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Complete assignments of ^{1}H and ^{13}C NMR spectroscopic data for three new stigmastane glycosides from *Vernonia cumingiana*

Maorong Suo* and Junshan Yang*

Three new steroidal saponins, Vernoniosides S1 (1), Vernoniosides S2 (2) and Vernoniosides S3 (3) were isolated from the stem of *Vernonia cumingian*. Their chemical structures were elucidated on the basis of MS, NMR spectroscopic and chemical analysis. Complete assignment of ^{1}H and ^{13}C NMR spectroscopic data were achieved by 1D and 2D NMR experiments (HMQC, HMBC, ROESY).

Keywords: NMR; ^{1}H NMR; ^{13}C NMR; 1D NMR; 2D NMR; *Vernonia cumingian*; steroidal saponins; Vernoniosides **S1**; Vernoniosides **S2**; Vernoniosides **S3**

Introduction

Vernonia cumingiana Benth.(Compositae) was mainly distributed in Guangxi, Yunnan and Guangdong provinces in China, as a known Chinese folk medicine, it was extensively used for the treatment of malaria, larynx aches, cold, tooth aches and rheumatism.[1] Members of *Vernonia* family were well known for the production of sesquiterpene lactone and stigmastane type glycosides.[2–5] In the course of searching for new and bioactive constituents from traditional Chinese medicines, three new stigmastane glycosides, named Vernonioside **S1** (**1**), Vernonioside **S2** (**2**), Vernonioside **S3** (**3**), were isolated from the stems of *V. cumingiana*. This paper describes the isolation and structure elucidation of these stigmastane glycosides from *V. cumingiana*. This is the first report that sugar unit is linked to C-28 of aglycone of stigmastane glycosides in the *Vernonia* family.

Results and Discussion

Compound **1** was obtained as colorless powder, and the Liebermann-Burchard and Molisch reaction of **1** were positive, indicating **1** to be steroidal or triterpenoids glucosides. The molecular formula-of **1** was deduced as $C_{41}H_{66}O_{15}$ by HR–FAB–MS (*m/z* 821.4282 $[M + Na]^+$), and the degree of unsaturation was 9. The UV spectrum of **1** showed absorption bands at 235.4 and 242.4 nm, suggesting the presence of a conjugated diene chromophore. The infrared (IR) spectrum displayed strong absorptions of hydroxyls (3600−3100 cm^{-1}) and carbonyl group (1699 cm^{-1}).

The FAB–MS (positive ion) showed the loss of two hexoses (162 mass units) from the positive ion $[M + Na]^+$ at *m/z* 821. The ^{13}C NMR spectrum of **1** displayed 29 carbon signals for the aglycon moiety ascribable to an stigmastane nucleus including two oxymethines (77.0 ppm, CH, C-3), (81.3 ppm, CH, C-28), and a carboxyl(ic) group (178.7 ppm, C-21). The ^{1}H NMR spectra for the aglycon part of **1** were typical of a sterol structure displaying two angular methyl singlets at δ 0.81 and 0.82, three secondary methyl proton signals at δ 1.09 (d, $J = 7.0$, H-26), 1.22 (d, $J = 7.0$, H-27) and 1.41(d, $J = 6.5$, H-29), and two characteristic multiplet at δ 3.93 (H-3) and 4.30 (H-28), which was shifted downfield by glycoslation.[5] Besides that, two olefine protons at δ 5.35 (br s) and 5.41 (br d $J = 6.0$) were displayed in the ^{1}H NMR spectrum of **1**. An unsaturated functionality evident from the ^{13}C NMR spectrum were two trisubstituted double bond (120.9 and 136.3, 144.2 and 118.5 ppm), situated between C-7 and C-8, C-9 and C-11, as indicated by analysis of the HMQC and HMBC correlations. These data suggested that **1** is based on a 3, 28-dihydroxy-stigmastane skeleton, possessing a $\Delta^{7,9(11)}$ diene system.[6] Key correlations peaks were observed between H-26, 27, 29 and C-24 (δ 76.5) by the analysis of DEPT, HMQC and HMBC spectra (Fig. 1) of **1**, indicating that there was a hydroxyl linked at C-24. The NMR data for the sugar moiety (Table 1) linked at C-3 and C-28 of the aglycon revealed the presence of two β-glucopyranosyl units. Their β-linkages were shown by the coupling constant values ($J = 8.0$, 7.5 Hz) of two anomeric proton signals at δ 5.00, 4.92 and by their chemical shifts in the ^{13}C NMR spectra. NMR, HMQC, DEPT and HMBC experiments allowed the assignments of all proton and carbon signals and the identification of two substituted β-glucopyranosyl residues. The configuration of the sugar units was assigned after acid hydrolysis, the hydrolysate was trimethylsilated, and gas chromatography (GC) retention times of sugar were compared with authentic samples. In this way, the sugar units of **1** were determined to be D-glucose.

