# A New *ent*-Kaurane Diterpenoid Glycoside from the Leaves of *Cussonia bojeri*, a Malagasy Endemic Plant

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**A new *ent*-kaurane diterpene glycoside, β-D-glucopyranosyl 17-hydroxy-*ent*-kauran-19-oate-16-*O*-β-D-glucopyranoside (4) was isolated from the dried leaves of *Cussonia bojeri* SEEM., together with four known compounds identified as 16β,17-dihydroxy-kauran-19-oic acid (1), β-D-glucopyranosyl 16β,17-dihydroxy-(−)-kauran-19-oate (2), paniculoside IV (3), and rutin (5). The structure of 4 was deduced on the basis of chemical and spectroscopic evidence.**

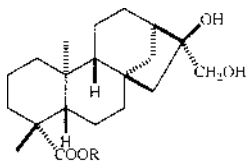
**Key words** *Cussonia bojeri*; Araliaceae; *ent*-kaurane; diterpenoid glycoside

*Cussonia bojeri* SEEM. (Araliaceae) is an endemic plant distributed in central and eastern of Madagascar. Its leaves are used in Malagasy folk medicine to treat acne, diarrhea, stomach ulcers, and syphilis. A decoction of the leaves is used as an antispasmodic.[1] Previous phytochemical investigations isolated flavonoids from this species.[2] In a continuation of our investigations of endemic Malagasy medicinal plants to search for novel biologically active compounds, we have isolated a new *ent*-kaurane glycoside (**4**) along with four known compounds (**1**—**3**, **5**) from the leaves of the plant. Compound **1** has been reported to show significant activity against HIV replication in H9 lymphocyte cells with an $EC_{50}$ value of 0.8 μg/ml.[3] This paper reports the structural elucidation of **4**.

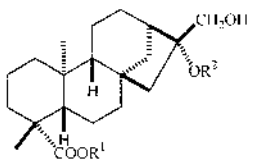
## Results and Discussion

Repeated column chromatography of the water-soluble fraction from the methanol extract of the dried leaves of *C. bojeri* SEEM. yielded 16β,17-dihydroxy-kauran-19-oic acid (**1**), β-D-glucopyranosyl 16β,17-dihydroxy-(−)-kauran-19-oate (**2**),[3] paniculoside IV (**3**),[4] β-D-glucopyranosyl 17-hydroxy-*ent*-kauran-19-oate-16-*O*-β-D-glucopyranoside (**4**), and rutin (**5**).[5] The structures of compounds (**1**—**3**, **5**) have been determined by comparison of their spectral data with the reported data. These *ent*-kaurane compounds (**1**—**4**) have been isolated from this plant for the first time.

Compound **4** was obtained as white amorphous powder having $[\alpha]_D^{21}$ −48.3° ($c$=0.18, pyridine). Its molecular formula was determined to be $C_{32}H_{52}O_{14}$ by negative high resolution (HR)-FAB-MS ($m/z$: $[M-H]^-$ obsd. 659.3318, calcd. 659.3278). The presence of an anomeric proton signal at δ 4.99 ($J$=7.8 Hz) and an anomeric signal showing a typical chemical shift of an ester linked β-glucopyranosyl at δ 6.21 ($J$=8.0 Hz) in the $^1$H-NMR spectrum suggested that two different types of glucosidic linkage were present in the molecule. The $^{13}$C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra exhibited 32 carbon signals, 12 of which could be assigned to those of sugar units and the




1: R= H
2: R= β-D-glucopyranosyl




3 R1= β-D-glucopyranosyl, R2 = H
4 R1= β-D-glucopyranosyl, R2 = β-D-glucopyranosyl
6 R1= H, R2 = H

Table 1. $^1$H- and $^{13}$C-NMR Spectral Data for Compounds **3** and **4** in Pyridine-$d_5$ (δ in ppm, 400 MHz and 100 MHz, Respectively)

