A New Leishmanicidal Saponin from *Brunfelsia grandiflora*

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A new furostan-type saponin (1) was isolated from the methanolic extract of *Brunfelsia grandiflora* leaves, together with four known compounds. The chemical structure of 1 was determined by spectroscopic analysis and chemical reaction to be 26-*O*-β-D-glucopyranosyl 22α-methoxyfurost-3β,26-diol 3-*O*-β-D-xylopyranosyl(1→3)-{β-D-glucopyranosyl(1→2)}-β-D-glucopyranosyl(1→4)-β-D-glucopyranoside. Compound 1 showed potent leishmanicidal activity *in vitro* against *Leishmania major*.

Key words *Leishmania*; *Brunfelsia grandiflora*; Solanaceae; chiricsanango; furostan

Brunfelsia grandiflora D. DON (Solanaceae), local name "chiricsanango", is widely cultivated in South America as an ornamental plant, and is also used in medicine. Its root is used against rheumatism and syphilis, and also has febrifuge activity. The leaf decoction is taken orally against arthritis and rheumatism in the Peruvian Amazon.[1)] In the Amazon region, this plant is also used as an additive in the preparation of "ayahuasca". Although a few constituents of *B. grandiflora* have been reported,[2,3)] the chemical details of this plant have not yet been investigated thoroughly.

Leishmaniasis is endemic in tropical regions, and currently affects 12 million people in 88 countries.[4)] This disease is transmitted by small biting sandflies (*Phlebotomus* sp.). The first-line drugs for the treatment of leishmaniasis are pentavalent antimonials such as *N*-methylglucamine antimonate (Glucantime) and sodium stilbogluconate (Pentostam). However, these drugs are toxic and generally expensive.

In a screening of South American medicinal plants for leishmanicidal activity, we found that *B. grandiflora* showed potent activity. In this report, we describe the chemical constituents of *B. grandiflora* and their leishmanicidal activities.

Results and Discussion

A new furostan-type saponin, **1**, was isolated from the methanolic extract of *B. grandiflora* leaves, together with three known compounds: chlorogenic acid, scopoletin and 3-*O*-β-D-glucopyranosyl, 28-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl betulinate (cirensenoside P).

Compound **1** was determined to have the chemical formula $C_{57}H_{96}O_{28}$ by high-resolution FAB-MS. The IR spectrum revealed the presence of hydroxyl groups (3407 cm^{-1}). In the ^{13}C-NMR spectrum, 57 signals were observed, 26 of which were derived from oxygenated methines. Furthermore, five anomeric proton signals were observed in the ^{1}H-NMR spectrum at δ 4.83 (d, 7.8), 4.87 (d, 7.6), 5.17 (d, 7.8), 5.22 (d, 7.8), and 5.55 (d, 7.3). Thus **1** was thought to be a pentasaccharide. The ^{13}C- and ^{1}H-NMR spectra revealed the presence of a methoxyl group (δ_C=47.3 ppm, δ_H= 3.23 ppm). Acid hydrolysis of **1** with 3% hydrochloric acid gave an aglycone (**1-a**) and a mixture of sugars, D-glucose and D-xylose. The ^{13}C-NMR data of **1-a** were identical to those of neotigogenin,[5)] which has a spirostanol structure. Based on a comparison of the ^{13}C-NMR data of **1** and **1-a**, the genuine aglycone was deduced to be furostanol-type because of large differences in the chemical shifts from C-22 to 27, and this was confirmed by 2D-NMR (DQFCOSY, HMQC, and HMBC). Furthermore, enzymatic hydrolysis by β-glucosidase (from almond) gave **1-b**. Since **1-b** lacked 1 mol of glucose compared with **1** in the ^{13}C-NMR spectrum (Table 1), the latter molecule was surmised to possess a terminal glucose. A furostanol-type saponin bearing a sugar moiety at C-26 can release the sugar upon hydrolysis to form a spirostanol-type compound, and therefore a terminal glucose was determined to be located at C-26 by the correlation between C-26 (δ_C=74.9 ppm) and H′-1 (δ_H=4.82 ppm) in the HMBC spectrum. The anomeric configurations were deduced from the *J* values of the anomeric protons, and these values suggested the presence of four β-D-glucose units and one β-D-xylose unit. However, attempts to determine the glycosyl linkage of the sugar moiety at C-3 of **1** or **1-b** based on the 2D-NMR spectra did not succeed because of hard overlapping signals in key correlations. To determine the glycosyl linkage, the method reported by Jansson *et al.*[6)] was applied. Compound **1** was converted to a permethylated compound (**1-c**) by Hakomori's method.[7)] Compound **1-c** was subjected to acid hydrolysis and reduction with sodium borohydride followed by acetylation *in situ*, and the final reaction mixture was analyzed by GC-MS. In the GC-MS analysis, acetates of 2,3,4-trimethyl xylitol, 2,3,4,6-tetramethyl glucitol, 2,3,6-trimethyl glucitol, and 4,6-dimethyl glucitol were detected (see Experimental). Based on the results of GC-MS analysis, 1,4-disubstituted, 1,2,3-trisubstituted, and 1-monosubstituted glucoses and 1-monosubstituted xylose were deduced to be part of the sugar component of **1**. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) MS analysis of **1** and **1-b** using product ion scan experiments with various collision energies were examined. The results revealed the presence of a terminal xylose (Xyl-) (M-132), a terminal glucose (Glc-) (M-162), an inner glucose combined with a glucose, and a xylose [(Glc)Xyl-Glc-] (M-456) and an inner glucose combined with two glucoses and a xylose [Xyl-(Glc)Glc-Glc] (M-618), however, a terminal diglucosyl fragment (Glc-Glc-) (M-324) was not found in **1-b**. In addition, electron impact (EI)-MS analysis of acety-

