A TRITERPENE ESTER SAPONIN FROM THE SEED OF *Barringtonia asiatica*

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ABSTRACT

A triterpene ester saponin has been isolated from the seed of Barringtonia asiatica and its structure has been established by extensive application of high-resolution FABMS and two dimensional NMR techniques to be 3-O-{[β-D-galactopyranosyl(1→3)-β-D-glucopyranosyl(1→2)]-β-D-glucuronopyranosyloxy}-22-O-[2-methylbutyroyloxy]-15,16,28-trihydroxy-(3β,15α,16α,22α)-olean-12-ene.

Keywords: *Barringtonia asiatica, Lecythidaceae, triterpenoid*.

INTRODUCTION

Barringtonia is an important old world genus of the family Lecythidaceae, and consists of 20 species including *B. asiatica* that are distributed from tropical Africa to Formosa, Polynesia, and northern Australia [1]. The seeds of *B. asiatica* are used as a fish poison by native fisherman in these regions for centuries to enhance their harvest [2].

Preliminary work on the saponins from *B. asiatica* showed that the seeds of which contained a mixture of saponins (A_1-barrinin) [3]. On treatment with 5% HCl in methanol, A_1-barrinin produced a mixture of sapogenins (A_1-barrigenin), together with a mixture of several sugar that were later shown to be fructose, glucose, glucuronic acid, and galactose in a ratio 1:2:2:2 by paper chromatography [4]. On basic hidrolysis, A_1-barrigenin gave two aglycons (A_1-barrigenol and A_2-barrigenol) together with tiglic acid. The structure of A_1-barrigenol (**1**) had earlier been deduced by ^{1}H NMR spectroscopy to be 3β,16α,22α,28-tetrahydroxyoelan-12-ene [5] and A_2-barrigenol was found to be identical to camelliagenin A (**2**), whose structure had been deduced previously as 3β,15α,16α,22α,28-pentahydroxyolean-12-ene [6], and latter confirmed by an extensive 1D and 2D NMR studies [7]. In previous papers, we reported three saponins (**3**, **4**, **5**) from the seeds of *B. asiatica* [2,8].

As part of our continuing investigation on structure elucidation of glycoside compounds including saponin, we wish to describe in this contribution, the isolation and structural elucidation of a triterpene ester saponin (**5**) from the seeds of *B. asiatica* by means of extensive NMR studies. The term ester saponin is used here to describe those glycosides which are acylated on the aglycone or on the sugar chain with an acid moiety [9]. The ester saponins belong to the group of the most complicated saponins, the isolation of which is very difficult as a result of easy acyl migration or deacylation [10]. The formation of artifacts during hydrolysis reactions is also common. In fact, numerous ester saponins have only been incompletely characterized and the locations of their acyl groups still remain to be determined [11]. It is also certain that a number of characterized saponins are actually ester saponins and that during the extraction/isolation process the ester linkage is cleaved. Structure elucidation of ester saponin is often problematic and substitution positions of acyl groups are difficult to establish with certainty. However, the extensive use of 1D and 2D NMR techniques may be necessary for unambiguous structure determination [8,12]. Therefore, this paper describes the isolation and structure elucidation of an ester saponin from the seeds of *B. asiatica*. Structure elucidation of saponins is important since it enables one to understand what comes their bioactivity. Knowledge of the structure also enables the parent compound to be chemically modified and potentially increase its activity [8].

RESULTS AND DISCUSSION

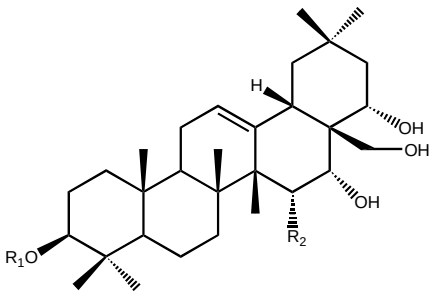
The dry powdered seeds of *B. asiatica* (1 kg) were extracted with petroleum ether and methanol. The methanol extract after evaporation (760 g) was then suspended in water and extracted with n-butanol. The n-butanol layer was evaporated and the residue was dissolved in methanol (50 ml) and diethyl ether (300 ml) added to precipitate the mixture of saponins and other polar constituents. This mixture was filtered to yield 4.35 g of crude saponin. The separation and purification of the crude saponin mixture (4.35 g) were performed using several steps of reverse phase-high performance liquid chromatography (see Experimental section) to obtain compound **5**.

