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Hydroxylation of the diterpenes *ent*-kaur-16-en-19-oic and *ent*-beyer-15-en-19-oic acids by the fungus *Aspergillus niger*

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ABSTRACT

The diterpenes *ent*-kaur-16-en-19-oic acid (**1**) and *ent*-beyer-15-en-19-oic acid (**2**) are the major constituents of a spasmolytic diterpenic mixture obtained from the roots of *Viguiera hypargyrea*, a Mexican medicinal plant. Microbial transformation of **1** and **2** was performed with *Aspergillus niger*. Two metabolites, *ent*-7α,11β-dihydroxy-kaur-16-en-19-oic acid (**4**) and *ent*-1β,7α-dihydroxy-kaur-16-en-19-oic acid (**5**), were isolated from the incubation of **1**, and one metabolite, *ent*-1β,7α-dihydroxy-beyer-15-en-19-oic acid (**6**), was isolated in high yield (40%) from **2**. The structures were elucidated on the basis of spectroscopic analyses and confirmed by X-ray crystallographic studies. Compounds **1**–**4** and **6** and methyl ester derivatives **4a** and **6a** were evaluated for their ability to inhibit the electrically induced contraction of guinea-pig ileum. Compounds **1**, **3**, **4**, **4a** and **5** were significantly active. These results showed that dihydroxylation of **1** at 7β, 11α-, and 1α, 7β-positions resulted in a loss of potency.

1. Introduction

Viguiera hypargyrea L. (Asteraceae), popularly known as "plateada" is a perennial herb that grows wild in the Durango state of México (Blake, 1918). Its roots are a reputed folk remedy for the treatment of gastrointestinal disorders (Martínez, 1969). Previous phytochemical studies on the roots established the presence of the triterpene oleanolic acid, as well as mono- and bi-desmoside oleanolic acid saponins (Alvarez et al., 2003), and a mixture of diterpenes (12.5%) *ent*-kaur-16-en-19-oic acid (**1**), *ent*-beyer-15-en-19-oic acid (**2**) and *ent*-kaur-9(11),16-dien-19-oic acid (**3**). This mixture, and its principal components **1** and **2**, showed inhibition of the electrically induced contraction of guinea-pig ileum (Zamilpa et al., 2002). While the *ent*-Kaurene diterpenes are encountered in the *Viguiera* genus (Alvarez et al., 1985; Marquina et al., 2001; Tirapelli et al., 2002), *ent*-beyerane diterpenes are rare and little-studied biologically thus far. Of these, beyerenoic acid (BA, **2**) has been described to display antimicrobial and spasmolytic activities (Zamilpa et al., 2002; McChesney et al., 1991), and kauradienoic acid (**3**) has been reported to display inhibitory action on the spontaneous contractility of rat, guinea pig and human uterus (Enriquez et al., 1984). Kaurenoic acid (KA, **1**) was also shown to possesses a wide spectrum of bioactivities (García et al., 2007). Thus, between them, the antispasmodic and relaxant actions on smooth muscle have received great attention (Zamilpa et al., 2002; Tirapelli et al., 2002; Bejar et al., 1984; Campos-Lara et al., 1990; Page et al., 1992; Campos-Bedolla et al., 1997; Tirapelli et al., 2003, 2004, 2005; Muller et al., 2003; Cunha et al., 2003; Ambrosio et al., 2004).

Biotransformation is today considered to be an economically competitive technology by synthetic organic chemists in search of new production routes for fine chemical, pharmaceutical and agrochemical compounds (Davis and Boyer, 2001). From the different transformations catalyzed by enzymatic systems, the selective hydroxylation of non-activated carbon atoms is particularly interesting, because this transformation is difficult to achieve by classical methods (Lehman and Stewart, 2001; Hanson, 1992). The introduction of hydroxyl groups in non-hydroxylated diterpenoids may enhance existing properties or lead to new biological activities. Microbial transformations have been used to introduce hydroxyl groups at positions remote from the functional group on diterpenoid molecules, such as dehydroabietic acid (Van Beek et al., 2007), stemodane (Chen et al., 2005; Fraga et al., 2004.), steviol (Yang et al., 2007) and manoyl oxides (Garcia-Granados et al., 2004), among others. Although several microbial hydroxylations on beyerane derivatives (Diaz et al., 1985; Ali et al., 1992; García-Granados et al., 1994, 1997; Yang et al., 2004) and the biologically important *ent*-16-ketobeyeran-19-oic acid (isosteviol) (Bearder et al., 1976; De Oliveira et al., 1999; Hsu et al., 2002; Akihisa et al., 2004; Lin et al., 2007) have been described, to the best of our knowledge, there are no reports about biotransformation of beyerenoic acid (**2**). The biotransformation of **1** by *Cunninghamella blakesleeana* caused

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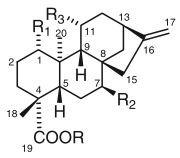
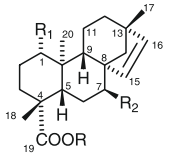
monohydroxylation at positions 7β and 16α, as well as dihydroxylation at 16α,17- and 7β,16α-positions (El-Emary et al., 1976), and the biotransformation by *Rhizopus stolonifer* caused hydroxylation at 7α, and hydroxylation/dehydrogenation at 12β/9(11) positions (Silva et al., 1999).

