PERGAMON

Phytochemistry 61 (2002) 367–372

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

ent-Kaurane diterpenoid glycosides from a Malagasy endemic plant, *Cussonia vantsilana*

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Received 16 April 2002; received in revised form 24 June 2002

Abstract

Four *ent*-kaurane diterpenoid glycosides, cussovantosides A–D, were isolated from the dried leaves of *Cussonia vantsilana* Baker, together with six known compounds. The structures of the compounds were deduced on the basis of their physical and spectral data.

Keywords: *Cussonia vantsilana*; Araliaceae; Cussovantosides A–D; Cussovantonins A and B; *ent*-kaurane diterpene glycosides; Malagasy endemic plant

1. Introduction

In previous phytochemical investigations (Harinantenaina et al., 2002a,b) of one species of the genus *Cussonia*, *Cussonia racemosa*, clerodane, labdane and *ent*-kaurane diterpenoid glycosides were isolated. The isolation of a sweet *ent*-kaurane diterpene glycoside, cussoracoside C, from *C. racemosa*, prompted the investigation of other species such as *Cussonia vantsilana* Baker from the same genus. *C. vantsilana* Baker is a tree widely distributed in the center and the South-East of Madagascar. The presence of saponins having ursolic acid as aglycone has been reported from this species (Chazan, 1971). From the leaves of the plant we have isolated five known compounds (**1–5**) and a large amount of cussoracoside C (**6**) together with four *ent*-kaurane diterpenoid glycosides (**7–10**) named cussovantosides A–D, respectively. The present study deals with the identification and structural elucidation of these compounds.

2. Results and discussion

Chromatographic separation followed by systematic HPLC purification of the concentrated hot methanol extract of the dried leaves of *C. vantsilana* Baker led to the isolation of a sweet compound named cussoracoside C (**6**) previously isolated from *C. racemosa* (Harinantenaina et al., 2002b) and four *ent*-kaurane diterpenoid glycosides (**7–10**) together with five known compounds identified as two known flavonol glycosides: rutin (**1**) and kaempferol rutinoside (**2**) (Harborne and Mabry, 1982), *ent*-kaur-16-en-19-oic acid (**3**) (Henrick and Jefferies, 1964), β-D-glucopyranosyl *ent*-16β,17-dihydroxykauran-19-oate (**4**) (Cheng et al., 1993), paniculoside IV (**5**) (Yamasaki et al., 1976) by comparison of their physical and spectral data with literature.

The molecular formula of cussovantoside A (**7**) was deduced as $C_{26}H_{42}O_{10}$ from HR FAB-MS data. The ^1H NMR spectrum showed signals of two quaternary methyl groups at δ 1.28 and 1.63 (each singlet), a methine bearing an oxygen atom at δ 3.35 (*dd*, $J = 12.4$, 3.9 Hz), and an anomeric proton of a β-glucopyranosyl ester at δ 6.19 (*d*, $J = 7.8$ Hz). Enzymatic hydrolysis of **7** gave a new aglycone named cussovantonin A (**7a**, $[\alpha]_D^{23}$ -23.2° $c = 0.4$, in pyridine) and D-glucose (see experimental). Inspection of the NMR spectral data suggested

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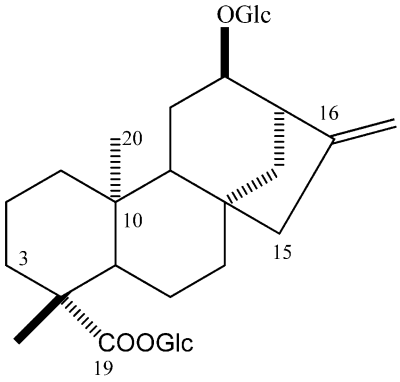
0031-9422/02/$ - see front matter
PII: S0031-9422(02)00263-7



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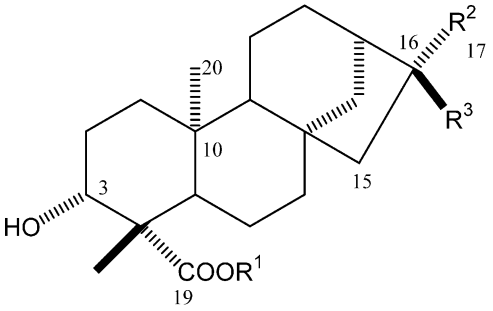
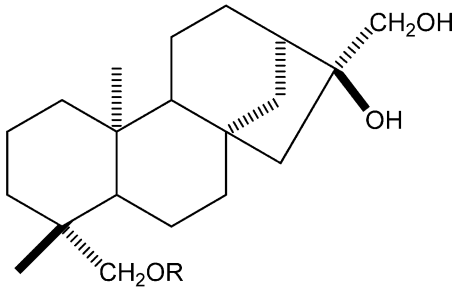


