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NEW OLEANANE-TYPE TRITERPENE SAPONINS FROM *MILLETTIA SPECIOSA*

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Abstract ——— The 70% ethanol extract of the roots of *Millettia speciosa* gave two new oleanane-type saponins along with two known pterocarpans, medicarpin and maackiain. The structures of new compounds were established on the basis of chemical and spectroscopic evidences. Medicarpin and maackiain showed cytotoxicity against leukemia cells (HL-60) and inhibited leukotrine secretion from RBL-2H3 cells.

About two hundred species of *Millettia* (Fabaceae) were distributed in ubtropical and tropical Africa, Asia and Australia, and many plants of the species were used as medicinal drugs, insecticide, or for stupefying fish in China.[1] Plants of the genus are well known to be a good source of flavonoids and isoflavonoids[2] and their roots are rich source of starch and used as a tonic and for making wine. In connection with a search of biologically active substances from traditional medicinal plants,[3] we report the isolation and structural elucidation of two novel oleanane-type triterpene saponins, named Millettiasaponins A (**1**) and B (**2**), along with two known pterocarpans, medicarpin (**3**)[4] and maackiain (**4**)[5] from 70% extract of the roots of *M. speciosa* collected in Vietnam (Chart 1). This is the first report of the isolation of oleanane-type saponins from this plant.

Structures of Millettiasaponin A (1) and Millettiasaponin B (2)

Millettiasaponin A (**1**) was obtained as a white amorphous powder, $[\alpha]_D$ – 3.2° (*c*=1.0, MeOH). Its negative HR-FAB-MS spectrum showed a quasimolecular ion peak at *m/z* 1013.493 $[M-H]^-$