| Position | H | C | |
|---|---|---|---|
| | **4** | **3** | **4** |
| 1 | Uncl. | 40.9 | 41.0 |
| 2 | Uncl. | 19.5 | 19.4 |
| 3a | 2.30 br d (14.0) | 38.5 | 38.3 |
| 3b | 0.95[a] | | |
| 4 | | 44.2 | 44.0 |
| 5 | 1.02 br d (9.8) | 57.5 | 57.2 |
| 6 | Uncl. | 22.5 | 22.1 |
| 7a | 1.30 ddd (13.7, 10.1, 4.9) | 43.8 | 42.2 |
| 7b | 1.60 br d (13.7) | | |
| 8 | | 44.9 | 44.1 |
| 9 | 0.92[a] | 56.3 | 56.5 |
| 10 | | 40.1 | 40.8 |
| 11 | Uncl. | 19.0 | 18.8 |
| 12 | Uncl. | 26.7 | 26.5 |
| 13 | 2.63 br s | 45.9 | 40.0 (−5.9) |
| 14 | Uncl. | 37.6 | 37.7 |
| 15a | 1.68 d (12.4) | 53.7 | 48.4 (−5.3) |
| 15b | 1.95 d (12.4) | | |
| 16 | | 81.7 | 87.0 (+5.3) |
| 17a | 3.78 d (10.0) | 66.5 | 66.6 |
| 17b | 3.80 d (10.0) | | |
| 18 | 1.24 s | 28.6 | 28.6 |
| 19 | | 176.9 | 176.9 |
| 20 | 1.26 s | 15.9 | 15.7 |
| 16-*O*-β-D-Glucopyranosyl | | | |
| 1′ | 4.99 d (7.8) | | 99.8 |
| 2′ | | | 75.1 |
| 3′ | | | 78.8 |
| 4′ | | | 71.9 |
| 5′ | | | 78.4 |
| 6′ | | | 63.0 |
| 19-*O*-β-D-Glucopyranosyl | | | |
| 1″ | 6.21 d (8.0) | 95.8 | 95.7 |
| 2″ | | 74.1 | 74.1 |
| 3″ | | 79.3 | 79.3 |
| 4″ | | 71.2 | 71.0 |
| 5″ | | 79.3 | 79.1 |
| 6″ | | 62.2 | 62.1 |

Assignments based on HSQC; *a*) Overlapping signals; uncl.: unclear correlations in HSQC.

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remaining 20 (five quaternary, three methines, 10 methylenes, and two methyl carbons) to a diterpenoid aglycone.

Inspection of the $^1$H- and $^{13}$C-NMR spectra of **4**, which are very similar to those of **3** (Table 1), demonstrated that the aglycone of **4** was also a kaurane-type diterpene with a primary hydroxyl group [at $\delta_C$ 66.6, $\delta_H$ 3.78 (1H, d, $J$=10 Hz), $\delta_H$ 3.80 (1H, d, $J$=10 Hz)], a tertiary hydroxyl group ($\delta_C$ 87.0), and a carboxyl ($\delta_C$ 176.9) group. On comparison of the $^{13}$C-NMR spectral data of **4** with those of **3**, additional signals of one $\beta$-glucopyranosyl unit, and a downfield shift of the signal due to C-16 (+5.3) and upfield shifts of the signals due to C-13 and C-15 (−5.9 ppm and −5.3 ppm, respectively) were observed. These suggest that the site of glucosylation was in the hydroxyl group at position 16.[6]

The position of the functions as well as the attachments was confirmed by heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond connectivity (HMBC) experiments. It was observed in the HMBC spectrum that the anomeric proton signal of the glucopyranosyl at $\delta$ 4.99 showed a long-range correlation with the carbon at the C-16 position ($\delta$ 87.0). The *ent*-nature of (**4**) was confirmed since (**6**) was obtained from enzymatic hydrolysis.[7] 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-glucopentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate, methyl 2-(L-glucopentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate] were prepared by the previously reported method.[8] Two spots were observed on TLC of the derivative from D-glucose (0.43, 0.51) due to the C-2 epimers, while only one single spot (0.46) was shown for the derivative from L-glucose. The water-soluble fraction from acid hydrolysis of (**4**) was treated in the same way as the standard samples and examined by TLC (see Experimental). D-Glucose was determined by comparison of the *Rf* values with those of standard samples. Thus the structure of **4** was determined to be $\beta$-D-glucopyranosyl-17-hydroxy-*ent*-kauran-19-oate-16-*O*-$\beta$-D-glucopyranoside.