7: R^1=Glc, R^2= CH_2OH, R^3= OH
7a: R^1=H, R^2= CH_2OH, R^3= OH
8: R^1=Glc, R^2= OH, R^3= CH_2OH
8a: R^1=H, R^2= OH, R^3= CH_2OH



9: R= Glc^6–Api
10: R= Glc^2–Rha

Glc: β-D-Glucopyranosyl
Api: β-D-Apiofuranosyl
Rha: α-L-Rhamnopyranosyl

that **7a** is an *ent*-kaurane diterpenoid with an equatorial hydroxyl group at C-3 [δ_H 3.34 (*dd*, J=4.4, 11.8 Hz)], an axial carboxyl group at C-19 (δ_C 181.0), a hydroxyl group at C-16 (δ_C 79.7), and an AB quartet (2H) with a doublet centered at δ 3.76 and δ 3.83 (each *d*, J=10.7 Hz). The allocation of the hydroxyl group at C-3 and C-16, the hydroxymethylene at C-17 and the carboxyl group at C-19 were confirmed by H–H COSY, NOESY, HSQC and HMBC experiment. The COSY correlations were observed from H-1 to H-3. The absence of further coupling for H-17 showed that the second hydroxyl group must be attached at C-16. No NOE correlation was shown between H-18 and H-20 methyl groups. Furthermore, in the HMBC spectrum, long-range correlations were observed between H-3 and C-1, C-2, C-18. The stereochemistry of C-16 was concluded as follow. In the ^{13}C NMR spectra, the signal of the C-16 appeared at δ 79.7, appropriate for β-hydroxylated C-16 (Cheng et al., 1993). Thus the structure of cussovantonin A was deduced as *ent*-3α,16β,17 trihydroxykauran-19-oic acid. The characteristic chemical shift of the anomeric carbon signal due to ester linked β-glucopyranosyl (δ_C 95.9) in **7** confirmed the C-19 glucosylation. Therefore cussovantoside A was assigned as shown.

Compound **8** (cussovantoside B, $C_{26}H_{42}O_{10}$ by HR FAB-MS spectrometry) has the same molecular formula as **7**. The ^1H and ^{13}C NMR spectral data (Tables 1 and 3), which are very similar to those of **7**, suggested that it is also a kaurane diterpenoid glycoside. On enzymatic hydrolysis, **8** gave D-glucose (see Experimental) and a new aglycone named cussovantonin B (**8a**, $[\alpha]_D^{23}$ −56.6° c=0.6, in pyridine). The ^{13}C NMR spectrum of **8a** was

Table 1
^{1}H NMR spectral data for compounds **7** and **8** (400 MHz in pyridine-d_5)

H	7	8
1a	0.92 *ddd* (13.5, 13.5, 3.7)	0.94 *ddd* (13.6, 13.6, 3.5)
1b	1.85 *brd* (13.5)	1.90 *brd* (13.5)
2a	1.92 *m*	1.95 *m*
2b	2.55 *m*	2.50 *m*
3a	3.35 *dd* (12.4, 3.9)	3.37 *dd* (12.6, 3.8)
3b		
5	1.02 *dd* (12.6, 2.9)	1.10 *dd* (12.4, 3.0)
6a	2.01 *m*	1.98 *m*
6b	2.37 *m*	2.32 *m*
7ab	1.47 *m*	1.45 *m*
7b	1.75 *m*	1.75 *m*
9	0.95 *dd* (7.3, 3.9)	1.02 *dd* (7.5, 3.6)
11	1.49 *m*	1.48 *m*
12a	1.42 *m*	1.41 *m*
12b	1.85 *m*	1.90 *m*
13	2.35 *brs*	2.34 *brs*
14a	1.99[a]	1.98[a]
14b	2.12 *brd* (12.1)	2.14 *brd* (12.1)
15a	1.65 *m*	1.63 *m*
15b	1.82 *m*	1.79 *m*
17a	4.03 *d* (10.8)	3.77 *d* (10.8)
17b	4.10 *d* (10.8)	3.72 *d* (10.8)
18	1.63 *s*	1.66 *s*
20	1.28 *s*	1.32 *s*
19-*O*-	Glc	Glc
1′	6.19 *d* (7.8)	6.20 *d* (7.8)

Glc: β-D-glucopyranosyl.
[a] Overlapped signals.