In the ROESY spectrum of **1**, the key correlation peaks between H-3 (δ 3.93) and H-5 (δ 1.32), H-17 (δ 2.25) and H-14 (δ 2.08), H-20 (δ 2.48) and H-18 (δ 0.81), H-28 (δ 4.30) and H−2″ (δ 4.04) were observed. Thus, compound **1** was established as 3β,24,

* *Correspondence to: Maorong Suo and Junshan Yang, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100094, China. E-mail: suomaorong@163.com; junshanyang@sina.com*

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100094, China

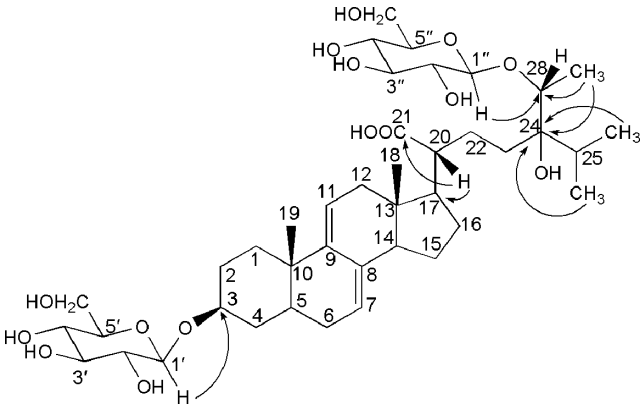

Figure 1. Structure and the key HMBC correlations of compound **1**.

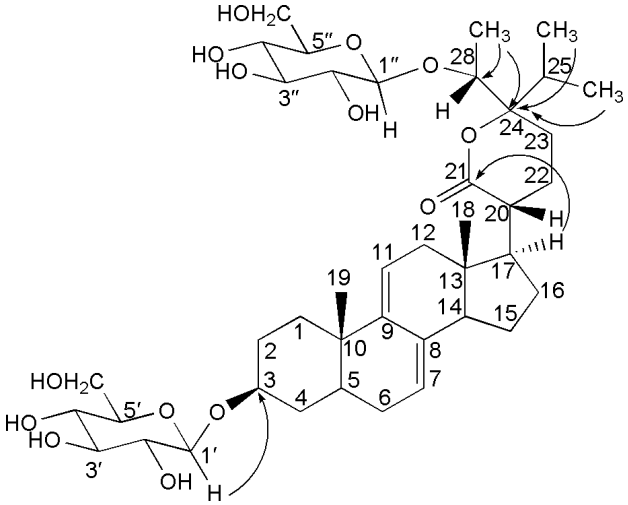

Figure 2. Structure and the key HMBC correlations of compound **2**.

28- trihydroxy-stigmastane-7(8),9(11)-dien-21-oicacid-3,28-dioxy-β-D-glucopyranoside, named Vernonioside **S1**.

