Phytochemistry Letters 5 (2012) 596–599

Contents lists available at SciVerse ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Stigmastane derivatives from the roots of *Vernonia guineensis* and their antimicrobial activity

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ARTICLE INFO

Article history:
Received 12 February 2012
Received in revised form 7 June 2012
Accepted 8 June 2012
Available online 21 June 2012

Keywords:
Vernonia guineensis
Asteraceae
Stigmastane derivatives
Vernoguinoside A
Antimicrobial activity

ABSTRACT

Chemical investigation of the roots of *Vernonia guineensis* (Asteraceae) afforded a new stigmastane derivative, vernoguinoside A (**1**) and the known vernoguinoside (**2**), stigmasterol 3-*O*-β-D-glucoside (**3**) and sitosterol 3-*O*-β-D-glucoside (**4**). Their structures were elucidated by spectroscopic analysis. Antimicrobial activities of **1**–**3** and CH_2Cl_2–MeOH (1:1) extract were evaluated against three bacteria species (*Salmonella typhi*, *Staphylococcus aureus* and *Shigella flexneri*) and three yeasts species (*Candida albicans*, *Candida parapsilosis* and *Cryptococcus neoformans*). Compounds **1** and **2** exhibited both antibacterial and antifungal activities that varied between the microbial species (MIC = 7.81–125 μg/mL) while *S. flexneri* and *C. albicans* were sensitive to all the tested compounds.

1. Introduction

Chemical studies of the *Vernonia* species have shown this genus to be a rich source of stigmastane glycosides, flavonoids, sesquiterpenoids, triterpenoids, alkaloids and cardiac glycosides (Cioffi et al., 2004; Igile et al., 1995; Jisaka et al., 1992, 1993; Ponglux et al., 1992; Sanogo et al., 1998). *Vernonia guineensis* Benth. (Asteraceae), is a small tree growing in the savannah region of West Africa. The plant is used in Cameroon as anthelmintic, aphrodisiac, antidote to poison, to treat malaria, jaundice and venereal diseases (Tchinda et al., 2002). We have previously reported the isolation and characterization of several stigmastane derivatives and isovaleryl sucrose esters (Tchinda et al., 2002, 2003) from this plant. In continuation of our investigation of this species for new antimicrobial agents, we herein report on the isolation and structure elucidation of a new stigmastane derivative, vernoguinoside A (**1**). Furthermore, some other antimicrobial substances were isolated.

2. Results and discussion

The CH_2Cl_2–MeOH (1:1) extract of the roots of *V. guineensis* was submitted to silica gel column chromatography and subsequently gel permeation through Sephadex LH-20 to afford vernoguinoside A (**1**), along with the known vernoguinoside (**2**), stigmasterol 3-*O*-β-D-glucoside (**3**), and sitosterol 3-*O*-β-D-glucoside (**4**). Their structures (Fig. 1) were elucidated by extensive 1D and 2D NMR spectroscopic analysis, FABMS and by comparison of the data with those reported in the literature (Tchinda et al., 2002; Kang et al., 2003; Wang et al., 2009).

Compound **1** was obtained as yellow powder from acetone, $[\alpha]_D^{20}$ −1.5 (c. 0.731, MeOH). The molecular formula $C_{35}H_{52}O_{12}$ was deduced from the HRFABMS which displayed a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 687.3354 (Calcd for $C_{35}H_{52}O_{12}Na$: 687.3356), in conjunction with the NMR data. The IR spectrum showed absorptions at 3404–3454 (OH groups), 1772 (lactone) and 1454 cm^{-1} (C=C–C=C). The ^1H and ^{13}C NMR data of **1** (Table 1) were typical of a steroid glycoside (Igile et al., 1995; Jisaka et al., 1993). The ^{13}C NMR spectrum showed 35 signals, of which 29 were assigned to the stigmastane nucleus and six were in the glycosidic region corresponding to a hexose unit. These signals were sorted out by HMQC and DEPT 135° spectra as five methyl, nine