Table 2
^{1}H NMR spectral data for compounds **9** and **10** (400 MHz in pyridine-d_5)

H	9	10
1a	0.72 *ddd* (13.5, 13.5, 4.2)	0.69 *ddd* (13.3, 13.4, 4.3)
1b	1.62–1.75[a]	1.55–1.70[a]
2a	1.25[a]	1.24[a]
2b	1.62–1.75[a]	1.55–1.70[a]
3a	0.82 *m*	0.85 *m*
3b	2.00 *m*	2.05 *m*
5	1.01 *brd* (11.4)	1.05 *dd* (11.4, 3.5)
6a	1.28[a]	1.26 *m*
6b	1.53 *m*	1.45 *m*
7ab	1.25 *ddd* (14.0, 9.8, 4.8)	1.30 *ddd* (14.0, 9.8, 4.5)
9	0.65 *brd* (8.2)	0.68 *brd* (8.1)
11ab	1.54 *m*	1.48[a]
12a	1.45 *m*	1.42 *m*
12b	2.20 *m*	2.15 *m*
13	2.32 *brs*	2.22 *brs*
14a	1.02[a]	1.03[a]
14b	1.92 *brd* (12.1)	1.87 *brd* (12.3)
15a	1.56[a]	1.49[a]
15b	1.60[a]	1.58 *d* (12.5)
17a	3.72 *d* (10.7)	3.55 *d* (10.8)
17b	3.78 *d* (10.7)	3.65 *d* (10.8)
18	1.13 *s*	1.06 *s*
19ab	3.49 *d* (12.0)	3.34 *d* (12.0)
20	1.09 *s*	0.63 *s*
19-*O*-	Glc	Glc
1′	4.68 *d* (7.8)	4.77 *d* (7.8)
	Api	Rha
1″	5.62 *d* (2.1)	6.40 *s*
6″		1.52 *d* (6.2)

Glc: β-D-glucopyranosyl; Api: β-D-apiofuranosyl; Rha: α-L-rhamnopyranosyl.
[a] Overlapped signals.

correlated with that of **7a** except for the signal due to the quaternary carbon bearing a hydroxyl group. The carbon signal due to C-16 position of **8a** resonated at δ_C 81.6 while it was at δ_C 79.7 in **7a**. These data suggested that the structural difference between the two aglycones is due to the stereochemistry of C-16. The α-orientation of **8a** was confirmed by the downfieldshift of the AB quartet of H_2-17 (centered at δ 4.05 and δ 4.12) as compared to that of **7a** (centered at δ 3.76 and δ 3.83) in the ^{1}H NMR spectrum (Etse et al., 1987). The attachment of the β-D-glucopyranosyl unit to the aglycone was assigned to the 19 position since a long-range correlation was observed between the anomeric proton at δ 6.20 (*d*, $J = 7.8$ Hz) and C-19 (δ 176.4). Thus the structures of **8a** and **8** were deduced as shown.

Cussovantoside C (**9**) was determined as $C_{31}H_{52}O_{12}$ by HR FAB-MS. The ^{1}H NMR spectrum displayed signals of two anomeric protons at δ 4.68 (d, $J = 7.8$ Hz) and 5.62 (*d*, $J = 2.1$ Hz), two tertiary methyl groups at δ 1.09 and δ 1.13 (each singlet), an AB doublet (2H) at δ 3.49 (*d*, $J = 12.0$ Hz) and an AB quartet (2H) with doublets centered at δ 3.72 and 3.78 (*d*, $J = 10.7$ Hz) of two hydroxymethylene. The ^{13}C NMR spectrum (Table 3) indicated 31 carbons, 11 of which could be assigned as one terminal β-apiofuranosyl and one 6-linked β-glucopyranosyl units. The 20 remaining signals (identified as two methyls, two hydroxylated methylenes, nine methylenes, three methines and four quaternary carbons by DEPT experiment) were in agreement with those of *ent*-kaurane-16β,17,19 triol (Wu et al., 1996). The presence of a cross-peak between the anomeric proton of the 6-linked β-glucopyranosyl (δ 4.68) and the hydroxymethylene at δ_C 72.8 (C-19) and a cross-peak between the anomeric proton of the β-apiofuranosyl (δ 5.62) and the signal at δ_C 68.7 (C-6′) in the HMBC spectrum indicated that glycosylation was at the C-19 position and the β-apiofuranosyl at C-6′. From the above evidence, the structure of **9** was elucidated as shown.