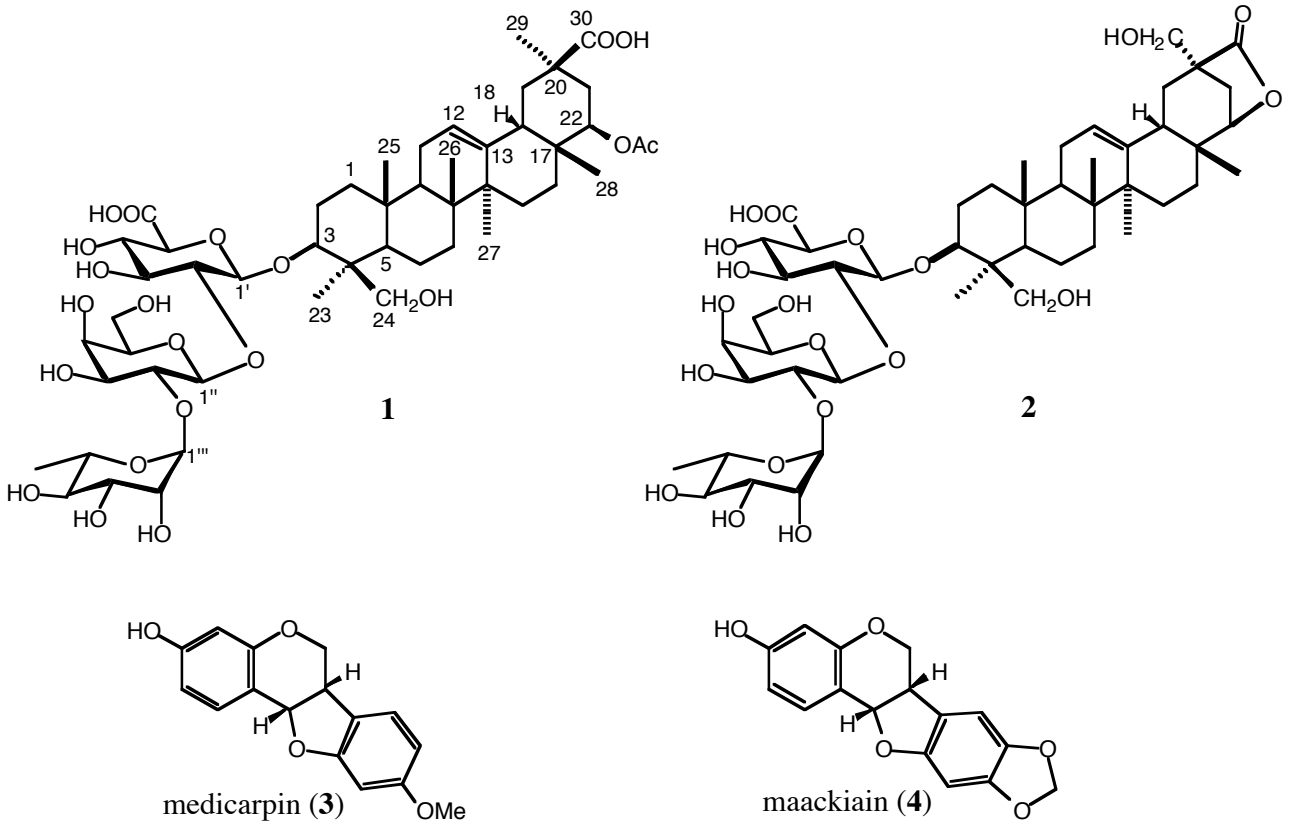

Chart 1

corresponding to the molecular formula $C_{50}H_{77}O_{21}$, and indicating eleven degrees of unsaturation. The IR spectrum of **1** showed the absorption bands at 3400-2900 and 1715 cm^{-1} suggesting the presence of carboxylic acid and ester groups. The ^{1}H- and ^{13}C-NMR (Table 1) spectra of **1** disclosed the presence of seven tertiary methyls [δ_H 0.69, 0.91, 0.98, 1.31, 1.32, 1.42, 2.00 (each s)], a secondary methyl [δ_H 1.72 (d, *J*=6.5 Hz)], an oxygen-bearing methylene [δ_H 3.22, 4.21 (each d, *J*=11.0 Hz)], two oxygen-bearing methines [3.39 (d, *J*=8.0 Hz), 4.83 (br s)], a double bond [δ_H 5.53 (br s)], three carboxyl groups (δ_C 170.3, 172.4, 179.4) and three sugars [δ_C 101.9, 102.4, 105.4; δ_H 4.92 (overlapped with solvent), 5.69 (d, *J*=7.5 Hz), 6.19 (br s) for anomeric]. On acid hydrolysis with 1M HCl in 1,4-dioxane, **1** liberated D-glucronic acid, D-galactose and L-rhamnose (1:1:1 molar ratio), which were identified by GLC analysis of their trimethylsilyl thiazolidine derivatives.[6] Tese spectral data and chemical reaction suggested that **1** was a triterpene saponin. As shown in Figure 1, the HMBC spectrum of **1** displayed the cross peaks due to the long-range couplings between the following proton and carbon signals: δ 1.32 (H-29) and 40.8 (C-20), 179.4 (C-30); 2.00 (-OCOC*H*$_3$) and 170.3 (-O*C*OCH$_3$); 4.83 (H-22) and 21.4 (C-28), 44.1 (C-18), 40.8 (C-20), 170.3 (-O*C*OCH$_3$); 2.90 (H-18) and 21.4 (C-28), 40.8 (C-20), 122.9 (C-12); 5.53 (H-12) and 47.7 (C-9); 0.69 (H-25) and 38.5 (C-1), 47.7 (C-9); 3.39 (H-3) and 38.5 (C-1), 63.6 (C-24); 1.42 (H-23) and 91.2 (C-3), 63.6 (C-24); 4.92 (H-1') and 91.2 (C-3); 5.69 (H-2'') and 78.5 (C-2'); 6.19 (H-1''') and 76.7 (C-2''). Thus, the plane structure of aglycone moiety of **1** should be an oleanane-type