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Table 1. ^{13}C- and ^{1}H-NMR Chemical Shifts for Sugar Moieties of **1** and **1-b** (Pyridine-d_5)

	1	**1-b**			**1**	**1-b**
	δ_C	δ_C	δ_H [a)]		δ_C	δ_C
Glc				C-1	37.2	37.1
1	102.4	102.4	4.88 d (7.3)	2	29.9	29.8
2	73.2	73.2	4.41 t (7.8)	3	77.4	77.3
3	75.6	75.5	4.08—4.14	4	34.8	34.8
4	79.9	79.9	4.60—4.62	5	44.7	44.6
5	75.3	75.3	4.0—4.05	6	28.9	28.9
6	60.6	60.6	4.7 dd (16.1, 10.1)	7	32.1	32.4
			4.21—4.24	8	35.2	35.2
Glc′				9	54.4	54.3
1	105.1	105.2	5.2 d (7.8)	10	35.8	35.7
2	81.3	81.4	4.45 t (8.7)	11	21.2	21.2
3	86.7	86.6	4.18 t (8.7)	12	40.0	40.1
4	70.4	70.4	3.83 t (9.2)	13	41.0	40.7
5	77.7	77.7	3.87—3.90	14	56.3	56.4
6	62.9	63.0	4.01—4.05	15	32.4	32.1
			4.5—4.55	16	81.3	81.2
Glc″				17	64.3	62.8
1	104.8	104.8	5.58 d (7.4)	18	16.5	16.6
2	76.2	76.2	4.11 t (9.2)	19	12.3	12.3
3	78.6	78.6	4.03—4.10	20	40.5	42.4
4	71.0	70.9	4.21—4.24	21	16.3	14.9
5	78.7	78.7	3.90—3.93	22	112.6	109.7
6	62.4	62.4	4.6 dd (11.9, 2.7)	23	31.0	26.2
			4.36 dd (11.9, 5.9)	24	28.2	26.3
Xyl				25	34.4	27.5
1	104.9	104.9	5.24 d (7.8)	26	74.9	65
2	75.0	75.0	3.96 td (8.2, 3.7)	27	17.5	16.3
3	77.6	77.6	4.07—4.13	OCH_3	47.3	
4	70.7	70.7	4.05—4.13			
5	67.3	67.3	3.67 t (10.6)			
			4.21—4.24			
Glc‴						
1	105.0					
2	75.2					
3	78.6					
4	71.6					
5	78.5					
6	62.8					

All spectra recorded at 800 MHz. *a*) *J* values (in Hz) parentheses.

lated **1** showed no fragment 619, which was derived from a terminal diglucose heptaacetate [Glc(OAc)$_4$-Glc(OAc)$_3$-]. Based on this information, the glycosyl linkage position of **1-b** was determined by 1D-HOHAHA technique and 2D-NMR (800 MHz) (Fig. 1). The orientation of a methoxyl group at C-22 of **1** was determined to be α because the nuclear Overhauser effect (NOE) correlation between a methoxyl group and H-16 was observed. Thus the chemical structure of **1** was established as shown in Fig. 2. To our knowledge, this compound has not been reported previously.

