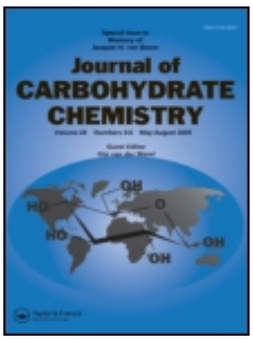

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New stigmastane type of steroidal glycosides from the roots of *Vernonia cumingiana*

Guo-Xu Ma[a], Wei Feng[b], Zhong-Hao Sun[a], Peng-Fei Li[a], Nai-Liang Zhu[a], Jun-Shan Yang[a], Xu-Dong Xu[a], and Hai-Feng Wu[a]

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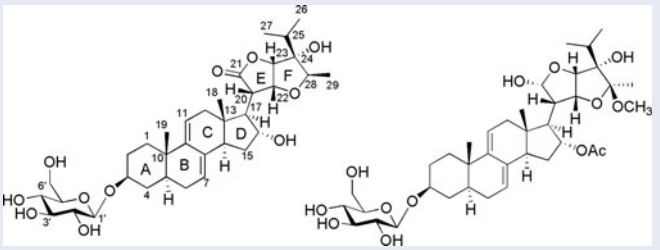
ABSTRACT

Two new stigmastane type of steroidal glycosides, vernoniacums A and B (1 and 2), with a $\triangle^{7,9(11)}$ steroidal core were isolated from the roots of *Vernonia cumingiana*. Their structures were elucidated based on various spectroscopic techniques, including IR, HR-FAB-MS, and 1D and 2D NMR. Both compounds were evaluated for their cytotoxicity against HeLa and HCT-8 cells, and compound 1 showed mild activity against the tested cell lines with IC50 values of 15.8 and 35.7 pounds were evaluated for their cytotoxicity against HeLa and HCT-8 cells, and compound **1** showed mild activity against the tested cell lines with IC_{50} values of 15.8 and 35.7 μM, respectively.

GRAPHICAL ABSTRACT



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Cytotoxic activity; steroidal glycoside; *vernonia cumingiana*

Introduction

Vernonia cumingiana Benth is a member of the genus *Vernonia* in the Compositae family and is widely distributed in the Guangxi, Yunnan, and Guangdong provinces of China.[1] As a traditional Chinese medicine, this plant has been used historically for the treatment of rheumatic arthritis, lumbocrural pain, fracture, and

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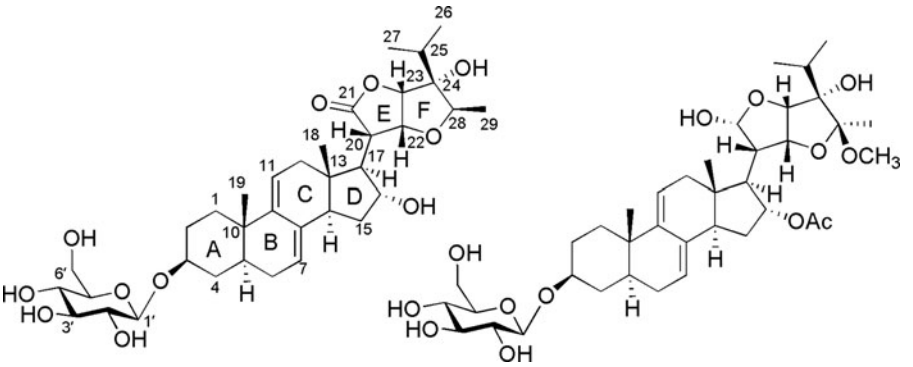

Figure 1. Structures of compounds 1–2.

malaria in southern China.[2] Previous phytochemical investigations on this genus have disclosed the presence of sesquiterpene lactones and steroidal glycosides that have extensive biological activities, such as cytotoxic, antibacterial, and antimalarial effects, etc.[3–10] Among these components, steroidal glycosides possessing the $\triangle^{7,9(11)}$ steroidal core with typical UV characteristics and representative ^{1}H and ^{13}C NMR data for the respective conjugated diene system were not common in reported natural products. For the purpose of finding new bioactive agents from medicinal plants, our team examined the methanol extract of *V. cumingiana* and isolated two new stigmastane type of steroidal glycosides, vernoniacums A and B (**1** and **2**, Fig. 1) with the $\triangle^{7,9(11)}$ steroidal core. In this paper, we reported the isolation and structure elucidation of the new compounds and their cytotoxic activities.