Compound **5**, a white amorphous solid, being a triterpene glycoside as evident from a positive Liebemann-Burchard reaction, exhibited a molecular formula $C_{53}H_{86}O_{22}$ (MW 1074) based on its ^{13}C-NMR (DEPT) and mass spectra, in which a quasimolecular ion $[M - H]^-$ at *m/z* 1073 was detected in the negative FABMS. The infrared spectrum of **5** displayed bands indicating hydroxyl group (ν 3427 cm^{-1}), carbonyl (ν 1709 cm^{-1}), carbon-carbon double bond (1638 cm^{-1}), and glycosidic linkages (ν 1100 – 1000 cm^{-1}).

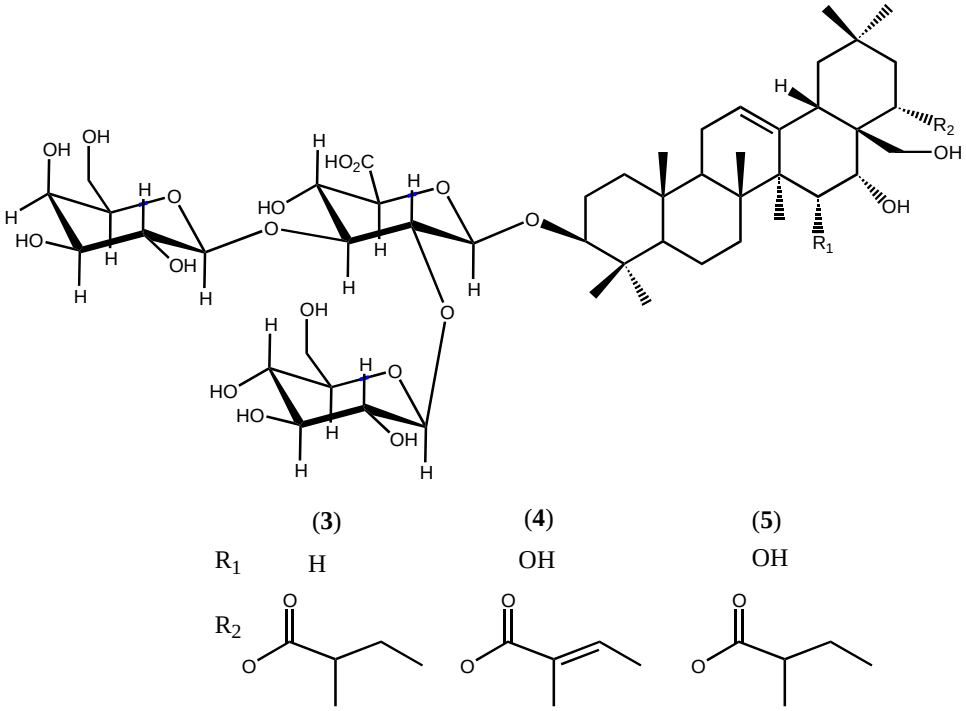
The ^{1}H and ^{13}C NMR spectra of the aglycone moiety of **5** which are presented in Table 1, showed seven methyl group signals at δ 0.83, 1.03, 1.08, 1.10, 1.24, 1.26, 1.87 ppm and 15.8, 16.7, 17.5, 21.3, 25.2, 27.9, 33.5 ppm, one olefinic proton at δ 5.46 ppm, two olefinic carbons at δ 124.8 and 144.5 ppm, two methyl protons at δ 3.59 and 3.78 ppm (AB d, 10.5) attached to carbon at δ 62.8 ppm, indicating that the aglycone moiety of **5** was probably an olean-12-ene with hydroxymethylene carbon (28-CH_2OH).