The IC_{50} values of the leishmanicidal activities of **1** against *L. major*, *L. guyanensis*, and *L. panamensis* were 0.3, 5.5, and 8.0 μg/ml, respectively.

In conclusion, a new compound, **1**, was isolated together with three known compounds. **1** was found to show leishmanicidal activity against *L. major*, *L. guyanensis*, and *L. panamensis*, and was especially potent against *L. major*. Compound **1** possesses a methoxyl group at C-22, which was thought to be inserted during extraction with methanol.

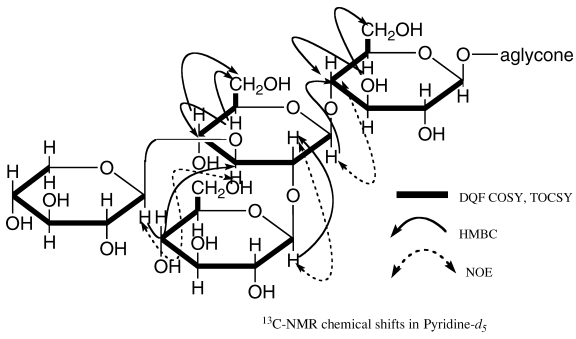

Fig. 1. DQF COSY, TOCSY, HMBC and NOESY Correlations for the Sugar Moiety of **1-b**

Experimental

Plant Material *Brunfelsia grandiflora* material was collected in the Peruvian Amazon region and identified by Dr. Elsa Rengifo (The Peruvian Research Institute of the Amazon, Iquitos, Peru). A voucher specimen (No. P07-01) is on file at the Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation (1–2 Hachimandai, Tsukuba, Ibaraki 305–0843, Japan).

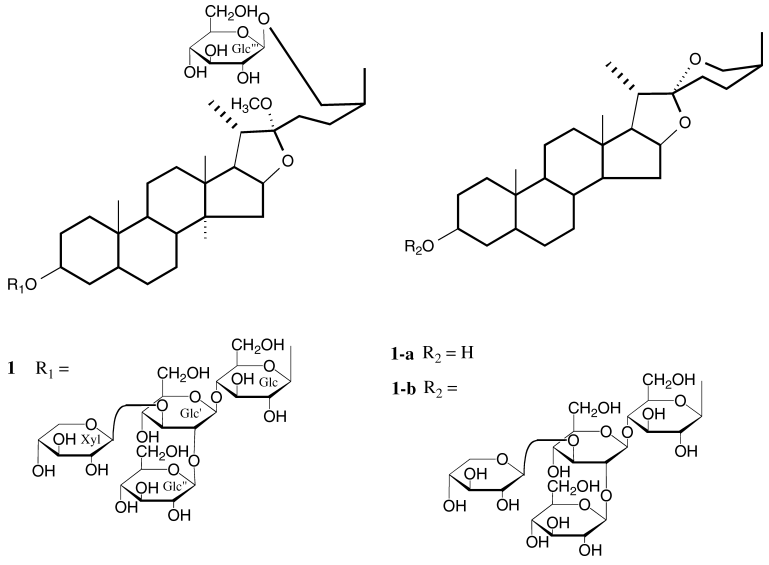

Fig. 2. Chemical Structures of **1**, **1-a** and **1-b**

General Experimental Procedures Melting points were determined by Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were taken with a JASCO DIP-370 automatic polarimeter. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL Alpha 500 spectrometer (500 MHz) and JEOL ECA 800 (800 MHz). IR spectra were recorded on a JASCO FTIR-5300 spectrometer. GC-MS and LC-MS were performed with a SHIMADZU QP 5050A and an Applied Biosystems QSTAR XL with an APCI, and ESI ion source apparatus, respectively. FAB-MS was measured by JEOL HX110 spectrometer. HPLC was run on Shimadzu LC-10VP system with JASCO OR-2090 Plus chiral detector.