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http://dx.doi.org/10.1016/j.phytol.2012.06.002

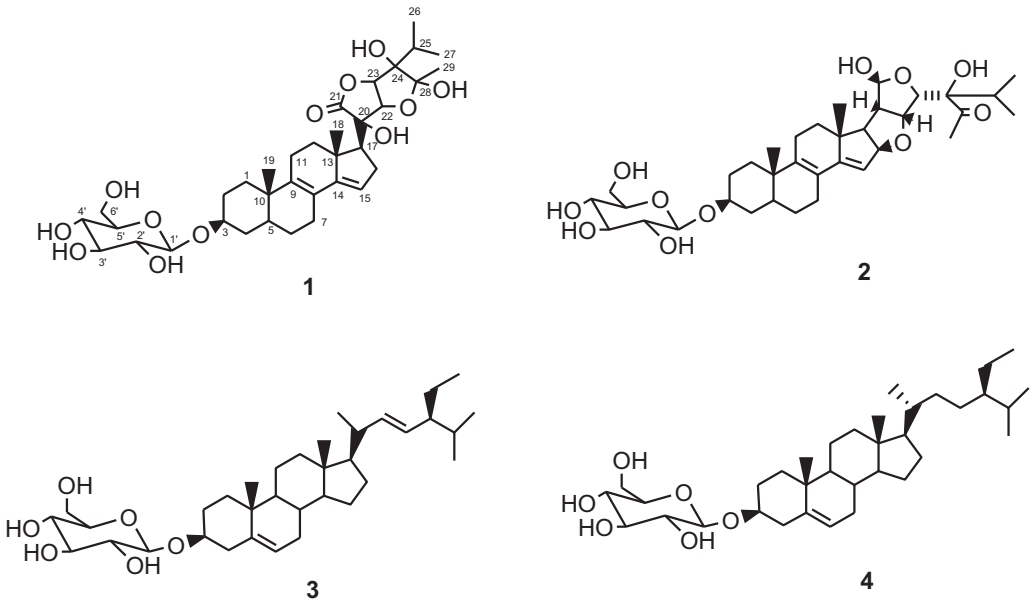

Fig. 1. Chemical structures of compounds **1**–**4**.

methylene, twelve methine and nine quaternary carbon atoms. The steroidal nature of the aglycone moiety of **1** was indicated in the ^{1}H NMR spectrum by the angular methyl singlets at δ_H 0.98 (Me-18) and 1.16 (Me-19) and the characteristic oxymethine multiplet at δ_H 3.66 (H-3) (Cioffi et al., 2004; Tchinda et al., 2003). This spectrum also showed signals of a quaternary methyl group at δ_H 1.37 (Me-29), an olefinic proton at δ_H 5.35 (brm, H-15) and two additional oxymethine protons at δ_H 4.72 (J = 4.9 Hz, H-22) and 4.82 (J = 4.9 Hz, H-23). The presence of an isopropyl unit was indicated in the spectrum by the signals at δ_H 0.95 (d, J = 6.7 Hz, Me-27), 1.09 (d, J = 6.7 Hz, Me-26) and 2.13 (m, H-25). In the ^{13}C NMR spectrum, signals of a conjugated diene system were observed at δ_C 117.3 (C-15), 122.9 (C-8), 140.9 (C-9) and 149.6 (C-14). This spectrum also exhibited signals assignable to the side chain of the steroidal nucleus, including the carbonyl of a lactone group at δ_C 176.6 (C-21), the isopropyl unit at δ_C 14.9 (C-27), 17.8 (C-26) and 28.2 (C-25), two hydroxylated quaternary carbons at δ_C 78.6 (C-20) and 81.2 (C-24), and a quaternary carbon of a hemiacetal, characteristic of most *Vernonia* stigmastane derivatives at δ_C 107.8 (C-28) (Igile et al., 1995; Jisaka et al., 1993). The comparison of the chemical shifts of the sugar carbon signals with the reported data confirmed the presence of glucose (Jisaka et al., 1993; Tchinda et al., 2003). The ^{1}H NMR spectrum showed the characteristic signal of an anomeric proton as a doublet at δ_H 4.44 (H-1′); the coupling constant J = 7.4 Hz indicated the β-configuration of the sugar residue (Ahmed et al., 1992; Jisaka et al., 1993). The anomeric carbon signal of the glucose moiety appeared at δ_C 101.1 (C-1′) and the downfield chemical shift value of C-3 of the aglycone at δ_C 78.5 showed the linkage of sugar unit at this carbon (Ahmed et al., 1992). This was further confirmed in the HMBC spectrum (Table 1) by the correlation between H-3 and C-1′. HMBC correlations between the olefinic proton and carbons C-8, C-13, C-14 and C-17 were in agreement with the position of the diene in the steroid nucleus (Tchinda et al., 2003). Further HMBC correlations between the proton at δ_H 2.44 (H-17) and carbons at δ_C 78.6 (C-20) and 82.6 (C-22) enabled us to attach the side chain to the steroid nucleus at C-17. Acid hydrolysis of **1** yielded a free sugar that was identified as D-glucose by measurement of the optical rotation ($[\alpha]_D^{20}$ +41.0 (c. 0.044, H_2O)). The angular Me-18 and Me-19 as well as the side chain at C-17 were β-oriented as reported for