Results and discussions

Compound **1** was obtained as an amorphous solid that had $[\alpha]_D^{20} + 58.4$ ($c = 0.07$, MeOH). Its positive reaction with the Liebermann–Burchard and Molisch reagents indicated that it might be a saponin.[11–12] Its HR-FAB-MS in the positive-ion mode gave a quasi-molecular ion peak at *m/z* 671.3471 $[M + Na]^+$ (calcd. 671.3407 for $C_{35}H_{52}O_{11}Na$). In conjunction with the analysis of ^{1}H and ^{13}C NMR (Table 1) spectra, the formula of compound **1** was deduced as $C_{35}H_{52}O_{11}$. Fragment ion at *m/z* 509.27 indicated the loss of a hexose moiety. UV spectrum displayed absorptions at 234, 243, and 250 nm, as reported for the $\triangle^{7,9(11)}$ stigmastane type of glycoside.[13] IR spectrum indicated the presence of a carbonyl group (1,764 cm^{-1}) and a double bond (1,641 cm^{-1}). The ^{1}H NMR spectrum (Table 1) of **1** showed the angular signals at δ_H 0.89 (s, H_3-18) and 1.22 (3H, s, H-19), two olefinic protons signals at δ_H 5.43 (1H, t, $J = 1.8$ Hz, H-7) and 5.59 (1H, t, $J = 2.4$ Hz, H-11), and the distinctive H-3 proton signal at δ_H 3.95 (m), as well as a set of proton signals for the sugar moiety with its anomeric proton at δ_H 5.01 (1H, d, $J = 8.4$ Hz, H-1′). The ^{13}C NMR spectrum (Table 1) displayed 35 carbon signals including six for a sugar moiety and 29 for the aglycone unit, which exhibited a conjugated diene consisting of two trisubstituted carbon signals at δ_C 120.4 (C-7) and 119.8 (C-11), two

Table 1. NMR data (600 MHz, in pyridine-d_5) for compounds **1** and **2**.

