz, H-12), 4.91 (1H, bs, H-17a) and 5.05 (1H, bs, H-17b), 5.36 (1H, d, J = 3.8Hz, H-11). ^{13}C NMR: Table 1. EIMS 70 eV, *m/z* (rel. int.): 316 [M]$^+$ ($C_{20}H_{28}O_3$) (100), 301 [M–Me]$^+$ (73), 283 [301-H_2O]$^+$ (45), 237 [283-HCOOH]$^+$ (29), 201 [M–$C_6H_{11}O_2$]$^+$ (42).

3.8. *Ent-16β,17-dihydroxy-kauran-19-oic acid (**4**)*

White solid. M.p. 268.4–270.1°C (lit. Etse et al. (1987) [264–266°]); IR ν_{max} cm^{-1}: 3450, 3280 (OH), 1700 (CO_2H). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.23 and 1.38 (3H each, s, Me), 4.10 and 4.20 (2H, AB system, J = 10.8 Hz, 2H-17). ^{13}C NMR: Table 1. EIMS 70 eV, *m/z* (rel. int.): 336 [M]$^+$ ($C_{20}H_{32}O_4$), 318 [M–H_2O]$^+$ (3), 305 [M–$C_2H_3O_2$]$^+$ (42), 287 [318-C_2HO]$^+$ (13), 259 [305-HCOOH]$^+$ (22).

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