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.

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**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, cussoaracside 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 cussoaracside 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 cussoaracside 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-D-glucopyranosyl ester at δ 6.19 (d, J = 7.8 Hz). Enzymatic hydrolysis of 7 gave a new aglycone named cussovantonin A (**7a**, [α]_{D}^{23} = −23.2° c = 0.4, in pyridine) and D-glucose (see experimental). Inspection of the NMR spectral data suggested
that 7a is an *ent*-kaurane diterpenoid with an equatorial hydroxyl group at C-3 \( \delta_H 3.34 \ (dd, J = 4.4, 11.8 \text{ Hz}) \), an axial carboxyl group at C-19 \( \delta_C 181.0 \), a hydroxyl group at C-16 \( \delta_C 79.7 \), and an AB quartet \( 2H \) with a doublet centered at \( \delta 3.76 \) and \( \delta 3.83 \) (each \( d, J = 10.7 \text{ Hz} \)). 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 \( ^{13} \text{C} \) NMR spectra, the signal of the C-16 appeared at \( \delta 79.7 \), appropriate for \( \beta \)-hydroxylated C-16 (Cheng et al., 1993). Thus the structure of cussovantobin A was deduced as *ent*-3a,16\( \beta \),17 trihydroxykauran-19-oic acid. The characteristic chemical shift of the anomeric carbon signal due to ester linked \( \beta \)-glucopyranosyl \( \delta_C 95.9 \) in 7 confirmed the C-19 glucosylation. Therefore cussovantonoside A was assigned as shown.

Compound 8 (cussovantoside B, \( \text{C}_{26}\text{H}_{42}\text{O}_{10} \) by HR FAB-MS spectrometry) has the same molecular formula as 7. The \( ^1 \text{H} \) and \( ^{13} \text{C} \) 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 cussovantobin B (8a, [\( \alpha \])\text{D} = \text{−}56.6 \text{c} = 0.6 \text{, in pyridine}). The \( ^{13} \text{C} \) NMR spectrum of 8a was
Table 1
1H NMR spectral data for compounds 7 and 8 (400 MHz in pyridine-
\textit{d}_6)

<table>
<thead>
<tr>
<th>H</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>0.92 \textit{ddd} (13.5, 13.5, 3.7)</td>
<td>0.94 \textit{ddd} (13.6, 13.6, 3.5)</td>
</tr>
<tr>
<td>1b</td>
<td>1.85 \textit{brd} (13.5)</td>
<td>1.90 \textit{brd} (13.5)</td>
</tr>
<tr>
<td>2a</td>
<td>1.92 \textit{m}</td>
<td>1.95 \textit{m}</td>
</tr>
<tr>
<td>2b</td>
<td>2.55 \textit{m}</td>
<td>2.50 \textit{m}</td>
</tr>
<tr>
<td>3a</td>
<td>3.35 \textit{dd} (12.4, 3.9)</td>
<td>3.37 \textit{dd} (12.6, 3.8)</td>
</tr>
<tr>
<td>3b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.02 \textit{dd} (12.6, 2.9)</td>
<td>1.10 \textit{dd} (12.4, 3.0)</td>
</tr>
<tr>
<td>6a</td>
<td>2.01 \textit{m}</td>
<td>1.98 \textit{m}</td>
</tr>
<tr>
<td>6b</td>
<td>2.37 \textit{m}</td>
<td>2.52 \textit{m}</td>
</tr>
<tr>
<td>7ab</td>
<td>1.47 \textit{m}</td>
<td>1.45 \textit{m}</td>
</tr>
<tr>
<td>9</td>
<td>0.95 \textit{dd} (7.3, 3.9)</td>
<td>1.02 \textit{dd} (7.5, 3.6)</td>
</tr>
<tr>
<td>11</td>
<td>1.49 \textit{m}</td>
<td>1.48 \textit{m}</td>
</tr>
<tr>
<td>12a</td>
<td>1.42 \textit{m}</td>
<td>1.41 \textit{m}</td>
</tr>
<tr>
<td>12b</td>
<td>1.85 \textit{m}</td>
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<td>2.35 \textit{brs}</td>
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<tr>
<td>14a</td>
<td>1.99\textsuperscript{a}</td>
<td>1.98\textsuperscript{a}</td>
</tr>
<tr>
<td>14b</td>
<td>2.12 \textit{brd} (12.1)</td>
<td>2.14 \textit{brd} (12.1)</td>
</tr>
<tr>
<td>15a</td>
<td>1.65 \textit{m}</td>
<td>1.63 \textit{m}</td>
</tr>
<tr>
<td>15b</td>
<td>1.82 \textit{m}</td>
<td>1.79 \textit{m}</td>
</tr>
<tr>
<td>17a</td>
<td>4.03 \textit{d} (10.8)</td>
<td>3.77 \textit{d} (10.8)</td>
</tr>
<tr>
<td>17b</td>
<td>4.10 \textit{d} (10.8)</td>
<td>3.72 \textit{d} (10.8)</td>
</tr>
<tr>
<td>18</td>
<td>1.63 \textit{s}</td>
<td>1.66 \textit{s}</td>
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<tr>
<td>20</td>
<td>1.28 \textit{s}</td>
<td>1.32 \textit{s}</td>
</tr>
<tr>
<td>19-O- Glev</td>
<td>Glev</td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{v}</td>
<td>6.19 \textit{d} (7.8)</td>
<td>6.20 \textit{d} (7.8)</td>
</tr>
</tbody>
</table>