	1		**2**	
Position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	35.0, CH_2	1.21, m1.91, m	35.3, CH_2	1.21, m1.80, m
2	30.3, CH_2	1.81, m2.12, m	30.5, CH_2	1.81, m2.11, m
3	77.1, CH	3.95, m	77.4, CH	3.96, m
4	34.5, CH_2	1.41, m2.00, m	34.8, CH_2	1.38, m2.04, m
5	39.3, CH	1.31, m	39.5, CH	1.35, m
6	30.2, CH_2	1.82, m2.10, m	30.5, CH_2	1.80, m2.12, m
7	120.4, CH	5.43, t (1.8)	122.1, CH	5.34, t (1.8)
8	136.4, C		135.4, C	
9	144.1, C		144.3, C	
10	36.2, C		36.5, C	
11	119.8, CH	5.59, t (2.4)	119.1, CH	5.47, t (2.4)
12	42.3, CH_2	2.29, dd (14.4, 2.4)3.43, d (14.4)	42.4, CH_2	2.30, dd (15.6, 2.4)2.51, d (15.6)
13	42.6, C		43.5, C	
14	50.4, CH	2.31, m	49.3, CH	2.62, m
15	37.1, CH_2	1.90, m2.55, m	35.2, CH_2	1.82, m2.54, m
16	72.1, CH	4.94, m	78.8, CH	5.57, m
17	45.2, CH	3.61, dd (8.4, 6.6)	48.6, CH	2.82, dd (9.6, 7.8)
18	13.6, CH_3	1.22, s	14.7, CH_3	0.63, s
19	19.6, CH_3	0.89, s	19.8, CH_3	0.81, s
20	52.8, CH	2.55, m	49.4, CH	2.24, m
21	178.2, C		99.4, CH	5.86, d (4.8)
22	81.1, CH	5.78, dd (4.8, 3.6)	80.4, CH	4.47, dd (4.8, 3.6)
23	86.8, CH	4.88, d (4.8)	92.1, CH	4.80, d (4.2)
24	85.1, C		82.3, C	
25	28.7, CH	2.32, m	32.8, CH	2.32, m
26	17.0, CH_3	1.07, d (7.2)	17.7, CH_3	1.28, d (7.2)
27	18.0, CH_3	1.28, d (7.2)	18.9, CH_3	1.20, d (7.2)
28	85.0, CH	4.52, q (7.2)	113.1, C	
29	15.9, CH_3	1.35, d (7.2)	17.9, CH_3	1.63, s
1′	102.3, CH	5.01, d (7.8)	102.7, CH	5.02, d (7.8)
2′	75.7, CH	4.04, m	75.7, CH	4.04, m
3′	78.9, CH	4.28, m	79.0, CH	4.29, m
4′	72.1, CH	4.26, m	72.1, CH	4.25, m
5′	78.5, CH	3.96, m	78.9, CH	3.99, m
6′	63.3, CH_2	4.42, m4.57, m	63.3, CH_2	4.38, m4.58, m
16-O$\underline{C}$OCH_3			170.9, C	
16-OCO$\underline{CH_3}$			22.2, CH_3	2.21, s
28-OCH_3			51.6, CH_3	3.31, s

tetrasubstituted ones at δ_C 136.4 (C-8) and 144.1 (C-19), and two angular carbons at δ_C 19.6 (C-18) and 13.6 (C-19). All of the proton signals were assigned to the corresponding carbons through direct ^1H and ^{13}C correlations in the HMQC spectrum. The above-mentioned data proved that compound **1** was a $\triangle^{7,9(11)}$ stigmastane type of glycoside.[14–15] Excluding the seven degrees of unsaturation due to the $\triangle^{7,9(11)}$ stigmastane type of steroidal skeleton and the sugar unit, three degrees of unsaturation remained for the $C_{10}H_{15}O_4$ side chain which included three methyl, five methine, and two quaternary carbons including an ester carbonyl group (δ_C 178.2). For the side chain, the Heteronuclear Multiple Bond Correlation (HMBC) correlations (Fig. 2) between δ_H 2.55 (H-20) and C-21 (δ_C 178.2) or C-22 (δ_C 81.1), between δ_H 5.78 (H-22) and C-20 (δ_C 52.8) or C-23 (δ_C 86.8), between δ_H 4.88 (H-23) and C-21 (δ_C 178.2) or C-24 (δ_C 85.1), and between δ_H 4.52 (H-28) and C-22 (δ_C 81.1), C-23 (δ_C 86.8) or C-24 (δ_C 85.1) indicated the presence of butyrolactone and furan rings, which were linked by C-22 and C-23. This partial structure was also deduced

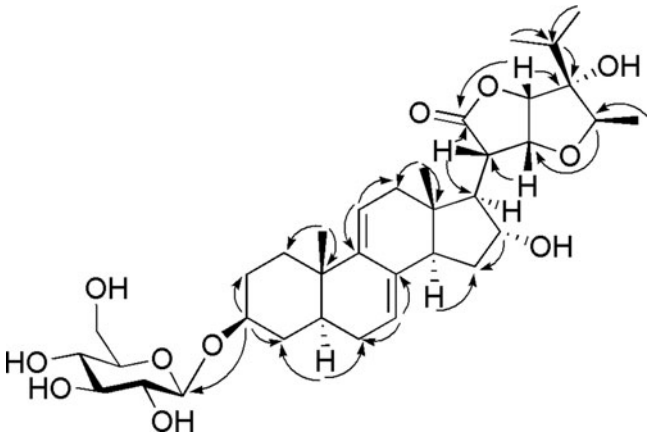

Figure 2. Key HMBC correlations of compound 1.