An AMX system signal of a shielded oxygen bearing methine proton at δ 3.29 ppm (H-3, dd, *J* = 5, 12 Hz) that in HMQC showed directly connected to a carbon at δ 89.4 ppm, which was coupled, from DQCOSY, to two protons on C-2 at δ 1.81 and 2.16 ppm. Comparison with published data for an olean-12-ene saponin indicated that the methine proton at δ 3.29 ppm very likely belonged to H-3 of the aglycone, and thus the carbon at δ 89.4 ppm was designated as C-3. The downfield shift of about 10 ppm of C-3, suggested the sugar unit was attached at C-3 (δ 89.4 ppm) of the aglycone. This conclusion was confirmed by the analysis of HMBC spectrum (Figure 1).

Two broad signals at δ 4.24 (d, 5.5 Hz) and 4.49 (m) which were directly connected, from HMQC, to two downfield shift carbons at 67.5 and 74.7 respectively, were also observed in **5** that correlated to each other in DQCOSY and HMQC-TOCSY spectra, indicated the protons attached to C-15, C-16. The downfield shifts of C-15 and C-16 indicated that they were hydroxylated positions. Moreover, the downfield shift of methyl-27 (δ 1.87 ppm) is characteristic of the 16α-hydroxyoleananes[2]. Meanwhile, the coupling constant of H-15 was 5.5 Hz indicates a β-axial proton. Thus, the hydroxyl substituents attached to C-15 and C-16 were α configurations (15α-OH, 16α-OH). The proton attached to C-28 was identified as one isolated AB system at δ 3.58 and 3.77 ppm (*J* = 10.5 Hz). The one deshielded doublet of doublets at δ 6.15 ppm (*J* = 12 and 5.5 Hz) whose multiplicity was caused by coupling to methylene protons at δ 1.94 ppm (*m*) and 2.80 (*t*, *J* = 12.0 Hz), indicating it was attached to C-22. The deshielding of H-22 suggested that C-22 was esterified. Based on the coupling constant of H-22 it can be assigned that the ester moiety should had α (equatorial) configuration. This NMR analysis enabled us to assign the A1-barrigenol structure (**1**) to the parent aglycone. The presence of a 2-methylbutyroyl group in **5** was indicated by the signals of a carbonyl carbon at δ 176.2 ppm and two methyl groups at δ 0.84 ppm (t, 7.5 Hz) and 1.10 (d, 7.0 Hz), which correlated to an AX system signal of a methine proton at δ 2.29 (sextet, 7.0 Hz). This suggestion was confirmed by comparison with literature data.[13] The observation of HMBC spectra showed correlation between H-22 at δ 6.15 ppm and a carbonyl at δ 176.2 ppm suggested that the 2-methylbutyroyl residue was attached at C-22 of the aglycone (Figure 1). Consequently, the structure of the aglycone part of **5** including the acid residue was 22-*O*-(2-methylbutyroyl)-A_1-barrigenol. This sapogenin had been isolated and charaterized by Higuchi et al. from the leaves of *Pittosporum undulatum* [13,14].



	R_1	R_2
(1)	OH	OH
(2)	OH	H



(**3**) (**4**) (**5**)

R_1 H OH OH

R_2

The sugar part of **5** consisted of three residues giving signals, in ^{1}H and ^{13}C-NMR spectra, for three anomeric protons at δ 4.93 ppm (d, 7.5 Hz), 5.31 ppm (d, 7.0 Hz), 5.66 ppm (d, 7.5 Hz) and two anomeric carbons at δ 103.8 and 105.2 ppm (Table 2). The two anomeric carbons overlapped at δ 105.2 ppm were observed by using an HMQC experiment. From HMQC, the anomeric proton at δ 5.68 ppm was connected to a carbon at δ 103.8 ppm, and two anomeric protons at δ 4.93 and 5.32 ppm connected with two overlapping carbons at δ 105.2 ppm. Consequently, a sugar part of **5** was identified as trisaccharide unit.