Thus based on the knowledge that KA (**1**) and BA (**2**) exert spasmolytic activity and that both compounds show slight differences in their chemical structures, we decided to submit these compounds to biotransformation with *Aspergillus niger* and to evaluate the antispasmodic activity of their transformation products. Biotransformation of *ent*-kaur-16-en-19-oic acid (**1**) with *A. niger* afforded metabolites **4** and **5** while biotransformation of *ent*-beyer-15-en-19-oic acid (**2**) yielded metabolite **6**. The present contribution describes the production, isolation, structure elucidation and spasmolytic activity of these metabolites. The structures of **4** and **6** were confirmed by X-ray crystallographic analyses.



	R	R_1	R_2	R_3
1	H	H	H	H
4	H	H	OH	OH
4a	CH_3	H	OH	OH
5	H	OH	OH	H
5a	CH_3	OH	OH	H



	R	R_1	R_2
2	H	H	H
6	H	OH	OH
6a	CH_3	OH	OH

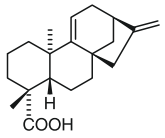


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Table 2
^{13}C NMR chemical shifts of compounds **4**–**6** and their methyl derivatives **4a**–**6a** ($CDCl_3$, δ values in ppm)[a].

Position	4	4a	5	5a	6	6a
1	42.0[b]	42.3	68.5	67.6	79.4	79.8
2	19.2	19.3	39.2	38.0	28.3[b]	28.4
3	37.9	37.9	31.6	41.6	22.7	23.2
4	43.2	43.6	42.5	44.5	42.1	42.7
5	47.6	47.8	45.0[b]	45.8	45.6	46.1
6	28.7	28.8	28.6	28.6	28.6[b]	35.5
7	76.7	76.9	80.0	78.6	72.0	72.9
8	49.2	49.8	47.5	46.1	54.5	54.6
9	53.3	53.3	50.7	54.4	46.0	46.2
10	40.6	40.5	45.7	40.8	43.1	43.2
11	69.8	70.1	18.6	17.6	29.7	30.0
12	42.2[b]	42.7	30.5	32.7	32.8	32.9
13	43.1	43.1	42.2	43.8	43.6	44.0
14	38.6	38.6	37.2	39.8	56.6	56.3
15	44.9	45.0	45.0[b]	45.4	133.0	132.1
16	154.8	154.6	155.7	158.1	137.2	137.8
17	103.3	103.8	106.2	105.1	24.4	24.4
18	29.1	29.1	28.4	29.5	28.3	28.6
19	180.7	178.7	180.7	179.8	179.4	177.0
20	16.2	16.1	17.5	17.5	8.0	7.9
OCH_3		51.4		51.9		52.9

[a] Assignments based on DEPT, HMQC and HMBC. Signal multiplicity and coupling constants (Hz) are in parentheses.
[b] Assignments are interchangeable.

2. Results and discussion

The less polar metabolite obtained from biotransformation of **1** with *A. niger* was **4** (20% yield) and displayed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 335.2202 corresponding to the molecular formula $C_{20}H_{30}O_4$, indicating a metabolite structure containing two more oxygen atoms than **1**. The location and orientation of the hydroxyl groups was confirmed by detailed analyses of 1D NMR, and 2D NMR (^1H NMR, NOE, ^{13}C NMR, HMBC and HSQC) spectroscopic data (Tables 1 and 2). The HSQC spectrum showed

Table 1
^1H NMR chemical shifts of metabolites **4**–**6** and their methyl ester derivatives **4a**–**6a** ($CDCl_3$, δ values in ppm)[a].