by ^{1}H-^{1}H COSY spectrum. Meanwhile, the proton signals at δ_H 1.07 (3H, d, J = 7.2 Hz, H_3-26), 1.28 (3H, d, J = 7.2 Hz, H_3-27), 1.35 (3H, d, J = 7.8 Hz, H_3-29), and 2.32 (1H, m, H-25), together with three methyl resonances at δ_C 17.0 (C-26), 18.0 (C-27), and 15.9 (C-29), and a methenyl carbon signal at δ_C 28.7 (C-25) revealed the presence of an isopropyl moiety and a methyl group. These two groups were determined to be attached to C-24 and C-28, respectively, on the basis of HMBC correlations between δ_H 2.32 (H-25) to C-24 (δ_C 85.1) and δ_H 1.35 (H-29) to C-28 (δ_C 85.0). The sugar moiety was located at C-3 on the basis of the correlation between the proton signal at δ_H 3.95 (1H, m, H-3) and the anomeric carbon at δ_C 102.3. The type and absolute configuration of the sugars was identified as D-glucose on the basis of comparison with authentic monosaccharide ($CHCl_3$:MeOH:H_2O = 3:2:0.2, visualized with ethanol-5% H_2SO_4 spraying) by Thin Layer Chromatography (TLC) method, followed by gas chromatography. The stereochemistry of the different ring junctions and substituents was determined via a 2D Nuclear Overhauser Effect Spectroscopy (NOESY) experiment. The cross-peaks observed between H-5/H-1α, H_3-19/H-1β, H-14/H-15α, H_3-18/H-12β, H-20/H-22, H-22/H-23 established the *trans* fusion of the A/B and C/D rings junction, and the *cis* fusion of the E/F ring junction. Additionally, the NOE correlations between H-3 and H-5 and between H-16 and H_3-18 indicated the β-orientation of sugar moiety at C-3 and the α-orientation of the hydroxyl group at C-16. The large coupling constant (J = 7.8 Hz) between H-1′ and H-2′ also supported the beta-linkage of the sugar moiety. Furthermore, the association of H-22 with H_3-29 and H-23 with H-25 suggested that the methyl group at C-28 was β-oriented and the hydroxyl group at C-24 was α-oriented. Therefore, the structure of compound **1** was established as shown in Figure 1 and named as vernoniacum A.

Compound **2** was isolated as a white amorphous powder which had $[\alpha]_D^{20}$ + 37.9 (c = 0.08, MeOH). Its molecular formula was calculated as $C_{38}H_{58}O_{13}$ based on the analysis of its HR-FAB-MS data (*m/z*: 745.3813 $[M + Na]^+$, calcd. 745.3775 for $C_{38}H_{58}O_{13}Na$). Its ^{1}H NMR spectrum (Table 1) displayed two methyl signals at δ_H 0.63 (3H, s, H_3-18) and 0.81 (3H, s, H_3-19), two olefinic protons signals at δ_H 5.34