In order to map out the spin systems of the sugar moiety of **5**, the HMQC-TOCSY experiment was employed. The HMQC part leads to direct (one-bond) ^{1}H and ^{13}C correlations [15], and the TOCSY part then was used to obtain correlations for all of the protons of an isolated spin network [16]. For example, from the cross-peak between the anomeric proton at δ 5.66 ppm (1H, d, 8.0 Hz) and the anomeric carbon at δ 103.8 ppm, four carbons at δ 78.4, 77.7, 76.3, 72.4 ppm, which were later assigned to C-3, C-5, C-2, C-4 respectively, were found to correlate to this anomeric proton. Meanwhile, four protons at δ 4.23 (1H, m), 4.15 (1H, m), 4.09 (1H, dd, 8.0, 9.0 Hz), 3.81 ppm (1H, ddd, 4.0, 5.0, 5.0 Hz) correlated to that anomeric carbon. Those protons were assigned to H-3, H-4, H-2, H-5 respectively. Further correlation occurred between the methylenic protons at δ 4.33 and 4.45 ppm with C-5, C-4, and C-3, indicating the presence of a large vicinal coupling among ring protons due to a *trans* diaxial orientation of a hexose sugar, suggesting a *gluco* configuration. Meanwhile, the $^{3}J_{H\text{-}1,H\text{-}2}$ value for this sugar was 8.0 Hz, indicating a β-anomeric configuration. Therefore this sugar residue was assigned to β-glucopyranose. The protocols of the complete assignments for the two monosaccharide units were similar, enable us to establish β-galactopyranose and β-glucuronopyranose, respectively for these two monosaccharides. The relayed correlations observed in HMQC-TOCSY spectra are shown in Table 3. Acid methanolysis [5] followed by per-trimethylsilylation was used to confirm the sugar identities for **5** using gas chromatography. The GC profile of the products was compared with reference sugars treated under the same conditions and this indicated that D-galactose, D-glucose and D-glucuronic acid were present in approximately equal amounts. The D- configuration has been assumed for these sugars in keeping with Massiot and Lavaud's assertion [12], "The enantiomers of these sugars are not found in plants, a fact used as a clue in the determination of these sugars".

The linkages of trisaccharide chain were decided by HMBC as well as the linkage to the aglycone. The inter-glycosidic long-range carbon correlations arising from the anomeric protons are shown in Figure 1.

Table 1 ^{1}H and ^{13}C NMR data for aglycone moiety of **5** (Pyridine-d_5)[a].

Position	δ C (ppm)	DEPT	δ H (ppm), multiplicity, *J* (Hz)
1	38.9	CH_2	1.38 *dm*, 12.5; 2.06 dm, 12.5
2	27.2	CH_2	1.80 *dm*, 12.0; 2.15 *dm*, 12.0
3	89.4	CH	3.30 *dd*, 3.5, 11
4	39.6	C	-
5	55.5	CH	0.80 *m*
6	18.8	CH_2	1.39 br *d*, 12.5; 1.60 br *d*, 12.5
7	36.9	CH_2	2.07 *m*, 2.15 *m*
8	41.5	C	-
9	47.1	CH	2.52 *m*
10	36.7	C	-
11	23.9	CH_2	1.74 *dm*, 11.0; 1.87 *dm*, 11.0
12	124.8	CH	5.46 *t*, 3.2
13	144.5	C	-
14	47.4	C	-
15	67.5	CH	4.24 *d*, 5.5
16	74.7	CH	4.49 *m*
17	45.2	C	-
18	41.7	CH	3.03 dd, 4.0, 14.0
19	47.0	CH_2	1.33 m; 2.87 *t*, 13.5
20	31.9	C	-
21	41.6	CH_2	1.95 *m* 2.78 *dd*, 11.5, 12.0
22	72.0	CH	6.15 *dd*, 5.5, 12.0
23	27.9	CH_3	1.24 *s*
24	16.8	CH_3	1.10 *s*
25	15.8	CH_3	0.83 *s*
26	17.5	CH_3	1.03 *s*
27	21.3	CH_3	1.87 *s*
28	62.8	CH_2	3.59 *d*, 10.5 3.78 *d*, 10.5
29	25.2	CH_3	1.07 *s*
30	33.5	CH_3	1.26 *s*

[a] Assignment was also based upon DQCOSY, HMQC, HMBC, HMQC-TOCSY.