Position	4	4a	5	5a	6	6a
1α	2.70, *m*	2.65, *m*	β3.19, *dd* (11.5, 5)	β3.34, *dd* (11.6, 5)	β3.28, *dd* (11.2, 4.8)	β3.44, *dd* (11.4, 5.0)
	0.82, *m*	0.62, *dt* (12.8, 3.7)				
2	1.80, *dd* (13.2, 2.0)	1.4, *dt* (14.2, 2.8)	1.6, *m*	1.38, *m*	1.70, *ddd* (14, 3.6)	1.74, *dt* (10.6, 2.4)
	1.38, *br d* (14)	1.82, *m*[b]		1.78, *m*	1.45, *m*[b]	1.4, *m*[b]
3	1.07, *dd* (5.6, 2)	1.07, *t* (4.0)	2.0, *m*[b]	2.03, *m*	1.98, *dt* (13.6, 3.6)	2.09, *m*[b]
	2.24, *br d* (2.4)	2.2, *d* (2)		2.26, *dd* (12.4, 2)	2.05, *dd* (13.6, 2.0)	1.5, *br s*[b]
5	1.93, *dd* (12.4, 2.4)	1.88, *dd* (12.4, 2.4)	1.8, *m*	1.84, *m*	1.59, *dd* (13.2, 2.2)	1.96, *m*
6	2.15, *d* (2.4)	2.15, *d* (2.4)	2.0–2.2, *m*	2.09, *m*	β2.05, *dd* (13.6, 1.6)	1.90, *m*[b]
	1.96, *m*	1.97, *m*		2.15, *m*[b]	1.85, *dt* (14.0, 2.0)	2.09, *m*[b]
7	α3.62 *br s*	α3.59, *t* (3.4)	α3.64, *t* (3.4)	α3.93, *br s*	α3.47, *t* (3.4)	α3.73, *br s*
9	2.06 *dd* (13.2, 2.4)	2.07, *dd* (14, 2.4)	2.0–2.2, *m*	2.16, *m*[b]	1.47, *dd* (11.6, 5.2)	1.5, *m*[b]
11	β4.18 *ddd* (12.8, 6.4, 6.4)	β4.23, *ddd* (12.8, 6.4, 6.4)	1.7–1.9, *m*	1.5–1.8, *m*	1.73, *ddd* (3.2, 4, 11.2)	1.4, *m*[b]
			1.7–1.9, *m*	1.5–1.8, *m*	1.45, *m*[b]	
12	β1.85 *m*	β1.86, *dd* (12.4, 2.4)	1.2, *m*	1.05, *m*	1.40, *m*	1.24, *m*[b]
	1.42, *m*	1.4, *br d* (14.2)			1.15, *m*	0.83, *m*
13	2.71, *br s*	2.72, *br s*	2.78, *br s*	2.77, *m*		
14	2.09, *m*	2.0, *m*[b]	2.0, *m*[b]	1.84, *m*	1.41, *d* (8)	1.38, *d* (8)
	1.20, *m*	1.18, *m*		2.09, *m*	1.2, *d* (8)	1.24, *d* (8)[b]
15	2.28, *d* (2)	2.28, *d* (2)	2.2, *m*	2.24, *m*	5.44, *d* (6)	5.54, *d* (6)
	2.19, *t* (2.4)	2.19, *t* (2.4)		2.18, *m*		
16					5.40, *d* (6)	5.51, *d* (6)
17	4.74, *s*	4.86, *s*	4.69, *d* (2)	4.93, *s*	0.91, *s*	1.04, *s*
	4.63, *s*	4.75, *s*	4.57, *d* (2)	4.81, *s*		
18	1.16, *s*	1.16, *s*	1.17, *s*	1.17, *s*	1.03, *s*	1.07, *s*
20	1.04, *s*	1.03, *s*	1.02, *s*	1.03, *s*	0.61, *s*	0.62, *s*
OCH_3		3.65, *s*		3.66, *s*		3.64, *s*

[a] Assignments based on DEPT, HSQC and HMBC. Signal multiplicity and coupling constants (Hz) are in parentheses.
[b] Overlapping signals.

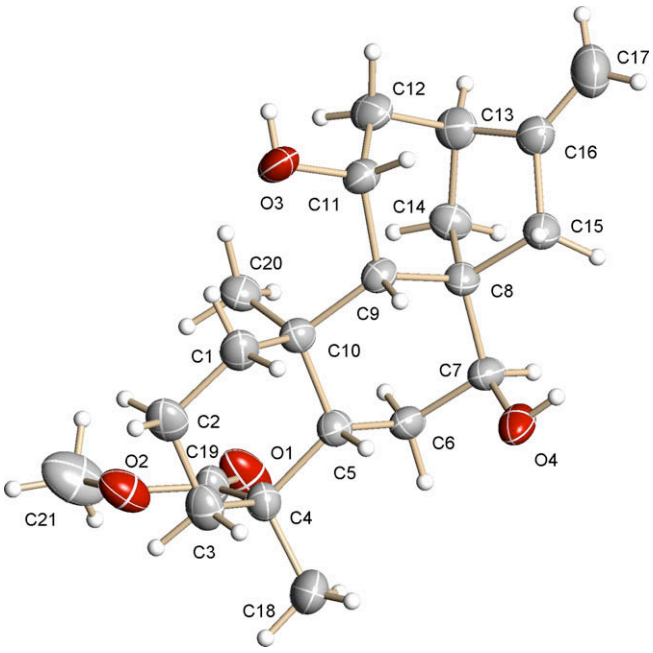

Fig. 1. Perspective drawing of the X-ray structure of **4a**.