Table 1. ^{13}C-NMR chemical shifts for **1** and **2**

Carbon	**1***	**2****	3-*O*-Glycosyl moiety	**1***	**2****
1	38.5	39.7	GlcA-1'	105.4	105.5
2	26.6	27.0	2'	77.5	77.3
3	91.2	92.2	3'	76.4	76.9
4	43.9	44.7	4'	73.8	74.1
5	56.0	57.2	5'	78.5	78.4
6	18.5	19.3	6'	172.4	176.6
7	32.8	34.2	Gal-1"	101.9	102.1
8	40.1	40.7	2"	77.7	78.2
9	47.7	49.3[a]	3"	76.7	76.3
10	36.4	37.5	4"	71.2	71.5
11	24.0	24.8	5"	76.5	76.3
12	122.9	126.3	6"	61.7	62.2
13	144.1	141.9	Rha-1'"	102.4	102.2
14	41.9	43.8	2'"	72.3	72.2
15	26.3	25.9	3'"	72.7	72.2
16	26.3	27.3	4'"	74.3	74.3
17	36.2	37.6	5'"	69.4	69.5
18	44.1	45.9	6'"	18.9	18.3
19	41.7	38.4			
20	40.8	49.8			
21	35.3	34.3			
22	78.1	86.5			
23	23.0	23.4			
24	63.6	64.3			
25	15.7	16.4			
26	16.8	17.4			
27	26.6	25.3			
28	21.4	23.9			
29	29.9	64.0			
30	179.4	181.0			
-C=O	170.3				
CH_3	21.0				

*Measurements performed in Pyridine-d_5 at 125 MHz

**Measurements performed in CD_3OD at 125 MHz

[a] This signal overlapped with solvent signal.

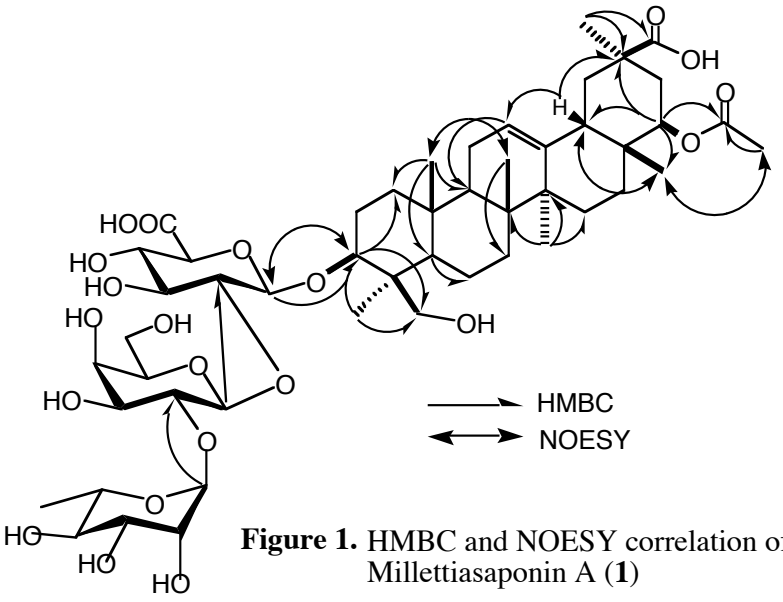

Figure 1. HMBC and NOESY correlation of Millettiasaponin A (**1**)

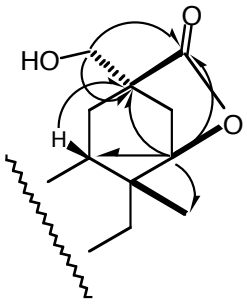

Figure 2. Selected HMBC correlation of Millettiasaponin B (**2**)

triterpene shown in Figure 1 and the sugar moiety was assigned as 3-*O*-α-L-rahmnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside. Finally, the relative stereochemistry of **1** was determined by analysis of the NOESY spectrum as shown in Figure 1.