Table 2 ^{1}H and ^{13}C NMR data of osidic moieties of **5** in pyridine-d_5.

	δC (ppm)	**δH** (ppm) multiplicity *J* (Hz)		**δC** (ppm)	**δH** (ppm) multiplicity *J* (Hz)
β-D-Glc-A			β-D-Gal		
1’	105.2	4.93 (d, 7.5)	1’’’	105.2	5.31 (d, 7.5)
2’	78.8	4.43 (m)	2’’’	72.9	4.48 (m)
3’	87.5	4.36 (m)	3’’’	75.3	4.14 (m)
4’	71.7	4.51 (m)	4’’’	70.0	4.46 (m)
5’	77.2	4.55 (m)	5’’’	77.2	4.16 (m)
6’	170.6* (Me ester)	-	6’’’	61.9	4.33 (m) 4.45 (m)
β-D-Glc			2-methylbutyroyl		
1’’	103.8	5.66 (d, 8.0)	1’’’’	176.2	-
2’’	76.3	4.09 (dd, 8.0, 9.0)	2’’’’	41.8	2.29 (dq, 7.0)
3’’	78.4	4.23 (m)	3’’’’	26.7	1.36 (ddt, 6.0, 7.0, 15.0) 1.68 (ddt, 6.0, 7.0, 15.0)
4’’	72.4	4.15 (m)	4’’’’	11.8	0.84 (s)
5’’	77.7	3.81 (ddd. 4.0, 5.0, 5.0)	5’’’’	16.7	1.10 (d, 7.0)
6’’	63.1	4.33 (m) 4.45 (m)			

*observed in the ^{13}C NMR of methylated **5**.

Table 3 Relayed correlations for sugar residues of **5**. One-bond correlations shown in bold.

Glucose		Galactose		Glucuronic acid	
No. C	No. H	No. C	No. H	No. C	No. H
C1	**H1**,H2,H3,H4,H5	C1	**H1**,H2,H3,H4	C1	**H1**,H2,H3,H4,H5
C2	H1,**H2**,H3,H4,H5	C2	H1,**H2**,H3	C2	H1,**H2**,H3,H4,H5
C3	H1,H2,**H3**,H4,H5,H6	C3	H1,H2,**H3**,H4	C3	H1,H2,**H3**,H4,H5
C4	H1,H2,H3,**H4**,H5,H6	C4	H1,H2,H3,**H4**,H5	C4	H1,H2,H3,**H4**,H5
C5	H1,H2,H3,H4,**H5**,H6	C5	H4,**H5**,H6	C5	H1,H2,H3,H4,**H5**
C6	H3,H4,H5,**H6**	C6	H5,**H6**	C6	—

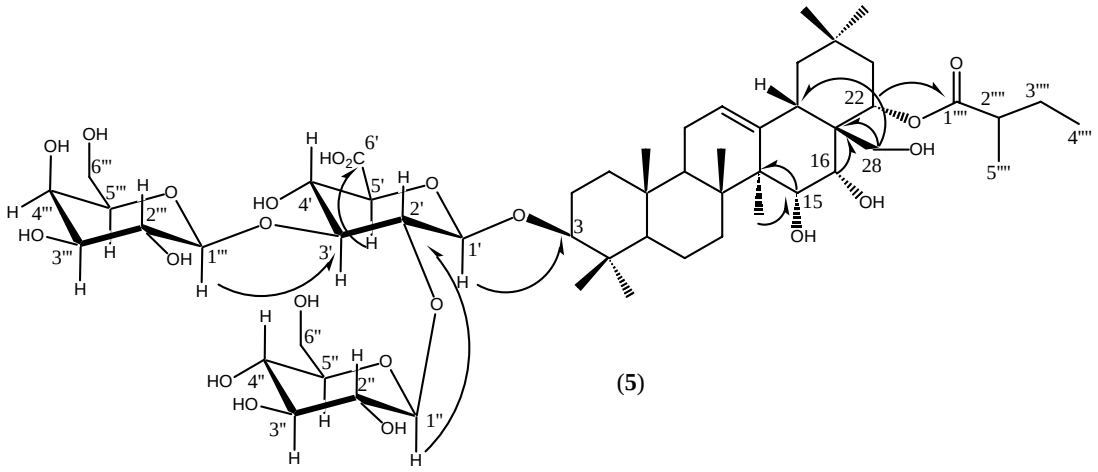


Figure 1 Selected HMBC correlations for establishing the sugar sequence and to determine the positions of attachment of the acid and sugar moieties of **5**.