Compound **2** was obtained as a colorless powder. Its HRFAB–MS at m/z 803.4163 $[M+Na]^+$ corresponded to the molecular formula of $C_{41}H_{64}O_{14}$, suggesting 10 degrees of unsaturation. Positive results from both Liebermann-Burchard and Molisch reactions indicated it to be stigmastane glycoside. The UV spectrum of **2** exhibited absorption bands at 242.8 and 235.6 nm, and its IR spectrum showed absorptions of hydroxyl (3421 cm^{-1}) and carbonyl groups (1718 cm^{-1}). Two D-glucose units were identified by GC analysis after acid hydrolysis as **1**.

The ^{1}H NMR spectrum of **2** exhibited two angular methyl singlets at δ 0.74 (s, H-18) and 0.83 (s, H-19), three secondary methyl proton signals at 0.91 (d, H-26), 1.01 (d, H-27), 1.38 (d, H-29), and two olefine protons δ 5.32 (br s) and 5.48 (br s). The analysis of the NMR data of **2** revealed the same aglycon as in **1** and two sugar chains comprised of two glucosides (Table 1). Comparison of the ^{13}C NMR spectrum of **2** with that of **1** showed the evident downfield shift of the signal ascribable to C-24 (δ 88.6 in **2** vs δ 76.5 in **1**), and

Table 1. ^{1}H NMR and ^{13}C NMR spectra data for compound 1–4[a]

	1		2		3	
Position	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
1	35.0	1.17 m 1.79 (br d J = 13.5)	35.0	1.17 m 1.79 m	35.0	1.27 m 1.84 (br d J = 13.5)
2	30.1	1.80 br s	30.1	2.05 br s 2.11 m	30.1	1.77 m
3	77.0	3.93 m	76.9	3.90 m	77.0	3.94 (d, J = 5.5)
4	34.5	2.01 br t J = 10.5	34.5	2.00 br t J = 10.5 2.08 m	34.5	1.99 m
5	39.2	1.32 m	39.1	1.24 m	39.2	1.28 m
6	30.4	2.09 br d J = 5.0	30.2	1.87 br s	30.1	1.77 m
7	120.9	5.35 br s	120.6	5.32 br s	120.8	5.35 br s
8	136.3		136.5		136.0	
9	144.2		144.1		144.2	
10	36.1		36.1		36.2	
11	118.5	5.41 (br d J = 6.0)	119.0	5.48 br s	118.5	5.46 br s
12	40.4	2.34 br d J = 17 2.52 br d J = 7.0	40.3	2.36 br s 2.40 br s	41.7	2.19 m
13	42.5		42.9		43.7	
14	53.3	2.08 br d J = 5.0	51.7	2.31 br s	49.4	2.75 m
15	23.0	1.69 br t J = 12	23.0	1.72 br t J = 10.5	36.1	2.11 m
16	26.8	2.00 m	26.0	1.99 m	74.5	4.58 br s
17	51.7	2.25 m	49.5	2.52 br s	62.6	2.05 m
18	11.6	0.81 s	12.4	0.74 s	13.5	0.67 s
19	19.4	0.82 s	19.5	0.83 s	19.5	0.82 s
20	49.7	2.48 br t J = 6.5	40.9	2.55 br s	41.9	1.90 br s
21	178.7		174.4		63.6	3.84(dd, J = 9.5, 4.5) 4.14 (br, d, J = 9.5)

Table 1. (*Continued*)