Table 1
^{1}H and ^{13}C NMR data for compound **1** (600 and 150 MHz, in $CDCl_3$), δ in ppm, J in Hz.

Position	δ_C	δ_H	HMBC (H → C)
1	34.6	1.79, m	C-3
		1.37, m	C-2, C-3
2	29.7	1.94, m	
		1.61, m	C-3
3	78.5	3.66, m	C-1′, C-2
4	35.4	1.87, m	C-3
		1.22, m	C-19
5	41.0	1.48, m	
6	25.5	1.37, m	
7	26.7	2.07, m	C-9
		2.36, m	C-8, C-14
8	122.9	–	
9	140.9	–	
10	36.8	–	
11	22.0	2.22, m	
12	31.6	2.96, m	C-14, C-15, C-17
		2.22, m	C-13, C-9, C-14
13	45.6	–	
14	149.6	–	
15	117.3	5.35, brm	C-8, C-12, C-13, C-14, C-17
16	37.4	2.36, m	C-8, C-14
		2.30, m	
17	53.4	2.44, m	C-12, C-13, C-16, C-18, C-20, C-22
18	18.3	0.98, s	C-13
19	17.6	1.16, s	C-10
20	78.6	–	
21	176.6	–	
22	82.6	4.72, d (4.9)	C-21, C-23
23	86.2	4.82, d (4.9)	C-22, C-28
24	81.2	–	
25	28.2	2.13, m	
26	17.8	1.09, d (6.7)	
27	14.9	0.95, d (6.7)	C-25
28	107.8	–	
29	24.7	1.37, s	C-28
1′	101.1	4.44, d (7.4)	C-2′, C-3′
2′	75.9	3.30, m	C-3′, C-4′
3′	76.5	3.42, m	C-2′, C-4′, C-6′
4′	70.3	3.45, m	C-2′, C-3′, C-5′, C-6′
5′	73.7	3.23, m	C-1′, C-2′
6′	62.0	3.85, dd (3.2, 12.0)	C-4′, C-5′
		3.76, dd (4.2, 12.0)	

Table 2
Inhibition parameters (MIC, MBC and MFC) of the CH_2Cl_2–MeOH (1:1) extract of *V. guineensis* and its constituents (μg/mL).