two new protons geminal to an alcohol function resonating at δ_H 3.62 (δ_C 76.7), and δ_H 4.18 (δ_C 69.8), respectively. According to the HMBC spectrum, the signal at δ_H 3.62 had connectivities with C-5 (δ 47.6), C-9 (δ 53.3) and C-14 (δ 38.6), indicating the introduction of a hydroxyl group at C-7. The β orientation of the 7-OH group was suggested from the cross-peaks of δ 3.62 (H-7) with H-6α, and CH_3-20 group in the NOESY experiment. The location of the second hydroxyl group at C-11 was deduced by HMBC correlations among δ_H 4.18 (H-11) and C-8 (δ 49.2), C-9 (δ 53.3), C-10 (δ 40.6), C-12 (δ 42.2) and C-13 (δ 43.1). The α-orientation of the hydroxyl group at C-11 was deduced from the multiplicity of the H-11 signal in the ^{1}HNMR spectrum, namely a double triplet (J = 12.8, 6.4 Hz) as well as cross-peaks between δ 4.18 (H-11) and H-9, H-12β and H-15), in the NOESY spectrum (Yang et al., 2007; Shigematsu et al., 1982). Thus, on the basis of the above evidence, it was determined to be *ent*-7α,11β-dihydroxykaur-16-en-19-oic acid (**4**). Methylation with diazomethane afforded the methyl ester **4a** whose structure was confirmed by an X-ray crystallographic study (Fig. 1, see Supplementary material). Compound **4** had been previously obtained by biotransformation of **1** with the *Gibberella fujikuroi* mutant B1-41a (Gaskin et al., 1984). We have now assigned the ^{1}H and ^{13}C NMR spectra of this compound utilizing two dimensional NMR spectroscopic data.

The second metabolite obtained in this biotransformation with a 5.8% yield was **5**, which was previously unreported. Metabolite **5** had a molecular formula of $C_{20}H_{31}O_4$ as determined from its positive ion HRFABMS $[M+H]^+$ at m/z 335.2219 as well as from its ^{13}C NMR spectrum, indicating a structure containing two more hydroxyl groups than **1**. The location of the hydroxyl groups was determined by a detailed analysis of the HMBC data. The chemical shift of the hydrogen at δ 3.64 showed connectivities with C-5 (δ 45.0), C-9 (δ 50.7) and C-14 (δ 37.2), indicating the presence of hydroxyl group at C-7 in **5**. The β-axial position of the 7-OH was suggested from the cross-peaks of δ 3.64 (H-7) with H-6α, and CH_3-20 group in the NOESY experiment. The chemical shift at δ_H 3.19 (*dd*, J = 11.5, 5 Hz) showed connectivities with C-3 (δ 31.6), C-5 (δ 45.0), C-10 (δ 45.7) and C-20 (δ 17.5) in the HMBC spectrum, supporting the position of the hydroxyl group at C-1. The α-orientation of this alcohol was suggested by the cross-peaks of H-1 (δ 3.19) with H-5 (δ 1.8) and H-9 (δ 2.0–2.2 m) in the NOESY experiment. On the basis of the above evidence, it was established as *ent*-1β,7α-dihydroxy-kaur-16-en-19-oic acid (**5**). Methylation with diazomethane afforded derivative **5a.**

Metabolite **6** was obtained with a 40% yield from the biotransformation of *ent*-beyer-15-en-19-oic acid (**2**) with *A. niger*, and was previously unreported. Its HRFABMS showed a quasi-molecular ion peak $[M+H]^+$ at m/z 335.2219, corresponding to a molecular formula of $C_{20}H_{31}O_4$. The ^{1}H NMR spectra was very similar to that of **5**, but now the signals of the protons geminal to the hydroxyl groups appeared at δ_H 3.28 (1H, *dd*, J = 11.2, 4.8 Hz) for H-1 and δ_H 3.47 (1H, *t*, J = 3.4 Hz) for H-7. Comparison of the ^{1}H and ^{13}C NMR spectra of **2** and **6** established that **6** had the same substitution pattern as for **5**. The location of the hydroxyl groups was confirmed by a detailed analysis of the HMBC data. The multiplicity of H-7 in the ^{1}H NMR spectrum, (t, J = 3.4 Hz), and cross-peaks of δ_H 3.47 with H-6α (1.85, *dt*, J = 14.0, 2.0 Hz) and CH_3-20 (δ 0.61, s), in the NOESY experiment, confirmed the β-axial position of the 7-OH group. The chemical shift at δ_H 3.28 (*dd*, J = 11.2, 4.8 Hz) showed connectivities with C-3 (δ 22.7), C-5 (δ 45.6), C-10 (δ 43.1) and C-20 (δ 8.0) in the HMBC spectrum, supporting the position of the second hydroxyl group at C-1. Its α-orientation was suggested from the cross-peaks of H-1 (δ 3.28) with H-5 and H-9 in the NOESY experiment. An X-ray crystallographic analysis confirmed structure **6** as *ent*-1β,7α-dihydroxy-beyer-15-en-19-oic acid (Fig. 2, see Supplementary material). Methylation with diazomethane afforded the methyl ester **6a**.