The sequence of trisaccharide chain was unambiguously determined by the following significant cross peaks in the HMBC spectra: anomeric proton H-1'' of glc at δ 5.66 ppm with C-2' (δ 78.8 ppm) of glc-A, the anomeric proton H-1''' of gal at δ 5.31 ppm with C-3' (δ 87.5 ppm) of the glc-A. As observed in the HMBC spectrum, a long range correlation between anomeric proton H-1' of glc-A at δ 4.93 ppm and C-3 (δ 89.4 ppm) of the A_1-barrigenol aglycone revealed the trisaccharide chain was linked to the C-3 position.

Based on the above descriptions, the structure of **5** was determined to be 3-*O*-β-D-glucopyranosyl(1-2)-β-D-galactopyranosyl(1-3)-β-D-glucuronopyranosyl 22-*O*-(2-methylbutyroyl)-A1-barrigenol. This compound as a mixture with the 22-*O*-3,3-dimethylacryloyl ester had been reported earlier by Higuchi *et al*. [13,14], and the ^{1}H and ^{13}C NMR data was not comprehensively reported in their paper.

Experimental Section

General Experimental Procedures

The IR spectrum was determined using a Perkin-Elmer 1800 FTIR spectrophotometer. Optical rotation was measured in a 1 decimetre path cell with a Perkin-Elmer 241 polarimeter. The FABMS was measured in a 3-nitrobenzyl alcohol matrix on a VG Analytical ZAB-SEQ2 Mass Spectrometer. ^{1}H and ^{13}C NMR spectra were recorded using a Varian INOVA instrument at 500 MHz (^{1}H) and 125 MHz [13,c]. All of the NMR data were measured in pyridine-d_5 at 25 ^{0}C and chemical shifts are expressed in δ (ppm). 2D experiments were performed using standard INOVA programs.

Two step semi-preparative HPLC was performed on YMC-Pack ODS-AQ, 5μm 120Å 250 μm columns of 10 and 20 mm internal diameter, thermostatted at 40 ^{0}C. The mobile phase was generated by blending A: MeOH/THF/H_2O/HOAc (9/1/90/0.05), B: MeOH/THF/HOAc (90/10/0.05) in 60%B of isocratic elution. Flow rates were 10 mL min^{-1} in 20 mm i.d. column (step 1) and 4 mL min^{-1} in 10 mm i.d. column (step 2). Instrumentation for HPLC consisted of two Waters 510 and 481 pumps, a Rheodyne 7125 injector fitted with a 4.4 mL sample loop, and a Waters 481 UV/visible detector fitted with a 2.3 mm path flow cell, monitoring absorbance at 210 nm.

GC was performed on a Varian-3400 instrument with an FID and a 12 metre 0.22 mm i.d., BP-1 (100% polydimethylsiloxane) column was used. The injector temperature was 250 ^{0}C, and the detector was 320 ^{0}C. The temperature program used was 50 ^{0}C (2 min hold) then heated at 10 ^{0}C min^{-1} to 300 ^{0}C and held 3 min at this temperature before cooling down. The retention times were compared with those of authentic sugars, treated under identical conditions.

Plant Material

The seeds of *Barringtonia asiatica* were collected on 1996 in Sangihe Talaud region, North Sulawesi province, Indonesia. This plant was identified by Mr Djuandi of the Herbarium of Department of Biology, Technology Institute of Bandung, Indonesia, where a voucher specimen has been deposited.