Millettiasaponin B (**2**) was obtained as a white amorphous powder, $[\alpha]_D$ – 15.4° (*c*=1.0, MeOH). Its negative HR-FAB-MS spectrum showed a quasimolecular ion peak at *m/z* 969.469 $[M-H]^-$ corresponding to the molecular formula $C_{48}H_{73}O_{20}$ and indicating eleven degrees of unsaturation. The IR spectrum of **2** showed the absorption band at 3400-2900 and 1762 cm^{-1} suggesting the presence of γ-lactone moiety. Acid hydrolysis of **2** followed by GC analysis showed that the sugar composition was the same with **1**. The ^1H- and ^{13}C-NMR (Table 1) spectra of **2** were very similar to those of **1** except for the appearance of an additional oxygen-bearing methylene [δ_C 64.0; δ_H 3.46, 3.65] and the disappearance of a tertiary methyl and an acetyl (CH_3CO-) groups. The HMBC spectrum of **2** (Figure 2) showed the cross peaks between the following proton and carbon signals. 1.97 (H-18) and 23.9 (C-28), 49.8 (C-20); 3.46, 3.65 (H-29) and 49.8 (C-20), 181.0 (C-30); 4.27 (H-22) and 23.9 (C-28), 45.9 (C-18), 49.8 (C-20), 181.0 (C-30). Thus, the structure of **2** was determined as lactonized derivative on E-ring of **1** as shown in Chart 1. Although each 50 μg/ml of Millettiasaponin A (**1**) and B (**2**) did not show the cytotoxicity against human leukemia (HL-60) cells and the inhibition of leukotriene secretion from rat basophilic leukemia (RBL-2H3) cells, pterocarpans, medicarpin (**3**) and maackiain (**4**) showed the cytotoxicity (IC_{50}= 18 μmol and 162 μmol) and the inhibitory activities (IC_{50}= 4.6 μmol and 24 μmol), respectively.

EXPERIMENTAL

Melting points were determined on a Yanagimoto micromelting point apparatus and uncorrected. Spectral data were obtained using the following apparatus; optical rotations with a JASCO DIP-360 digital polarimeter; FAB-MS with a JEOL JMS-GCMATE spectrometer using glycerol as a matrix. ^1H- and ^{13}C-NMR spectra were measured on a JEOL JNM lambda-500 spectrometer in $CDCl_3$ containing TMS as internal standard; and IR spectra were taken on a JASCO FT-IR 300E spectrometer. Column chromatography was carried out with Diaion HP-20 (Nippon Rensui) and Wakogel C-200 (Wako); TLC: SiO_2 gel 60 F_{254} plates (Merck), and detection was done by spraying with 5% H_2SO_4 in MeOH. HPLC separation was carried out with normal phase [Senshu Pak Pegasil silica 60-5, 250 x 10 mm (A)] and reverse phase [Shiseido CAPCELL PAK C_{18} MG120Å, 250 x 10 mm (B), Shiseido CAPCELL PAK C_{18} UG120Å, 250 x 20 mm (C)].

Plant material

The stem barks of *Millettia speciosa* were collected in Vietnam, 2000. The plant material was identified by Prof. Tetsuo Koyama (College of Bioresource Science, Nihon University) and a voucher specimen (VN005) was deposited in the herbarium of College of Pharmacy, Nihon University.

Extraction and Isolation of Millettiasaponin A (1) and Millettiasaponin B (2)