Compounds **1**–**4** and **6** and the methyl ester derivatives (**4a, 6a**) were evaluated for their ability to inhibit the electrically induced contraction of guinea-pig ileum. The results showed that none of the compounds with a beyerenoic acid skeleton (compounds **2** and **6**) showed spasmolytic activity (EC_{50} > 29 μmol/L, Table 3), while all the kaurenoic derivatives (**1**, **3**, **4**, **4a** and **5**) were significantly active. In particular, **1** (EC_{50} = 0.040 μmol/L) was more active than papaverin (EC_{50} = 0.054 μmol/L) (Table 3), as previously demonstrated (García et al., 2007). Compound **3** was included in this bioassay because it is the minor component of the spasmolytic diterpenic mixture obtained from the *n*-hexane extract of the roots of *V. hypargyrea* (Zamilpa et al., 2002) and had not been previously assayed in this bioassay. This compound displayed a minor activity than **1** (EC_{50} = 0.17 μmol/L). These results showed that dihydroxylation of **1** at 7β, 11α- (metabolite **4**, EC_{50} = 0.079 μmol/L), and 1, 7β- (metabolite **5**, EC_{50} = 0.11 μmol/L) positions resulted in a loss of potency. Metabolite **4** was the most active biotransformed product, and it is noteworthy that its methyl ester derivative (**4a**, EC_{50} = 0.097 μmol/L) displayed a similar effect to **4**, which indicated that the major solubility of compounds **1**, **3**, **4** and **5** did not influence activity in this bioassay, as previously demonstrated by Ambrosio et al. (2004).

3. Conclusion

The results of the incubations indicated that *A. niger* has the ability to perform *regio*- and stereoselective dihydroxylations at the 1α,7β-positions of **1** and **2**, and the 7β,11α-positions of **1**. The dihydroxylation of **2** was most efficient (41% yield), which provides an useful tool for preparing new beyerenoic acid derivatives dihydroxylated at these positions.

The hydroxylation of kaurenes and beyerenes at C-7 is a commun feature of microbial biotransformations. However, 7β,11α-dihydroxylation is novel which could give access to A and C rings, respectively. The dihydroxylation at C-1α and C-7β of **1** (20%) and **2** (41%) by *A. niger* gave better yields than did dihydroxylation at the same positions of *ent*-16β-hydroxybeyeran-19-oic acid (5% yield) (Yang et al., 2004), *ent*-16-ketobeyeran-19-oic acid (6.6% yield) (De Oliveira et al., 1999) and *ent*-13-hydroxykaur-16-

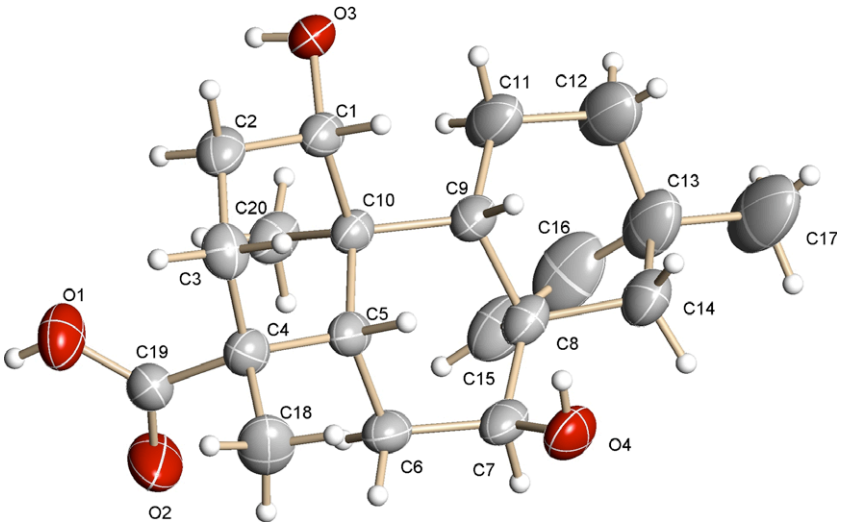

Fig. 2. Perspective drawing of the X-ray structure of **6**.