The molecular formula of cussovantoside D (**10**) was established as $C_{32}H_{54}O_{12}$ from HR FAB-MS analysis. Inspection of the ^{13}C NMR spectral data of **10** revealed a close similarity to those of **9** in the aglycone moiety, except for the presence of 12 signals attributable to glucopyranosyl and rhamnopyranosyl units in **10**. In addition, the two anomeric protons and a secondary methyl group observed in the ^{1}H NMR spectrum [δ 4.77 (*d*, $J = 7.8$ Hz), δ 6.40 (*s*) and δ 1.52 (*d*, $J = 6.2$ Hz)]

Table 3
^{13}C NMR spectral data for compounds **7–10** (100 MHz in pyridine-d_5)

C	7	7a	8	8a	9	10
1	39.7	39.8	39.6	39.6	40.7	40.5
2	28.9	29.2	28.8	29.1	18.6	18.8
3	78.4	78.3	78.3	78.3	36.6	36.0
4	49.9	49.1	49.8	49.0	38.2	38.3
5	56.6	56.4	56.2	56.1	57.7	57.6
6	21.9	22.3	22.2	22.7	20.6	20.2
7	42.4	42.3	42.6	42.6	42.7	42.5
8	43.6	43.7	44.6	44.6	43.8	43.8
9	56.9	56.5	56.8	56.3	57.0	57.1
10	39.7	39.6	39.5	39.5	39.5	39.4
11	19.6	19.5	19.0	19.0	19.1	19.1
12	27.3	27.5	26.6	26.6	27.4	27.4
13	41.6	41.6	45.8	45.7	41.7	41.7
14	38.2	38.4	37.4	37.6	38.4	38.4
15	53.1	53.1	53.5	53.5	53.3	53.3
16	79.7	79.7	81.6	81.6	79.6	79.6
17	70.4	70.4	66.4	66.4	70.4	70.4
18	24.3	24.6	24.2	24.5	28.2	28.4
19	176.4	181.0	176.4	181.0	72.8	72.6
20	15.7	16.0	15.8	16.0	18.3	18.5
18 or 19-O-Glc						
1′	95.9		95.9		104.9	103.5
2′	74.0		73.9		75.0	79.2
3′	79.7		79.5		78.6	78.1
4′	70.9		70.8		71.8	72.0
5′	79.0		79.0		76.9	77.3
6′	61.8		61.8		68.7	62.7
6′-O- Api or Rha						
					Api	Rha
1″					110.9	101.6
2″					77.7	72.1
3″					80.4	72.4
4″					75.1	74.1
5″					65.7	69.4
6″						18.6

Assignment based on HSQC.
Glc: β-D-glucopyranosyl, Api: β-D-apiofuranosyl, Rha: α-L-rhamnopyranosyl.

proved further the presence of β-glucopyranosyl and α-rhamnopyranosyl units. The downfieldshift of the signal due to C-2′ (δ 79.2) of glucose indicated that the glucopyranosyl unit was C-2′ substituted. The attachments were established by HMBC experiment. Long-range correlations were observed between the H-1′ (δ 4.77) and C-19 (δ 72.6), and between H-1″ (δ 6.40) and C-2′. Thus the structure of **10** was deduced as shown.

Although the absolute configuration of the aglycones of cussovantosides A–D were not established in this study, we assume that they belong to the *ent*-series like the other known *ent*-kauranes isolated from this plant. *C. vantsilana* Baker produced a sweet compound (cussoracoside C) in higher yield (0.11%). It was evaluated as being 13 times sweeter than sucrose (see Experimental).