Position	1		2		3	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
22	27.8	2.01 m 2.03 m	22.6	1.87 m	21.8	1.79 m
23	30.2	2.11 (br d $J = 8.0$)	21.9	2.11 (br d $J = 8.0$)	30.0	2.11 m
24	76.5		88.6		76.8	
25	33.9	2.16 m	34.5	2.10 m	33.8	2.17 m
26	17.8	1.09 d $J = 7.0$	17.2	0.91 d $J = 6.0$	17.8	1.09 d $J = 7$
27	17.8	1.22 d $J = 7.0$	17.5	1.01d $J = 6.0$	18.1	1.21d $J = 7$
28	81.3	4.30 m	78.6	4.28 t $J = 8.0$	80.7	4.37 m
29	16.1	1.41 d $J = 6.5$	14.9	1.38 d $J = 6.5$	15.7	1.50 d $J = 6.5$
1′	102.2	5.00 d $J = 8.0$	102.3	5.00 d $J = 7.5$	102.3	5.01 d $J = 7.5$
2′	74.7	4.00 br t $J = 7.5$	75.0	4.00 br t $J = 8.5$	75.0	3.99 (br dd, $J = 8, 6.5$)
3′	78.5	3.97 m	78.1	3.97 br s	78.4	3.93 m
4′	71.7	4.20 m	71.7	4.29 br d $J = 8.0$	71.7	4.17 br d $J = 8.0$
5′	78.5	3.97 m	78.6	4.20 t $J = 8.5$	78.6	4.22 t $J = 9.0$
6′	62.7	4.34 (dd $J = 10.5, 5.5$) 4.53 m	62.9	4.40 (dd $J = 10.5, 5.0$) 4.52 (br d $J = 11$)	62.7	4.31 (br, d, $J = 8.0$) 4.51 (br, d, $J = 10$)
1″	103.7	4.92 d $J = 7.5$	103.0	4.84 d $J = 7.5$	103.4	4.93 d $J = 7.5$
2″	75.3	4.04 br t $J = 8.0$	75.3	4.03 br t $J = 8.0$	75.3	4.03 br t $J = 8.0$
3″	78.6	4.28 t $J = 6.5$	78.5	3.99 m	78.5	3.98(d, $J = 8.5$)
4″	71.7	4.29 t $J = 9.0$	71.8	4.15 t $J = 10.5$	71.8	4.27(t, $J = 8.0$)
5″	78.5	4.22 br t $J = 8.0$	78.8	4.28 br t $J = 8.0$	78.8	4.26 (t, $J = 8.0$)
6″	62.8	4.39 (dd, $J = 10.5, 5.0$) 4.57 br d $J = 10$	63.1	4.34 (dd, $J = 10.5, 4.5$) 4.57 br d $J = 12$	62.9	4.41 (d, $J = 5.0$) 4.57 (br d $J = 9.5$)

[a] The experiments were carried out at 500 MHz for ^{1}H and 125 MHz for ^{13}C in C_5D_5N and TMS as reference (0.00 ppm).

the upfield shift of the signal ascrible to C-21 (δ 174.4 in **2** vs δ 178.7 in **1**) (Table 1), indicating C-24 was linked to C-21 by ester bonds. The cross peaks were observed between H-1′ (δ 5.00) and C-3 (δ 76.9), H-1″ (δ 4.84) and C-28 (δ 78.6) in the HMBC spectrum (Fig. 2), suggesting two D-glucopyranosyl units were linked at C-3 and C-28 respectively as that of **1**. Their β-linkage were shown by the coupling constant values (both $J = 7.5$ Hz) of two anomeric protons at δ 5.00 and 4.84. All proton and carbon signals were assigned by NMR, HMQC and HMBC data.

In the ROESY spectrum of **2**, significant cross peaks between H-3 (δ 3.90) and H-5 (δ 1.24), H-17 (δ 2.52) and H-14 (δ 2.31), H-20 (δ 2.55) and H-18 (δ 0.74) were observed. So, compound **2** was determined as 3β, 24, 28-trihydroxy-stigmastane-7(8), 9(11)-dien-21, 24-lactone-3, 28-dioxy-β-D-glucopyranoside, named as Vernonioside **S2**.

Compound **3**, was obtained as a colorless powder. Its molecular formula $C_{41}H_{68}O_{15}$ was deduced by HRFAB–MS $[M + Na]^+$ *m/z* 821.4282, and the degree of unsaturation was 8. The UV spectrum of **3** showed absorption at 242.2 and 235.4 nm. The IR spectrum displayed the presence of hydroxyls (3415 cm^{-1}) and double bonds (1458, 1379 cm^{-1}).