Table 3
Inhibition of electrically induced contractions of guinea-pig ileum by compounds **1**–**6**, **4a** and **6a**[a].

Compound	EC_{50} (μmol/L)	E_{max} (μmol/L)
1	0.040	0.085
2	>0.30	Nd[b]
3	0.17	0.030
4	0.079	0.217
4a	0.097	0.15
5	0.11	0.23
6	>0.29	Nd[b]
6a	>0.29	Nd[b]
Papaverin	0.054	0.25

[a] $P < 0.0001$ in the Student's *t*-test.
[b] Not determined.

en-19-oic acid (2.7% yield) (Yang et al., 2007), indicating that the oxygenated functions at C-16 and C-13 diminish the action of the enzymes of *A. niger*.

Hydroxylation at C-11α of *ent*-17,19-dihydroxy-16βH-kaurane by *Verticillum lecanii* (Vieira et al., 2002) and of isosteviol by *Cunninghamella bainieri* (Hsu et al., 2002) have been reported.

The larger amounts of *ent*-1β,7α-dihydroxy-beyer-15-en-19-oic acid (**6**), obtained from biotransformation of *ent*-beyer-15-en-19-oic acid (**2**) with *A. niger* will also provide a technical basis for studying its pharmacological properties. The isolated metabolites will be useful as reference standards for our continuing studies on structure modification and pharmacological evaluation of diterpenes.

4. Experimental

4.1. General

Melting points were determined on a Fisher-Johns Melting Point apparatus. Optical rotations were measured on a Perkin–Elmer 241 MC polarimeter. The IR spectra were measured on a Bruker Vector 22 spectrometer. All NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 MHz for ^{1}H NMR, ^{1}H–^{1}H COSY, HMBC, HSQC and ^{1}H–^{1}H NOESY and 100 MHz for ^{13}C NMR and ^{13}C DEPT using DMSO-d_6 and $CDCl_3$ as solvents. Chemical shifts are reported in ppm (δ) relative to TMS. HRFAB-MS in a matrix of glycerol were recorded on a JEOL JMX-AX 505 HA mass spectrometer. X-ray single crystal diffractions were measured on a Bruker-AXS APEX diffractometer equipped with a CCD area detector.

4.2. Substrates

ent-Kaur-16-en-19-oic acid (**1**), *ent*-beyer-15-en-19-oic acid (**2**), and *ent*-kaur-9(11)-dien-19-oic acid (**3**) were isolated from *V. hypargyrea* roots. Dry vegetal material (1.0 kg) was macerated with *n*-hexane (12 L × 3 times × 8 h each). The *n*-hexane extract (29.0 g) was evaporated to dryness at reduced pressure, and fractionated by vacuum liquid chromatography over silica gel eluting with *n*-hexane–EtOAc mixtures of increasing polarity to yield four fractions: Fr. 1, 5.3 g (100:0, 0.5 L); Fr. 2, 4.2 g (97:03, 0.5 L); Fr.3, 4.2 g (90:10 1 L) and Fr. 4, 4.9 g (0:100 1.5 L). An aliquot (3.2 g) of fraction 2, containing the diterpenic acid mixture, was subjected to an open column chromatography (CC) over reversed phase silica gel 60 (RP-18, 40–63 mm, 15 g) using an CH_3CN–H_2O gradient (9:1 to 10:0). A total of 48 fractions (10 mL each) were collected and analysed by HPLC using a Lichrosphere C-18 column (5 μm, 124 × 4 mm) with CH_3CN–H_2O (90:10) as isocratic eluting system, a flow rate of 1 mL/min, and a refractive index detector. Fraction III (eluates 10–13, CH_3CN/H_2O 9:1) afforded kauradienoic acid (**3**) (110 mg); fraction V (eluates 17–27, CH_3CN/H_20 9.5:0.5) gave kaurenoic acid (**1**) (248 mg) and finally, beyerenoic acid (**2**) (627 mg) was obtained from fraction VII which was eluted with 9.5:0.5 CH_3CN/H_2O mixtures. The retention times of these compounds were: 8.25, 10.5 and 12.7 min, respectively. The purity was determined to be >99% by ^{1}H NMR spectroscopic analysis.

4.3. Microorganisms and culture media

A. niger AN-1 was obtained from the Enviromental Biotechnology Laboratory, Universidad Autónoma del Estado de Morelos, México. Cultures of *A. niger* were maintained at 4 °C on Sabouraud-agar slants (per liter: 10 g peptone, 40 g glucose, 15 g agar) and subcultured periodically. In the transformation experiments a medium composed of maltose (20 g), peptone (10 g) and Sabouraoud-maltose extract (2 g) at pH 5.6 in H_2O (1 L) was used.