Glc: β-\textit{p}-glucopyranosyl.
\textsuperscript{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 δC 81.6 while it was at δC 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\textsubscript{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 \textit{1H} NMR spectrum (Etsel et al., 1987). The attachment of the β-\textit{p}-glucopyranosyl unit to the aglycone was assigned to the 19 position since a long-range correlation was observed between the anomic proton at δ 6.20 (\textit{d}, \textit{J} = 7.8 Hz) and C-19 (δ 176.4). Thus the structures of 8a and 8b were deduced as shown.

Cussovantoside C (9) was determined as C\textsubscript{31}H\textsubscript{52}O\textsubscript{12} by HR FAB-MS. The \textit{1H} NMR spectrum displayed signals of two anomic protons at δ 4.68 (\textit{d}, \textit{J} = 7.8 Hz) and 5.62 (\textit{d}, \textit{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 (\textit{d}, \textit{J} = 12.0 Hz) and an AB quartet (2H) with doublets centered at δ 3.72 and 3.78 (\textit{d}, \textit{J} = 10.7 Hz) of two hydroxymethylene. The \textit{13C} 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 \textit{ent}-kaurane-16β,17,19 triol (Wu et al., 1996). The presence of a cross-peak between the anomic proton of the 6-linked β-glucopyranosyl (δ 4.68) and the hydroxymethylene at δC 72.8 (C-19) and a cross-peak between the anomic proton of the β-apiofuranosyl (δ 5.62) and the signal at δC 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\textsubscript{32}H\textsubscript{54}O\textsubscript{12} from HR FAB-MS analysis. Inspection of the \textit{13C} 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 anomic protons and a secondary methyl group observed in the \textit{1H} NMR spectrum [δ 4.77 (\textit{d}, \textit{J} = 7.8 Hz), δ 6.40 (\textit{s}) and δ 1.52 (\textit{d}, \textit{J} = 6.2 Hz)]
proven further the presence of β-glucopyranosyl and α-rhamnopyranosyl units. The downfield shift 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 aglycons 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, 13C, HSQC, HMBC) were recorded in pyridine-d5 using a Jeol JNM A-400 spectrometer (400 MHz for 1H NMR and 100 MHz for 13C 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) CH2Cl2–MeOH–H2O (17:4:0.5 to 17:8:2), (II) 20–100% MeOH, (III) 30–100% MeOH, (IV) 30% CH3CN. The spray reagent used for TLC was 10% H2SO4 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-L101) 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 H2O, 30% CH3CN. The spray reagent used for TLC was 10% H2SO4 in H2O–EtOH (1:1).

3.4. Cussovantoside A (7)

Amorphous powder. [α]D 25 30.7° (pyridine; c 0.1); For 1H NMR: and; 13C 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°C). [z]_{D}^{21} = −55° (pyridine; c 0.2); For $^1$H 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. [z]_{D}^{21} = −131° (pyridine; c 0.2); For $^1$H 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. [z]_{D}^{21} = −7.2° (pyridine; c 0.6); $^1$H 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-glucopyranosyl)-thiazolidine-4(R)-carboxylate, methyl 2-(l-gluco-pentahydropentyl)-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_{2}Cl_{2}–MeOH–H_{2}O: 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-glucopyranosyl)-thiazolidine-4(R)-carboxylate, R_{f}: 0.43, 0.51 (C-2 epimers of thiazolidine) by TLC, CH_{2}Cl_{2}–MeOH–H_{2}O (25:10:1.5). The residue of the aqueous fraction obtained from 8 was prepared as the same way and methyl 2-(d-glucopyranosyl)-thiazolidine-4(R)-carboxylate has been observed on the TLC.

3.8.1. Cussovantonin A (7a)

[α]_{D}^{23} = −23.2° (pyridine; c 0.4); $^1$H 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). C NMR: (Table 3). Negative HR-FAB-MS, m/z: [M−H]− 351.2123 (C_{20}H_{31}O_{3} requires 351.2171).

3.8.2. Cussovantonin B (8a)

[α]_{D}^{23} = −56.6° (pyridine; c 0.6); $^1$H 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). C NMR: (Table 3). Negative HR-FAB-MS, m/z: [M−H]− 351.2171 (C_{20}H_{31}O_{3} requires 351.2171).

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

The test panel consisted of ten experienced testers from Maruzen Pharmaceuticals Co., Ltd. The tasters determined the intensity of sweetness of cussoracside C in 0.2% water solution. They concluded the relative sweetness of cussoracside 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 cussoracside 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.
References