3. Experimental

3.1. General

NMR spectra (^1H, ^{13}C, HSQC, HMBC) were recorded in pyridine-d_5 using a Jeol JNM A-400 spectrometer (400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR). MS were recorded on a Jeol JMS-SX 102 spectrometer. Optical rotations were measured with a Union PM-1 digital polarimeter. Preparative HPLC was carried out on columns of ODS (150×20 mm i.d., YMC) with a Tosoh refraction index (RI-8) detector, flow rate 6 ml/min. For CC, silica gel G 60 (Merck), RP-18 (50 mm, YMC) and highly porous copolymer of styrene and divinylbenzene (Mitsubishi Chem. Ind. Co. Ltd) were used. The solvent systems were: (I) CH_2Cl_2–MeOH–H_2O (17:4:0.5 to 17:8:2), (II) 20–100% MeOH, (III) 30–100% MeOH, (IV) 30% CH_3CN. The spray reagent used for TLC was 10% H_2SO_4 in H2O–EtOH (1:1).

3.2. Plant material

The plant material was collected in March 2000 from Ranomafana-Ifanadiana, Madagascar. The identity of the plant was confirmed by Dr. Armand Rakotozafy from Institut Malgache de Recherches Appliquées. A voucher specimen (CUSSVAT-LI01) has been deposited in the Herbarium of the Institute of Pharmaceutical Sciences, Hiroshima University Faculty of Medicine.

3.3. Extraction and isolation

The dried leaves (1.9 kg) of *C. vantsilana* Baker were extracted with hot MeOH (3×6 l, reflux). After removal of the solvent by evaporation, the residue (352 g) was suspended in water and extracted with *n*-hexane and EtOAc successively. The aqueous layer (144 g) was subjected to a column of highly porous copolymer of styrene and divinylbenzene, and eluted with H_2O, 30% MeOH, MeOH and Me_2CO, successively. The fraction eluted with MeOH was applied to a column of silica gel (system I), affording ten fractions. Fraction 2 was subjected to a CC on RP-18 and ODS-HPLC using system II and system IV, respectively to afford compounds **1** (1.7 g) and **4** (39 mg). Fraction 3 was further separated on column of RP-18 using system II to give compounds **6** (2.3 g), **7** (62 mg), **8** (57 mg), and **9** (18 mg). Fractions 4, 7 and 8 were successively subjected to a CC on RP-18 using system II to afford compounds **2** (26 mg) and **10** (15 mg) from fraction 4; **9** (20 mg) and **5** (43.9 mg) from fraction 7, and **3** (7 mg) from fraction 8.

3.4. Cussovantoside A (7)

Amorphous powder. $[\alpha]_D^{21}$ −30.7° (pyridine; *c* 0.1); For ^1H NMR: and; ^{13}C NMR: see Tables 1 and 3;

Negative HR - FAB - MS, *m/z*: $[M-H]^-$ 513.2737 ($C_{26}H_{41}O_{10}$ requires 513.2699).

3.5. Cussovantoside B (**8**)

Colorless needles (from MeOH; mp 165–172°). $[\alpha]_D^{21} = -55°$ (pyridine; *c* 0.2); For ^1H and ^{13}C NMR: see Tables 1 and 3; Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 513.2689 ($C_{26}H_{41}O_{10}$ requires 513.2699).

3.6. Cussovantoside C (**9**)

Amorphous powder. $[\alpha]_D^{21} = -131°$ (pyridine; *c* 0.2); For ^1H and ^{13}C NMR see Tables 2 and 3; Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 615.3411 ($C_{31}H_{51}O_{12}$ requires 615.3380).

3.7. Cussovantoside D (**10**)

Amorphous powder. $[\alpha]_D^{21} = -7.2°$ (pyridine; *c* 0.6); ^1H and ^{13}C NMR see Tables 1 and 2; Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 629.3577 ($C_{31}H_{51}O_{12}$ requires 629.3537).

3.8. Enzymatic hydrolysis of compounds 7 and 8

An aqueous solution of each sample (40 mg of **7** and 35 mg of **8**) and crude hesperidinase was incubated at 37 °C for 72 h. The solution was extracted with EtOAc to produce **7a** and **8a** and the aqueous layer was evaporated. The determination of the absolute configuration of the sugar (D-glucose) was established as follows. Standard samples of thiazolidine derivatives of D- and L-glucose [methyl 2-(D-gluco pentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate, methyl 2-(L-glucopentahydroxy pentyl)-thiazolidine-4(*R*)-carboxylate] were prepared by the method given in the literature (Hara et al., 1987). Two spots were observed on the TLC (CH_2Cl_2–MeOH–H_2O; 25:10:1.5) of the derivative from D-glucose (0.43 and 0.51), due to the C-2 epimers, while only one single spot (0.46) was shown for the derivative from L-glucose. In the same way, a mixture of pyridine (0.5 ml), L-cysteine methyl ester hydrochloride (5 mg) and the residue of the aqueous layer obtained from **7** were warmed at 60 °C for 1 h. After removal of the solvent, the residue was dissolved in water (1 ml) and extracted with *n*-BuOH (1 ml). The organic layer after evaporation was shown to contain methyl 2-(D-gluco pentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate, R_f: 0.43, 0.51 (C-2 epimers of thiazolidine) by TLC, CH_2Cl_2–MeOH–H_2O (25:10:1.5). The residue of the aqueous fraction obtained from **8** was prepared as the same way and methyl 2-(D-gluco pentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate has been observed on the TLC.