4.4. Biotransformation of ent-kaur-16-en-19-oic acid (*1*)

Mycelia of *A. niger* from agar slants were aseptically transferred to 250 mL Erlenmeyer flasks containing 80 mL of liquid medium. The fungus was incubated at 25 °C on a rotary shaker (180 rpm)

in the dark for 24 h to make a stock inoculum. Then 10 mL of the inoculum was added to each of the two 2-L flasks containing 800 mL of medium. After 36 h incubation, kaurenoic acid **1**, (220 mg) dissolved in DMSO (5 mL) were distributed equally among the two flasks. The incubation was allowed to continue for 13 additional days on the shaker. The cultures were then pooled and filtered. The filtrate was saturated with NaCl and extracted with EtOAc (5 × 500 mL), and the dried cell mass was extracted with MeOH (4 × 200 mL). The extracts were combined, dried over Na_2SO_4, and concentrated under vacuum at 60 °C to afford a brownish solid (754 mg). The extract was subjected to silica gel CC (50 g, 200–300 mesh, Ø 2 × 30 cm), eluted in 50 mL fractions with a gradient of *n*-hexane/EtOAc to afford four fractions. Further chromatography of fraction 2 (171.3 mg) over silica gel eluted with *n*-hexane–EtOAc (4:1), gave **1** (98 mg). Fraction 3 (*n*-hexane/EtOAc 2:3, 105.6 mg), was further subjected to silica gel CC eluted with CH_2Cl_2–MeOH (20:1) to give eight fractions. Fractions 4–7 were subjected to preparative thin layer chromatography impregnated with $AgNO_3$ 0.1 N, and eluted with *n*-hexane/EtOAc (3:2) to give *ent*-7α,11β-dihydroxykaur-16-en-19-oic acid (**4**) (15.7 mg, 20% yield), and *ent*-1β,7α-dihydroxykaur-16-en-19-oic acid (**5**) (5.4 mg, 5.8% yield). Fraction 8 was methylated with CH_2N_2, and the product was purified over silica gel eluted with *n*-hexane–EtOAc (7:3) to give **5a** (6.2 mg) and **4a** (25 mg).

4.4.1. *ent*-7α,11-Dihydroxykaur-16-en-19-oic acid (**4**)

White powder; IR (KBr) ν_{max} 3490, 3357, 1721, 1661, 1152 and 873 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRFABMS *m/z* 335.2202 $[M+H]^+$ ($C_{20}H_{31}O_4$, calc. 335.2223).

4.4.1.1. Methyl-*ent*-7α,11-dihydroxykaur-16-en-19-oate (**4a**).

Colorless crystals (MeOH); mp 223–225 °C; IR (KBr) ν_{max} 3500, 3359, 1717, 1661, 1154 and 874 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRFABMS *m/z* 349.2375 $[M+H]^+$ ($C_{21}H_{33}O_4$, calc. 349.2379).

4.4.1.2. The crystal data of **4a**.

$C_{21}H_{32}O_4$, orthorhombic, $P2_12_12_1$, *a* 7.9869(8) Å, *b* 11.2849(11) Å, *c* 21.030(2) Å, *V* 1895.5(3) $Å^3$; Z4, D_{calcd} 1.221 g cm^{-3}, *F*(0 0 0) 760, λ(Mo Kα) 0.71073 Å, *T* 273(2) K, 13562 reflections collected. Final GooF 1.062, final *R* indices R_1 0.0336, wR_2 0.0869, 231 parameter, μ 0.083 mm^{-1}, *R* indices based on 3343 reflections with $I > 2\sigma()$, absorption corrections applied.

4.4.2. *ent*-1β,7α-Dihydroxy-kaur-16-en-19-oic acid (**5**)

White powder; IR (KBr) ν_{max} 3490, 3395, 1720, 1680, 1150 and 874 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRFABMS *m/z* 335.2219 [M+H]+ ($C_{20}H_{31}O_4$, calc. 335.2223).

4.4.2.1. Methyl *ent*-1β,7α-dihydroxy-kaur-16-en-19-oate (**5a**).

White powder; IR (KBr) ν_{max} 3510, 3380, 1712, 1670, 1155 and 872 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRFABMS *m/z* 349.2365 $[M+H]^+$ ($C_{21}H_{33}O_4$, calc. 349.2379).