The anti-HIV activity of 16-hydroxylated *ent*-kaurane diterpenes has been reported.[3,9] Compound **1** (0.006%) has been shown to have the most anti-HIV activity (significant activity against HIV replication in H9 lymphocyte cells, with an $EC_{50}$ value of 0.8 $\mu$g/ml), while its 16-epimer showed significant inhibition of HIV reverse transcriptase. The screening of the activity of the *ent*-kaurane diterpenoid isolated from the genus *Cussonia* will be the subject of our next investigation.

## Experimental

**General Methods** NMR spectra ($^1$H, $^{13}$C, HSQC, HMBC) were recorded in pyridine-$d_5$ using a JEOL JNM A-400 spectrometer (400 MHz for $^1$H-NMR and 100 MHz for $^{13}$C-NMR). MS were recorded on a JEOL JMS-SX 102 spectrometer. Optical rotations were measured with Union PM-1 digital polarimeter. Preparative HPLC was carried out on columns of octadecyl silica (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 a highly porous copolymer of styrene and divinylbenzene (Mitsubishi Chemical Industries) were used. The solvent systems were: (I) $CH_2Cl_2$–MeOH–$H_2O$ (17 : 4 : 0.5 to 17 : 8 : 2), (II) EtOAc–$C_6H_{12}$ (2 : 8 to 10 : 0), (III) 50% $CH_3OH$, and (IV) 55% $CH_3OH$. The spray reagent used for TLC was 10% $H_2SO_4$ in 50% EtOH.

**Plant Material** The leaves of *C. bojeri* SEEM. (Araliaceae) were collected near Andasibe (140 km east of Antananarivo), Madagascar, in March 2000. The plant was identified at the Parc Botanique et Zooligique de Tsimbazaza, Antananarivo, Madagascar. A voucher specimen has been deposited in the Herbarium of the Institute of Pharmaceutical Sciences, Faculty of Medicine, Hiroshima University.

**Extraction and Isolation** The dried leaves (250 g) of *C. bojeri* SEEM. were extracted with MeOH. After removal of the solvent by evaporation, the residue (26.2 g) was suspended in water and extracted with hexane and EtOAc in succession. The aqueous layer (19.4 g) was subjected to a column of a highly porous copolymer of styrene and divinylbenzene, and eluted with $H_2O$, 30% MeOH, 100% MeOH, and $Me_2CO$ in succession. The fraction eluted with MeOH (6.2 g) was chromatographed on a column of silica gel (system I), affording seven fractions. Fraction 3 yielded compound **4** (34 mg) by precipitation with MeOH. Compounds **2** (137 mg) and **3** (48 mg) were obtained from fractions 2 and 4, respectively, by ODS-HPLC using systems III and IV. ODS HPLC of fraction 6 (system IV) afforded compound **5** (24 mg).

The EtOAc extract was subjected to CC on silica gel (system II). Fractions were combined according to their TLC pattern to yield four fractions. Compounds **1** (15 mg) and **2** (50 mg) were obtained by precipitation with MeOH of fractions 3 and 4, respectively.

**Enzymatic Hydrolysis of Compound (4)** An aqueous solution of **4** (20 mg) and crude hesperidinase (*ca.* 20 mg) was incubated at 37 °C for 72 h. The solution was extracted with $CH_2Cl_2$ and the aglycone produced was identified as **6** (7 mg) by comparison of its spectral data with those of 16$\alpha$,17-dihydroxy-kauran-19-oic acid (crystal, mp: 248—255°).[7] The aqueous layer was examined for identification of the component sugar, and glucose was identified by direct comparison on silica gel TLC with authentic sample.

**Acid Hydrolysis of Compound (4)** Ten milligrams of **4** in 10% $H_2SO_4$ (2 ml) was heated under reflux for 2 h. Water was added to the reaction mixture and extracted with EtOAc. The water-soluble fraction was evaporated to give the sugar fraction.

**Determination of the Absolute Configuration of the Sugar of (4)** A mixture of pyridine (0.5 ml), L-cysteine methyl ester hydrocloride (5 mg), and the sugar fraction was warmed at 60 °C for 1 h in the same way as described in the literature.[8] After removal of the solvent, the residue was dissolved in water and extracted with *n*-BuOH (1 ml). The organic layer after evaporation was shown to contain methyl 2-(D-glucopentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate, *Rf*: 0.43, 0.51 (C-2 epimers of thiazolidine) by TLC on $CH_2Cl_2$–MeOH–$H_2O$ (25 : 10 : 1.5).

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