The analysis of the NMR data of **3** revealed the same aglycon as in **1** and two sugar chains comprised of two glucosides (Table 1) and its aglyone possessed 3, 28-dihydroxy-stigmastane-7 (8), 9 (11)-dien. Comparison of the ^{13}C NMR spectrum of **3** with that of **1** showed the absence of carboxyl group at δ 178.7. Differences were also observed in the ^{13}C NMR of aglycone of **3** added an oxymethylene and an oxymethine at δ 63.6 (C-21) and 74.5 (C-16) than that of **1**, suggesting two hydroxyl was linked at C-21 and C-16 respectively, which was approved by the correlation peaks

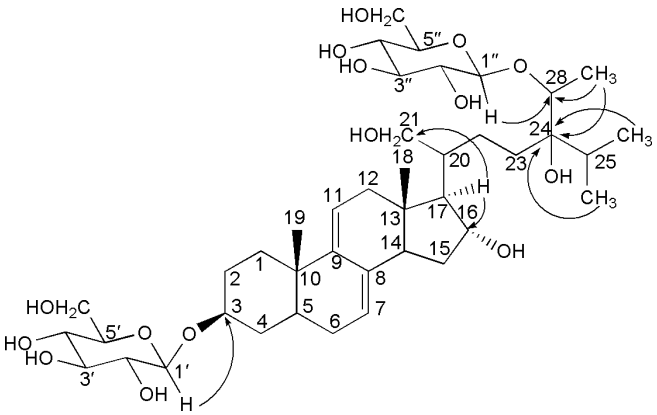

Figure 3. Structure and the key HMBC correlations of compound **3**.

between C-21 (δ 63.6) and H-17 (δ 2.05), C-20 (δ 41.9); C-14 (δ 49.4) and H-16 (δ 4.58) in the HMBC spectrum of (Fig. 3) **3**.

The ^{1}H NMR and ^{13}C NMR of **3** (Table 1) gave two sets of D-glucose signals, which were supported by GC analysis after the acid hydrolysis experiment. The coupling constant values of both anomeric protons (δ 5.01, 4.93) were 7.5 Hz, suggesting that the compound **3** was β-D-pyranglucosides. Similarly, two β-D-glucose were connected with C-3 and C-28 respectively, which were confirmed by the correlation peaks between H-1′ (δ 5.01) and C-3 (δ 77.0), H-1″ (δ 4.93) and C-28 (δ 80.7) in the HMBC of **3**. All proton and carbon signals were assigned by ^{1}H NMR, ^{13}C NMR, HMQC and HMBC data.

The relative stereochemistry of **3** was determined by evaluation of the cross-peak in the ROESY spectrum of **3**, the strong correlation peaks between H-3 (δ 3.94) and H-5 (δ 1.28), H-17 (δ 2.05) and H-14 (δ 2.75), H-16 (δ 4.58) and H-12 (δ 2.19) were observed. Thus, compound **3** was elucidated as 3β, 16α, 21, 24, 28-penta hydroxy-stigmastane-7 (8), 9 (11)-dien–3, 28–dioxy-β-D-glucopyranoside, named as Vernonioside **S3**.

Experimental

Methods

Melting points were determined on an X4 micro melting point apparatus and uncorrected. Optical rotations were measured on a Perkin-Elmer 341 digital polarimeter (Perkin-Elmer, Norwalk, CT, US) at 589 nm. UV spectra were recorded on Hitachi UV–2201 spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were recorded in KBr discs on an Impact 410 FTIR spectrophotometer. ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, HMBC and ROESY spectra of compound **1**, **2** and **3** were recorded on a Varian Inova-500 spectrometer with a 5-mm inverse probe at room temperature. The compound **1**, **2** and **3** were dissolved separately in 1-ml pyridine-d_5, and transferred to a 5-mm NMR tube. All chemical shifts are in ppm (δ), using TMS as internal standard and coupling constants (J) are in hertz. FAB–MS and EIMS were obtained on a VG-Autospec- 3000 spectrometer (Thermo Electron, Manchester, UK). GC analysis was carried out on Agilent 6890N gas chromatography using an HP-5 capillary column (28 m $\times$ 0.32 mm i.d.); detection, FID; detector temperature, 260 °C; column temperature, 180 °C; carrier gas, N2; flow rate, 40 ml/min. Silica gel (100—200, 300—400 mesh) and silica gel GF_{254} sheets (both from Qingdao Haiyang Chemical Group Co., Qingdao, Shandong Province, China) were used for column chromatography and thin layer chromatography (TLC), respectively.