4.5. Biotransformation of *ent*-beyer-15-en-19-oic acid (**2**)

Mycelia of *A. niger* from agar slants were aseptically transferred to Erlenmeyer flasks (250 mL) containing liquid medium (80 mL). The fungus was incubated at 25 °C on a rotary shaker (180 rpm) in the dark for 24 h to make a stock inoculum. Then inoculum (10 mL) was added to each of the two 2-L flasks containing medium (800 mL). After 36 h incubation, BA (**2**) (300 mg) dissolved in DMSO (5 mL) was evenly distributed among the two flasks. The incubation was allowed to continue for 13 additional days on the shaker. The cultures were then pooled and filtered through Whatman No. 1 filter paper. The filtrate was saturated with NaCl and extracted with EtOAc (5 × 500 mL), and the dried cell mass was extracted with MeOH (4 × 200 mL). The extracts were combined, dried (Na_2SO_4), and concentrated under vacuum at 60 °C to afford a brownish solid (880 mg). The extract was subjected to silica gel CC (50 g, 200–300 mesh, Ø 2 × 30 cm), eluted in 50 mL fractions with a gradient of CH_2Cl_2/MeOH to afford seven fractions. With further chromatography of the fraction 1 (125.3 mg) over silica gel eluted with *n*-hexane–EtOAc (4:1), **2** (75 mg) was recovered. Fraction 3 (CH_2Cl_2/MeOH 9:1, 167.9 mg), was further subjected to silica gel CC impregnated with $AgNO_3$ eluted with *n*-hexane–EtOAc (1:1) to give nine fractions. Fraction 5 was methylated with diazomethane and the residue was purified over silica gel eluted with *n*-hexane–EtOAc (7:3) to give 6**a** (12.2 mg). After recrystallization with MeOH, fraction 6 afforded 110.8 mg of *ent*-1β,7α-dihyroxykaur-16-en-19-oic acid (**6**) (41% total yield).

4.5.1. *ent*-1β,7α-Dihydroxy-beyer-15-en-19-oic acid (**6**)

Colorless crystals (MeOH); mp 225–228 °C; IR (KBr) ν_{max} 3404, 1682, 1158 and 993 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRFABMS *m/z* 335.2219 $[M+H]^+$ ($C_{20}H_{31}O_4$, calc. 335.2223).

4.5.1.1. The crystal data of **6**.

$C_{20}H_{30}O_4$, orthorhombic, $P2_12_12_1$, *a* 7.9977(17) Å, *b* 10.919(2) Å, *c* 21.261(5) Å, *V* 1856.7(7) $Å^3$; Z4, D_{calcd} 1.196 g cm^{-3}, *F*(0 0 0) 728, λ(Mo Kα) 0.71073 Å, *T* 298(2) K, 16836 reflections collected. Final GooF 1.049, final *R* indices R_1 0.0410, wR_2 0.1111, 223 parameter, μ 0.082 mm^{-1}, *R* indices based on 3275 reflections with $I > 2\sigma()$, absorption corrections applied.

4.5.2. Methyl *ent*-1β,7α-dihydroxy-beyer-15-en-19-oate (**6a**)

Oil; IR (KBr) ν_{max} 3498, 3323, 1715, 1672, 1120 and 864 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRFABMS *m/z* 349.2377 $[M+H]^+$ ($C_{21}H_{33}O_4$, calc. 349.2379).

4.6. Spasmolytic activity

Male guinea pigs (400–600 g) were sacrificed by a blow to the base of the skull and cervical dislocation and 1.5 cm pieces of the ileum were dissected from the ileum segment 10 to 20 cm proximal to the ileocecal valve. The tissue was mounted in a set of 3 ml chambers. One end of the tissue was tied to the bottom of the chamber while the other was tied throw a silk thread to a force transducer, which was connected to an acquisition system (MACLAB) and recorded in a Mac computer. Tissues were maintained at 37 °C in a Tyrode solution [composition (mM): 136.0 NaCl, 5.0 KCl, 0.98 $MgCl_2$, 2.0 $CaCl_2$, 0.36 NaH_2PO_4, 11.9 $NaHCO_3$, and 5.5 glucose], pH 7.4 and bubbled continuously with carbogen (O_2 95%, CO_2 5%). After 30 min of adaptation period, the tissue was electrically stimulated (25 V, 5 mS, 1 Hz, 5 S, every 2 min; with a Grass stimulator) by isolated tungstene electrodes connected to the end of the tissue. Induced contractions were recorded and after reach a homogeneous response, different concentrations (12.5, 25, 50 and 100 μg/L) of compounds under study (dissolved in PVP 10000) were added into the chamber and the ability for inhibiting the electrically induced contraction was evaluated. Means obtained from 6 repetitions were used for constructing concentration response curves and the EC_{50} (effective concentration at 50%) as well as the maximum effect (E_{max}) of every compound were determined.

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Appendix A. Supplementary data

CCDC 696754 (1) and CCDC 696755 (2) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax:+44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.09.005.

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