Extraction and Isolation

Plant material (3.0 kg) was extracted as described previously [2,8]. The crude saponin (4.35 g) was dissolved in methanol and then injected on to a large scale RP-HPLC column using step 1 conditions. Five fractions: LSF1 (t_R = 46.11), LSF2 (t_R = 50.67), LSF3 (t_R = 61.65), LSF4 (t_R = 65.64), LSF5 (t_R = 74.33) were obtained. Fractions LSF1 and LSF2 were combained and purified using step 1 of HPLC condition gave two subfractions, SSF1 and SSF2. Fraction SSF2 was further purified using step 2 of HPLC condition to obtain a single pure compound **5** (100 mg).

Compound 5: White amorphous solid, 100 mg; $[\alpha]^{25}_D$ +0.7^0 (3.06 mg mL^{-1} CH_3OH); IR (KBr) ν_{max} 3427, 2945, 1709, 1638, 1464, 1387, 1261, 1202, 1157, 1076, 1041, 1019 cm^{-1}; NMR (see Table 1 and 2); FABMS (see text); high-resolution negative FABMS *m/z* 1073.5619 $[M - 1]^-$ (calcd for $C_{53}H_{85}O_{22}$, 1073,5508); *anal*. C 55.6%, H 7.7% calcd for $C_{53}H_{86}O_{22}$ + CH_3COOH + $3H_2O$, C 55.5%, H 8.1%.

Acid hydrolysis of 5. The acid hydrolysis was performed according to Chapman and Kennedy[16] using 1 mg of **5**. The hydrolysate was dissolved in 100 μl pyridine, 100 μl BSTFA containing 1% TMCS added, then heated at 95 ^{0}C for 1 hour before GC analysis.

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REFERENCES

1. Hasan, C.M., Khan, S., Jabbar, A. and Rashid, M.A., 2000, *J. Nat. Prod*., 63, 410-411.
2. Rumampuk, R. J., Tarigan, P., Herlt, A. J. and Mander, L.N., 2001, *Proceedings of the International Seminar on Natural Products Chemistry and Utilization of Natural Resources*, June 5-7, Depok, Indonesia, pp 208-214.
3. Nozoe, T., 1934, *J. Chem. Soc. Jpn.* 55, 1106-1114.
4. Lin, Y.T., Lo, T.B. and Tai, W.T., 1954, *J. Chinese Chem. Soc*., (Taiwan), 1, 57-63.
5. Errington, S. G., White, D. E. and Fuller, M., 1967, W. *Tetrahedron Lett.* 1289-1294.
6. Ito, S., Kodama, M. and Konoike, M., 1967, *Tetrahedron Lett*. 591-596.
7. Rumampuk, R.J., Pongoh, E.J., Tarigan, P., Herlt, A.J. and Mander, L.N., 2003, *Presented at the International Symposium on Biomedicines*, Bogor Agryculture University, Bogor, September 18-19.
8. Rumampuk, R.J., 2001, Elusidasi struktur saponin dari biji *Barringtonia asiatica* (L.) Kurz. *Disertasi*, Universitas Padjadjaran, Bandung.
9. Hostettmann, K. and Marston, A., 1995, *Chemistry and Pharmacology of Natural Products : Saponins*, Cambridge University Press.
10. Nakayama, K., Fujino, H., Kasai, R., Tanaka, O. and Zhou, 1986, *J. Chem. Pharm. Bull*., 34, 2209-2213.
11. Kühner, K., Voigt, G. and Hiller, K., 1985, *Pharmazie*, 40, 576-578.
12. Massiot, G. and Lavaud, C., 1995, Structural elucidation of saponins, in *Studies in Natural Products Chemistry*, ed. Atta-ur-Rahman, Elsevier, 15, 187-224.
13. Higuchi, R., Komori, T., Kawasaki, T and Lassak, E.V., 1983, *Phytochemistry*, 22, 1235-1237.
14. Bax, A. and Subramanian, S., 1986, *J. Magn. Reson*., 67, 565-570.
15. Davis, D.G. and Bax, A., 1985, *J. Am. Chem. Soc*., 107, 2820-2821.
16. Chapman, M.F. and Kennedy, J.F., 1986, *Carbohydrate analysis – a practical approach*. IRL Press, pp. 25 & 78-79.