(1H, t, $J = 1.8$ Hz, H-7) and 5.47 (1H, t, $J = 2.4$ Hz, H-11), and one anomeric proton at δ_H 5.02 (1H, d, $J = 7.8$ Hz, Glu-1), corresponding to the $\triangle^{7,9(11)}$ stigmastane type of glycoside. Its ^{13}C NMR (Table 1) spectrum displayed 38 resonance signals due to one sugar moiety, five methyl groups (δ_C 14.7, 19.8, 17.7, 18.9, and 17.9), one acetyl group (δ_C 22.2, 170.9), one methoxy group (δ_C 51.6), two double bonds (δ_C 122.1, 135.4, 144.3, 119.1), and other 20 carbons. The NMR data for this compound were similar to that of **1**, except for the downfield-shifted C-16 (δ_C 78.8) and C-28 (δ_C 113.1) and the upfield-shifted C-21 (δ_C 99.4) for **2**, in contrast to the C-16 (δ_C 72.1), C-28 (δ_C 85.0), and C-21 (δ_C 178.2) for **1**. In the HMBC spectrum, the correlations between methine H-16 (δ_H 5.57) and δ_C 170.9 and between methoxyl group δ_H 3.31 and C-28 (δ_C 113.1) or C-29 (δ_C 17.9) suggested the presence of acetyl group at C-16 and methoxy and methyl groups at C-28 for **2**. Additionally, the HMBC correlations between C-21 (δ_C 99.4) and H-20 or H-22 displayed that the ester carbonyl at C-21 in compound **1** was reduced to hydroxyl group in compound **2**. The remaining correlations observed in the NOESY spectrum of **2** were in same as that of **1**, indicating that the *trans* fusion of the A/B and C/D rings and *cis* fusion of the E/F ring junctions. The NOE enhancements from H_3-29 to H-17 indicated that the methyl group at C-28 was α-oriented and the methoxyl group at C-28 was β-oriented. Accordingly, the structure of **2** was assigned as shown in Figure 1 and named as vernoniacum B.

The isolated compounds **1** and **2** were tested for cytotoxic activity against HeLa (cervical cancer) and HCT-8 (colon cancer) human cancer cell lines using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method with adriamycin as a positive control. The result showed that compound **2** was inactive against the two cell lines, while compound **1** showed mild cytotoxic activities towards Hela and HCT-8 cells with IC_{50} values of 15.8 and 35.7 μM, respectively, as compared to the IC_{50} value of adriamycin (0.86, 1.24 μM).

Conclusions

Two new stigmastane type of steroidal glycosides, vernoniacums A and B (**1** and **2**) with the $\triangle^{7,9(11)}$ steroidal core were isolated from the roots of *V. cumingiana*. Their structures were elucidated by extensive spectroscopic analysis. Compound **1** showed mild cytotoxic activities towards Hela and HCT-8 cells with IC_{50} values of 15.8 and 35.7 μM, respectively.

Experimental section

General experimental procedures

Optical rotation data were obtained using a Perkin-Elmer 341 digital polarimeter. UV and IR spectra were recorded on Shimadzu UV2550 and FTIR-8400S spectrometer, respectively. NMR spectra were obtained with a Bruker AV III 600 NMR spectrometer (chemical shift values are presented as δ values with TMS as the internal standard and the temperature is conducted at 300.0 K). HR-ESI-MS spectra were

performed on a LTQ-Obitrap XL spectrometer. C_{18} reversed-phase silica gel (40–63 μm, Merk, Darmstadt, Germany), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), MCI gel (CHP 20P, 75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan) and silica gel (100–200 and 300–400 mesh, Qingdao Marine Chemical plant, Qingdao, People's Republic of China) were used for column chromatography. And precoated silica gel GF_{254} plates (Zhi Fu Huang Wu Pilot Plant of Silica Gel Development, Yantai, People's Republic of China) were used for TLC. All solvents used were of analytical grade (Beijing Chemical Works).

Plant material

The roots of *V. cumingiana* were collected in September 2014 from Nanning city, Guangxi province, China, and identified by Prof. Jing-Quan Yuan, Department of Pharmaceutical Chemistry, Guangxi Botanical Garden of Medicinal Plant. A voucher specimen (NO. 140905) was deposited at the Guangxi Botanical Garden of Medicinal Plant.