The roots of *Millettia speciosa* (460 g) were crushed and extracted ultrasonically for 30 min with 70 % EtOH (8 L). The extracts were concentrated *in vauo* to give a crude extract (62 g). The crude extract was subjected on Diaion HP-20 CC (1.5 L) and eluted successively with 20% MeOH (4 L), 40 % MeOH (2 L), 60 % MeOH (3 L), 80 % MeOH (3 L), MeOH (3 L) and acetone (4 L) to afford six fractions [fr. 1 (45.5 g), fr. 2 (3.7 g), fr. 3 (2.2 g), fr. 4 (1.9 g), fr. 5 (1.7 g), and fr. 6 (2.2 g)]. Fraction 4 was subjected on to SiO_2 gel CC eluting successively with solvent of increasing polarity ($CHCl_3$: MeOH : H_2O = 7 : 3 : 0.3, 6 : 4 : 0.5, 6 : 4 : 1 and MeOH) to give 9 fractions (fr.s 4-1 ~ 4-9). Fractions 4-6 (136 mg) were purified by reverse phase (rp)-HPLC (25% CH_3CN, system B) to give millettiasaponin A (20 mg). Fraction 5 (1.69 g) was subjected on to SiO_2 gel CC eluting successively with solvent of increasing polarity ($CHCl_3$: MeOH : H_2O = 7 : 3 : 0.3, 6 : 4 : 1 and MeOH) to give 9 fractions (fr. 5-1 ~ 5-9). Fractions 5-8 (190 mg) were purified by rp-HPLC (35% CH_3CN, system B) to give millettiasaponin B (15 mg). Fraction 6 (2.24 g) was subjected on to SiO_2 gel CC eluting successively with solvent of increasing polarity (*n*-hexane : acetone = 9 : 1, 4 : 1, 2 : 1, 1 : 1, $CHCl_3$: MeOH = 9 : 1, 4 : 1, 2 : 1, 1 : 1 and MeOH) to give 12 fractions (fr. 6-1 ~ 6-12). Fractions 6-4 (187 mg) were purified by normal phase (np)-HPLC (*n*-hexane : ethyl acetate = 3 : 1) to give medicarpin (9 mg) and maackiain (15 mg).

Millettiasaponin A (**1**): amorphous powder; negative HR-FAB-MS $[M-H]^-$ *m/z*: 1013.493 (Calcd 1013.496 for $C_{50}H_{77}O_{21}$); $[\alpha]_D$ –3.2˚ (*c*=1.0, MeOH); IR (KBr): 3424, 2927, 1715, 1456, 1382, 1264, 1048 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) δ 0.69 (3H, s, H-25), 0.91 (3H, s, H-26), 0.98 (3H, s, H-28), 1.31 (3H, s, H-27), 1.32 (3H, s, H-29), 1.42 (3H, s, H-24), 1.72 (3H, d, *J*=6.5 Hz), 2.00 (3H, s, -OAc), 2.90 (m, H-18), 3.22 and 4.21 (each 1H, d, *J*=11.0 Hz), 3.39 (1H, br d, *J*=8.0 Hz, H-3), 4.83 (1H, br s, H-22), 4.92 (overlapped with solvent, GlcA H-1’), 5.53 (1H, br s, H-12), 5.69 (1H, d, *J*=7.5 Hz, Glc H-1’’), 6.19 (1H, br s, Rha H-1’’’); ^{13}C NMR see Table 1.

Millettiasaponin B (**2**): amorphous powder; negative HR-FAB-MS $[M-H]^-$ *m/z*: 969.4690 (Calcd 969.4694 for $C_{48}H_{73}O_{20}$); $[\alpha]_D$ –15.4˚ (*c*=0.78, MeOH); IR (KBr): 3426, 2925, 1762, 1416, 1382, 1298, 1127, 1075, 1046 cm^{-1}; ^{1}H-NMR (CD_3OD, 500 MHz) δ 0.89 (3H, s, H-25), 0.96 (3H, s, H-26), 0.96 (3H, s, H-28), 1.20 (3H, s, H-27), 1.26 (3H, s, H-23), 1.27 (3H, d, *J*=6.0 Hz, Rha H-6’’’), 1.97 (1H, dd, *J*=13.5, 6.0 Hz, H-18), 3.18 and 4.12 (each 1H, d, *J*=11.5 Hz, H-24), 3.40 (m, H-3), 3.46 and 3.65 (m, H-29), 4.27 (1H, d, *J*=5.5 Hz, H-22), 4.43 (1H, d, *J*=7.5 Hz, GlcA H-1’), 5.14 (1H, d, *J*=1.5 Hz, Rha H-1’’’), 5.30 (1H, br t, *J*=3.5 Hz, H-12),; ^{13}C NMR see Table 1.