The pulse conditions for (**1**) were as follows: for the ^{1}H NMR spectra: spectrameter frequency (SF) 499.745 MHz, acquisition time (AQ) 1.865 s, number of repetition (NR) 16, temperature (TE) 298.1 K, relaxation delay (RD) 4.000 s, flip angle (FA) 87.2°, spectral width (SW) 7998.4 Hz; for the ^{13}C NMR spectrum: SF 125.7 MHz, AQ 1.000 s, NR 1728, TE 298.1K, RD 1.000 s, FA 72.1°, SW 31 421.8 Hz; for the HMQC spectrum: the HMQC experiment for single-bond ^{1}H, ^{13}C chemical shift correlation spectra utilized GARP, ^{13}C decoupling. Two sets of 128 time increments were obtained in the phase-sensitive mode with 48 transients obtained per time increment, SF 499.745 MHz, AQ 0.203, TE 298.1, RD 1.000 s, SW 5039.4 Hz, 2D SW 23 809.5 Hz; for the HMBC spectrum: the HMBC experiment was performed with 32 scans for each of 320 F1 inceremets, 2048 data points in F2, SF 499.745, AQ 0.189 s, TE 298.1 K, RD 1.000 s, SW 5417.9 Hz, 2D SW 23 809.5 Hz; for the ROESY spectrum: the ROESY experiment was performed with 64 scans for each of 256 F1 increments, 4096 data points in F2, SF 499.745, AQ 0.206 s, TE 298.1 K, RD 1.600 s, mixing time (MT) 0.800 s, SW 4948.4 Hz, 2D SW 4948.4 Hz.

The pulse conditions for (**2**) were as follows: for the ^{1}H NMR and ^{13}C NMR spectra: the same as those of **1**; for the HMQC spectrum: two sets of 256 time increments were obtained in the phase-sensitive mode with 64 transients obtained per time increment, the same as those of **1** except for AQ 0.210 s, SW 4978.3 Hz, 2D SW 23 809.5 Hz; for the HMBC spectrum: 96 scans for each of 320 F1 inceremets, 2048 data points in F2, the same as those of **1** except for AQ 0.208 s, SW 4925.2 Hz; for the ROESY spectrum: eight scans for each of 200 F1 increments, 4096 data points in F2, the same as those of **1** except for AQ 0.207 s.

The pulse conditions for (**3**) were as follows: for the ^{1}H NMR and ^{13}C NMRspectrum: the same as those of **1**; for the HMQC spectrum: two sets of 256 time increments were obtained in the phase-sensitive mode with 48 transients obtained per time increment, the same as those of **1** except for SW 5050.8 Hz, 2D SW 19 431.6 Hz; for the HMBC spectrum: 80 scans for each of 320 F1 increments, 2048 data points in F2, SF 499.745, AQ 0.204 s, TE 298.1 K, RD 1.000 s, SW 5030.5 Hz, 2D SW 19 431.6 Hz; for the ROESY spectrum: eight scans for each of 200 F1 increments, 2048 data points in F2, the same as those of **1** except for AQ 0.207 s, RD 1.600 s, MT 0.800 s, SW 4952.0 Hz, 2D SW 4952.0 Hz.

Plant material

The stems of *V. cumingiana* were collected from Nanning, Guangxi Province, people's Republic of China, in September 2003. The plant was identified by the Advisor Chao-Liang Zhang in the Guangxi Subinstitute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher sample (NO. YA-02-0726) was deposited in the Herbarium of Institute of Medicinal Plant Development, Chinese Academy of Medical Science and Peking Union Medical College.