Extraction and Isolation The methanolic extract of *B. grandiflora* leaves was prepared in Peru by Dr. Victor Zorrilla (Institute of Tropical Medicine, Lima, Peru). The concentrated extract (367 g) was dissolved in methanol and the solution was passed through a column of activated charcoal (70 g). Methanol (10 l), 30% chloroform/methanol (10 l), and chloroform were then each used as an eluent to give 3 Fractions (FR. A—B). Each fraction was concentrated *in vacuo* to give a syrup. The fraction eluted with methanol (FR. A) was subjected to column chromatography on silica gel (developing solvent: gradient with chloroform–methanol system) to afford 20 Fractions (FR. 1—20). Fractions eluted with 30—40% methanol/chloroform (FR. 7—9) were combined and evaporated under reduced pressure, and the residue was crystallized from methanol to give ursolic acid (670 mg). Fractions eluted with 60—70% methanol/chloroform (FR. 12—16) were combined and concentrated then partitioned between *n*-buthanol and water. The *n*-buthanol layer was concentrated *in vacuo* and the residue was chromatographed on Sephadex LH-20 with 80% methanol/water as an eluent to give 83 fractions (FR. 1′—83′). FR. 67′—83′ were combined and concentrated to yield chlorogenic acid (565 mg). FR. 13′—33′ were combined and rechromatographed on silica gel with a mixture of chloroform–methanol–water (100 : 20 : 1) as an eluent to afford 294 fractions (FR. 1″—294″). FR. 148″—177″ were combined and the concentrated residue was subjected to HPLC (CapcelPak C-18, Shiseido, developing solvent: methanol/water (=3 : 1—4 : 1)) to afford cirensenoside P (12 mg). FR. 208″—242″ were combined and crystallized with methanol to yield **1** (1.08 g) as colorless fine needles. FR. B (eluted with 30% methanol/chloroform) was subjected to column chromatography on silica gel with a mixture of methanol and chloroform as an eluent then crystallized with chloroform–methanol to afford scopoletin (145 mg). **1**: colorless fine needles. mp 209—205 °C $[\alpha]_D^{23}$ −44.6° (c=1.0, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.59 (3H, s, 19-CH_3), 0.76 (3H, s, 18-CH_3), 1.02 (3H, d, J=6.9 Hz, 27-CH_3), 1.14 (3H, d, J=6.9 Hz, 21-CH_3), 3.23 (3H, s, 22-OCH_3), 4.83 (1H, d, J=7.8 Hz, 26-Glc-1), 4.87 (1H, d, J=7.9 Hz, Glc-1), 5.17 (1H, d, J=7.3 Hz, Glc′-1), 5.22 (1H, d, J=7.8 Hz, Xyl-1), 5.55 (1H, d, J=7.4 Hz, Glc″-1). IR (KBr) cm^{-1}: 3407, 2927, 1654, 1451, 1378, 1073. HR-FAB-MS (positive mode) m/z: 1229.6195 $[M+H]^+$ (Calcd for $C_{57}H_{97}O_{28}$: 1229.6166). FAB-MS (positive mode) m/z: 1251 $[M+Na]^+$. APCI-TOF-MS (negative mode): m/z 1227 $[M-H]^-$, 1095 $[M-Xyl]^-$, 1065 $[M-Glc]^-$, 933 $[M-Glc-Xyl]^-$, 771 $[M-2Glc-Xyl]^-$, 609 $[M-3Glc-Xyl]^-$.

Acid Hydrolysis of 1 Compound **1** (32 mg) was dissolved in 3% hydrochloric acid (12 ml) and the mixture was refluxed for 4 h. After cooling, the mixture was poured into ice water, and the precipitate was filtered to give neotigogenin (10 mg).[5] The filtrate was evaporated *in vacuo*, then a half residue was analyzed by HPLC with chiral detector [column: high-performance carbohydrate column (Waters), solvent system: 80% acetonitrile/water, flow rate: 1.0 ml/min, Peak A: 5.0 min(+), Peak B: 4.5 min(+), D-glucose standard: 5.0 min(+), D-xylose standard: 4.5 min(+)]. A trimethylsilylating reagent (TMS-HT, Tokyo Kasei Kogyo Co., Ltd.) was added to another residue and the supernatant solution was analyzed by GC-MS [column: ZB-1701 capillary column (I.D. 0.25 mm×30 m) (Zebron), column temperature program: initial temperature 100 °C (0 to 5 min), rising from 100 to 245 °C (5 to 65 min)]. In GC-MS analysis, 4 peaks (17.5, 18.5, 20.7, 22.3 min) were detected. They were identified to be a trimethylsilylated glucose (TMSGlc) and a trimethylsilylated xylose (TMSXyl) by comparison of retention times and MS fragment patterns with standard samples (TMSGlc: 20.7, 22.3 min, TMSXyl: 17.5, 18.4 min).