Acid Hydrolysis of 1 and 2

Each glycosides (1.5 mg) were heated at 90˚C in 1,4-dioxane (0.2 mL) and 1M HCl (0.3 mL) for 2 h. After cooling, the reaction mixture was neutralized with silver carbonate and then filtered. The filtrate was transferred to a Sep-Pak C18 catridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (4 mg) in pyridine (0.4 mL) at 60˚C

for 1 h. After the reaction, the solution was treated with TMS-HT (150 μL, hexamethyldisilazane and trimethylchlorosilane in pyridine) at 40˚C for 15 min. The reaction mixture was then subjected to GLC analysis to identify the derivatives of L-rhamnose (i), D-galactose (ii) and D-glucuronic acid (iii) from 1 and 2. GLC conditions: column, ULBON HR-1, 25 m x 0.25 mm (i.d.) capillary column; injector temperature, 250˚C; detector temperature, 280˚C; column temperature, 250˚C for 0.5 min 1.5˚C/min up to 270˚C; He flow rate, 24 cm/sec; t_R, i 6.4 min, ii 8.7 min, iii 9.0 min, respectively.

Cytotoxicity assay

HL-60 leukemia cells (purchased from Dainippon Pharmaceutical Co., Ltd.) were maintained in RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, sodium bicarbonate, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells (1 x 10^5 cells/mL) were incubate for 72 h at 37˚C with or without test compound ranging from 50 to 0.1 μg/mL, and cell growth was estimated by colorimetric measurement of stained living cells with Alamar Blue assay.[7] Optical density was determined at 595 and 570 nm on a microtiter plate reader (Bio Rad). A dose-response curve was plotted for each compound, and the concentration giving 50% inhibition (IC_{50}) was calculated.

Measurement of leukotriene secretion

The rat basophilic leukemia (RBL-2H3) cells obtained from NIHS (JCRB) (cell number JCRB0023) were grown in Dulbecco's modified Eagle's MEM (DMEM) (Sigma) supplemented with 10% FCS (GIBCO) in 5% CO_2 atmosphere. The RBL-2H3 cells were harvested by incubating them in Trypsin-EDTA (1 mM EDTA, 0.25% trypsin) for 2 min at 37˚C. RBL cells were suspended in complete DMEM at concentrations of 5 x 10^5 cells/mL, and plated on a 24-well plate at the density of 2 x 10^5 cells/well. Then the cells were sensitized with 1 μg/ml of anti-DNP IgE at 37˚C overnight. IgE-sensitized cells were washed with PBS and incubated in DMEM contaning 20 mM Hepes, pH 7.4 (Hepes-DMEM) and test samples at 37˚C for 30 min and the cells were stimulated with 1 μg/mL of DNP-BSA in Hepes-DMEM, and incubated at 37˚C for 30 min.

LTC_4 content in supernatants was determined by LTC_4 Enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. This assay is based on the competition between LTC_4 and an LTC_4-acetylcholinesterase conjugate for a limited amount of LTC_4 antiserum. Briefly, the assay plate well pre-coated with mouse anti-rabbit IgG samples was added with supernatants diluted with EIA buffer, LTC_4 standard, the LTC_4-acetylcholinesterase conjugate and rabbit LTC_4 antiserum, covered with plastic film and incubated for 18 h at rt. After washing five times with wash buffer, each well was added with Ellman's reagent containing acetylcholine, the substrat of acetylcholinesterase and 5,5'-dithio-bis-(2-nitrobenzoic acid). To develop the enzymatic reaction between acetylcholinesterase and the substrate, the plate was placed on a shaker for 60 min at rt in the dark. Hydrolysis of acetylcholine by acetylcholinesterase produces thiocholine. The nonenzymatic reaction of thiocholine and 5'5-dithio-bis-(2-nitrobenzoic acid) then produces 5-thio-2-nitrobenzoic acid,

which has a distinct yellow color and a strong absorbance at 412 nm. The absorbance at 415 nm was measured by a microtiter plate reader (Bio Rad). The intensity of the color (the absorbance at 415 nm) is proportional to the amount of LTC_4-acetylcholinesterase conjugate bound to the well, which is inversely proportional to the amount of free LTC_4 in the well. A dose-response curve was plotted for each compound, and the concentration giving 50% inhibition (IC_{50}) was calculated.

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