3.8.1. Cussovantonin A (**7a**)

$[\alpha]_D^{23} = -23.2°$ (pyridine; *c* 0.4); ^1H NMR spectral data (pyridine-d_5): δ 3.83 (1H, *d*, *J* = 10.7 Hz, H-17a), 3.76 (1H, *d*, *J* = 10.7 Hz, H-17b), 3.34 (1H, *dd*, *J* = 11.8 and 4.4 Hz, H-3), 2.45 (1H, *m*, H-2a), 2.39 (1H, *brs*, H-13), 2.0 (1H, *brd*, *J* = 13.5 Hz, H-1b), 1.93 (1H, *m*, H-2b), 1.66 (3H, *s*, CH_3-18), 1.21 (3H, *s*, CH_3-20), 1.17 (1H, *dd*, *J* = 12.4, 3.0 Hz, H-5), 0.90 (1H, *dd*, *J* = 7.5, 3.6 Hz, H-9), 0.86 (1H, *ddd*, *J* = 13.5, 13.5, 3.4 Hz, H-1a). ^{13}C NMR: (Table 3). Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 351.2180 ($C_{20}H_{31}O_5$ requires 351.2171).

3.8.2. Cussovantonin B (**8a**)

$[\alpha]_D^{23} = -56.6°$ (pyridine; *c* 0.6); ^1H NMR spectral data (pyridine-d_5): δ 4.12 (1H, *d*, *J* = 10.8 Hz, H-17a), 4.05 (1H, *d*, *J* = 10.8 Hz, H-17b), 3.33 (1H, *dd*, *J* = 12.4 and 3.9 Hz, H-3), 2.46 (1H, *m*, H-2a), 2.42 (1H, *brs*, H-13), 1.84 (1H, *brd*, *J* = 13.6 Hz, H-1b), 1.95 (1H, *m*, H-2b), 1.65 (3H, *s*, CH_3-18), 1.17 (3H, *s*, CH_3-20), 0.98 (1H, *dd*, *J* = 12.6, 3.0 Hz, H-5), 0.96 (1H, *dd*, *J* = 7.3, 3.9 Hz, H-9), 0.94 (1H, *ddd*, *J* = 13.5, 13.5, 3.6 Hz, H-1a), ^{13}C NMR: (Table 3). Negative HR-FAB-MS, m/z: $[M-H]^-$ 351.2123 ($C_{20}H_{31}O_5$ requires 351.2171).

3.9. Sensory evaluation (Darise et al., 1984) of cussoracoside C (**6**)

The test panel consisted of ten experienced testers from Maruzen Pharmaceuticals Co., Ltd. The tasters determined the intensity of sweetness of cussoracoside C in 0.2% water solution. They concluded the relative sweetness of cussoracoside C compared to 2.2–3% solution (w/v) of sucrose by testing its solutions at different concentrations and selecting the concentration at which the taste was approximately closest to that of the sucrose solution.

Analysis of the results indicated that the panel members recognized that cussoracoside C at a concentration 0.2% was equivalent in sweetness intensity to sucrose at 2.6% (w/v). Therefore, the relative sweetness of **6** was determined to be 13 times greater than that of sucrose.

Acknowledgements

Liva Harinantenaina is grateful to the Japanese Ministry of Education, Culture, Sports, Science and Technology for the award of a scholarship to study for a PhD at the Institute of Pharmaceutical Sciences, Hiroshima University, Japan. We also thank Dr. Kenji Mizutani of Maruzen Pharmaceuticals Co., Ltd. for the sweet taste evaluation, and the Research Center for Molecular Medicine, Hiroshima University, School of Medicine, Japan, for the use of its NMR facilities.

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