Extraction and isolation

The air-dried and pulverized stem of *V. cumingiana* Benth. (8.0 kg) was extracted two times with 95% EtOH for 1.5 h under reflux condition, and the extract (1.10 kg) was chromatographed with silica gel column (100—200 mesh), eluting with petroleum ether, chloroform, EtOAc, MeOH and H_2O respectively. The EtOAc fraction (205 g) was chromatographed on silica gel (300—400 mesh) column, eluting with gradient $CHCl_3$—MeOH to afford 40 fractions. Fraction 25—40 (1.0 g) were combined and chromatographed on silica gel (300—400 mesh) with gradient solvent ($CHCl_3$:MeOH:H_2O) and 200-mg eluent ($CHCl_3$:MeOH:H_2O = 5:1:0.1) was obtained. Then the eluent was further chromatographed on ODS gel with MeOH–H_2O as eluting solvent to yield compound **3** (18 mg) (MeOH:H_2O = 2:5). Fraction 8 (69 mg) was chromatographed over ODS gel to yield compound **1** (50 mg) (MeOH : H_2O = 1:2) and **2** (25 mg) (MeOH : H_2O = 2:3).

Vernonioside **S1** (**1**) colorless powder (MeOH), $[\alpha]_D^{20}$ +7.62 (*c* 0.92, MeOH);UV (MeOH) λ_{max} (logε): 242.4 (2.70), 235.4 (2.67), IR (KBr) cm^{-1}: 3406, 2943, 2873, 1699; ^{1}H NMR and ^{13}C NMR data (Table 1); HR–FAB–MS (positive) *m/z* 821.4282 [M + Na]$^+$ (calcd for $C_{41}H_{66}O_{15}Na$, 821.4299).

Vernonioside **S2** (**2**) colorless powder (MeOH), [a]$_D^{20}$ −6.31 (*c* 0.63, MeOH); UV (MeOH) λ_{max} (logε): 242.8 (3.14), 235.6 (3.10); IR (KBr) cm^{-1}: 3410, 2960, 2877, 1718; ^{1}H NMR and ^{13}C NMR data (Table 1); FAB–MS (positive) *m/z* 781[M + H]$^+$, HR–FAB–MS (positive) *m/z* 803.4163 [M + Na]$^+$ (calcd for $C_{41}H_{64}O_{14}Na$, 803.4193).

Vernonioside **S3** (**3**) colorless powder (MeOH), [a]$_D^{20}$ +10.14 (*c* 0.69, MeOH); UV (MeOH) λ_{max} (logε): 242.2 (2.97), 235.4 (2.93); IR (KBr) cm^{-1}: 3415, 2927, 2875, 1637, 1458, 1379, 1078, 1034; ^{1}H NMR and ^{13}C NMR data (Table 1); HR–FAB–MS (positive) *m/z* [M + Na]$^+$ 821.4282 (calcd for $C_{41}H_{68}O_{15}Na$, 821.4299).

Acid hydrolysis of 1-3

Compounds **1**–**3** (each 2 mg) were refluxed with 10% HCl in aq. MeOH (3 ml) for 6 h. Each reaction mixture was diluted with H_2O

and neutralized with Ag_2CO_3. The neutral hydrolysate revealed the presence of glucose by TLC detection comparison with an authentic sample.

Sugar identification by hydrolysis and GC analysis

Compounds **1**–**3** (each 3 mg) was heated in 2 ml of 10% HCl–dioxane (1 : 1) at 80 °C for 4 h. After the dioxane was removed, the solution was extracted with EtOAc (2 ml × 3). The aqueous fractions were evaporated and the residues were prepared to their derivatives for GC analysis according to the methods described in the literature.[7] The D-glucose was confirmed by the comparison of its retention time (t_R, 12.50 min) with that of authentic standards.[8]

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