Enzymatic Hydrolysis of 1 Compound **1** (30 mg) was suspended in citrate buffer solution (pH 4.25) (10 ml), β-glucosidase (from almond, Oriental Yeast Co., Ltd.) (30 mg) was added, and the mixture was stirred at 30 °C for 3 d. The mixture was poured into water, extracted with *n*-buthanol, and evaporated to afford **1-b** (10 mg). **1-b**: colorless amorphous powder. $[\alpha]_D$ −37.7° (c=0.19, MeOH). HR-FAB-MS (positive mode) m/z: 1035.5347 $[M+H]^+$ (Calcd for $C_{50}H_{83}O_{22}$ 1035.5376). FAB-MS (positive mode) m/z: 1058 $[M+Na]^+$. ESI-TOF-MS (negative mode) m/z: 1033 $[M-H]^-$, 901 $[M-Xyl]^-$, 871 $[M-Glc]^-$, 739 $[M-Glc-Xyl]^-$, 577 $[M-2Glc-Xyl]^-$, 415 $[M-3Glc-Xyl]^-$.

Permethylation, Acid Hydrolysis, Reduction and Acetylation of 1[8] A solution of sodium hydride (60% oil suspended) (100 mg) in dimethyl sulfoxide (dried with molecular sieves 4A) (7.5 ml) was heated at 65 °C with stirring for 60 min, and a solution of **1** (50 mg) in dimethyl sulfoxide (2.5 ml) was then added and the mixture was stirred for 1 h. Methyl iodide (0.75 ml) was added and the mixture was stirred at room temperature for 12 h. After the reaction, the mixture was poured into water and extracted twice with chloroform, and the organic layer was washed with water and evaporated *in vacuo*. The residue was subjected to preparative thin-layer chromatography (TLC) (development solvent system; chloroform/methanol (=20 : 1)) to give a permethylated compound (**1-c**). **1-c**: ^{1}H-NMR ($CDCl_3$) δ: 3.35, 3.37, 3.40, 3.44, 3.44, 3.48, 3.51, 3.52, 3.53, 3.56, 3.56, 3.58, 3.60, 3.61, 3.62, 3.62, 3.62 (each 3H, s), 4.19 (1H, d, J=7.3 Hz), 4.30 (1H, d, J=7.8 Hz), 4.70 (1H, d, J=7.3 Hz), 4.93 (1H, d, J=7.3 Hz), 4.99 (1H, d, J=7.8 Hz). **1-c** was dissolved in 0.5 N-sulfuric acid in 90% acetic acid (10 ml), and the solution was stirred at room temperature for 30 min then at 77 °C for 5 h. After being cooled, the reaction mixture was neutralized with silver carbonate and filtered. The filtrate was freeze-dried to give a residue. The residue was dissolved in water (5 ml) and sodium borohydride was added until halting foaming. The solution was stirred at room temperature for 2 h and then a few drops of acetic acid were added to stop the reaction. Methanol was added

and the solution was concentrated under reduced pressure to afford a residue. Acetic anhydride (2 ml) and pyridine (2 ml) were added to the residue and the mixture was heated at 70 °C for 2 h. After being cooled, water was added to the reaction mixture and the solution was stirred at room temperature for 30 min, extracted with chloroform, and evaporated *in vacuo* without heating to give a mixture of partially methylated alditol acetates. The mixture was analyzed by GC-MS. GC-MS analysis was performed with a DB-225 capillary column (I.D. 0.25 mm×30 m) (Agilent Technologies Co., Ltd.) at a column temperature of 170 °C. The relative retention times and mass fragmentation patterns of detected peaks were compared with the values in the literature.[6] The following peaks were detected and their mass fragmentation patterns were identical to those reported previously: 4,6-dimethylglucitol acetate (retention time; 3.29 min), 2,3,6-trimethylglucitol acetate (2.09 min), 2,3,4,6-tetramethylglucitol acetate (1.0 min), and 2,3,4-trimethylxylitol acetate (0.61 min).

Leishmanicidal Activity Assay Cultivation of *Leishmania* promastigotes and leishmanicidal activity assay were carried out as we previously described.[9] Amphotericin B was used as a positive control (IC_{50} 0.04 μg/ml). The moderate leishmanicidal activities for ursolic acid,[10] chlorogenic acid,[11] and scopoletin[12] have been reported in the literature. Activity for cirensenoside P was not carried out due to amount shortage.

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