Extraction and isolation

The roots of *V. cumingiana* (4.2 kg) were powdered and extracted three times with methanol. Removal of the methanol under reduced pressure yielded a methanol extract (523 g). The residue was dissolved in water and extracted with petroleum ether (3×1,000 mL), chloroform (3×1,000 mL), ethyl acetate (3×1,000 mL) and *n*-butanol (3×1,000 mL), respectively. The *n*-butanol-soluble fraction (68 g) was subjected to silica gel column chromatography using a $CHCl_3$-MeOH gradient (from 1:0 to 0:1) as eluent, to yield eleven fractions (Fr.VC1–VC5). The Fr. VC2 (8.6 g) was separated to ODS MPLC eluting with MeOH-H_2O (30:70; 50:50; 70:30; 80:20; 90:10; 100:0, v/v) to obtain six sub-fractions, VI_1–VI_6. Sub-fraction VI_4 was prepared by HPLC using MeOH-H_2O (60:40, v/v) on YMC-Pack ODS-A column to give compound **1** (6.7 mg, $t_R = 24.6$ min). Sub-fraction VI_5 was prepared by HPLC using MeOH-H_2O (58:42, v/v) on YMC-Pack ODS-A column to give compound **2** (7.9 mg, $t_R = 29.7$ min).

Vernoniacum A (1)

Amorphous solid; $[\alpha]_D^{20} + 58.4$ ($c = 0.07$, MeOH); UV (MeOH) λ_{max} (log ε) 234 (4.16), 243 (4.20), 250 (4.04) nm; IR (KBr) cm^{-1} 3,428, 1,764, 1,641; ^1H and ^{13}C NMR (Pyridine-d_5): see (Table 1); HR-FAB-MS *m/z* 671.3471 $[M + Na]^+$ (calcd. $C_{35}H_{52}O_{11}Na$ for 671.3407).

Vernoniacum B (2)

Amorphous solid; $[\alpha]_D^{20} + 37.9$ ($c = 0.08$, MeOH); UV (MeOH) λ_{max} (log ε) 234 (3.87), 243 (3.91), 250 (3.73); IR (KBr) cm^{-1} 3,457, 2,945, 2,874, 1,698, 1,656; ^1H

and ^{13}C NMR (Pyridine-d_5): see (Table 1); HR-FAB-MS *m/z* 745.3813 $[M + Na]^+$ (calcd. $C_{38}H_{58}O_{13}Na$ for 745.3775).

Acid hydrolysis of 1–2

Each compound (3.0 mg) was heated in 3 mol/L CF_3COOH (4 mL) for 3 h in a water bath. Each mixture was then extracted with EtOAc. The aqueous layer was evaporated to dryness with ethanol *in vacuo* at 50°C until neutral. The residues were determined in comparison with D-Glucose using TLC ($CHCl_3$: MeOH: H_2O = 3:2:0.2, visualization with ethanol-5% H_2SO_4 spraying). Furthermore, the absolute configurations of the sugars were determined by gas chromatography according to a method previously described.[13–14] By this method, L-cysteine methyl ester hydrochloride (0.06 mol/L) and hexamethyldisilazane-trimethylchlorosilane (HMDS-TMCS, 3:1) were added to the aqueous residue for derivatization. The solution was then centrifuged and the precipitate was removed. After these processes, n-hexane was used to extract derivate and analyzed by GC. D-Glucose (t_R = 25.3 min) was detected by comparing with authentic monosaccharide.

Cytotoxicity bioassays

Antiproliferative activity of compounds **1**–**2** were assessed by MTT method using HeLa and HCT-8 human cancer cell lines. Each cell was sent on to 96-well microtiter plates at a density of 6×10^4 cells/mL per well. Cells were preincubated for 24 h at 37°C in 5% CO_2 for 24 h. Then five different concentrations of each compound dissolved in dimethyl sulfoxide (DMSO) were added to each well. Each concentration was tested in triplicate. After 48 h, 10 μL of MTT (4 mg/mL) was added to each well and incubated for another 4 h. Then removed the liquid in the well and added DMSO (200 μL) to each well. The absorbance was recorded on a microplate reader at a wavelength of 570 nm.

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