Microorganisms	Parameters	Test substances				
		Extract	**1**	**2**	**3**	Reference antibiotics[a]
Bacteria						
Salmonella typhi	MIC	195.31	–	–	–	7.81
	MBC	390.60	–	–	–	7.81
	MBC/MIC	2	–	–	–	1
Staphylococcus aureus	MIC	781.25	62.50	125	–	3.90
	MBC	781.25	62.50	125	–	3.90
	MBC/MIC	1	1	2	–	1
Shigella flexneri	MIC	390.60	62.50	62.50	62.50	3.90
	MBC	390.60	62.50	62.50	62.50	3.90
	MBC/MIC	1	1	1	1	1
Yeasts						
Candida albicans	MIC	781.25	15.62	31.25	31.25	1.95
	MFC	1562.50	15.62	31.25	31.25	1.95
	MFC/MIC	2	1	1	1	1
Candida parapsilosis	MIC	1562.50	7.81	–	125	15.62
	MFC	1562.50	7.81	–	125	15.62
	MFC/MIC	1	1	–	1	1
Cryptococcus neoformans	MIC	1562.50	7.81	–	–	1.95
	MFC	1562.50	7.81	–	–	1.95
	MFC/MIC	1	1	–	–	1

–, not active at concentration up to 125 μg/mL; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration.
[a] Ciprofloxacin for bacteria and nystatin for yeasts.

stigmastane steroids (Hill et al., 1991). The stereochemistry on the side chain was not determined because of the absence of the effective cross peaks on the NOESY spectrum, the reason being the existence of equilibrium on the hemiacetal moiety. An attempt to convert this hemiacetal moiety into the methyl acetal in acidic conditions (2%HCl–MeOH) was not successful.

Thus, compound **1** was characterized as a new stigmastane glucoside named vernoguinoside A.

In addition to the above new stigmastane steroid, the known vernoguinoside (**2**), stigmasterol 3-*O*-β-D-glucoside (**3**) and sitosterol 3-*O*-β-D-glucoside (**4**) were isolated. Their structures were established by comparison of NMR data with those reported in the literature (Tchinda et al., 2002; Kang et al., 2003; Wang et al., 2009).

The antimicrobial activities of compounds **1**–**3** and that of the crude CH_2Cl_2–MeOH (1:1) extract were evaluated (Table 2). Compounds **1** and **2** exhibited both antibacterial and antifungal activities that varied between the microbial species (MIC = 7.81–125 μg/mL). Compound **1** with the same basic skeleton as compound **2** was more active. This could be due to the modifications on the side chain such as the nature of the carbonyl groups or the position of the hydroxyl groups. *Shigella flexneri* and *Candida albicans* were sensitive to all the tested compounds. It was also found that MIC values obtained were generally equal to the corresponding MBC or MFC values (Table 2), suggesting that a cidal effect of the crude extract and the isolated compounds could be expected on most of the tested microorganisms (Mims et al., 1993). From this study, we can conclude that the CH_2Cl_2–MeOH (1:1) extract of the roots bark of *V. guineensis* and its new stigmastane derivative, vernoguinoside A (**1**) possess interesting antibacterial and antifungal properties.

3. Experimental

3.1. General experimental procedures

IR spectra were recorded on a Shimadzu FTIR-8400S spectrometer. HRFABMS and FABMS were measured on Jeol JMS-700 spectrometer. Specific rotations were measured on JASCO P-1030 Polarimeter. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ and pyridine-d_5 with a Jeol JNM ECA-600 and AL-400 spectrometers, with the residual solvent signals as internal reference. Column chromatography was carried out on silica gel 60 (70–230 mesh; Merck; Darmstadt, Germany) and Sephadex LH-20. Analytical TLC was performed on silica gel 60 F_{254} precoated plates (Merck; Darmstadt, Germany). Spots were visualized at 254 nm and 365 nm, and by spraying with 50% H_2SO_4 followed by heating at 100 °C.

3.2. Plant material

The roots of *V. guineensis* were collected in Bamenda, in the North West Region of Cameroon, in November 2008. Identification was done by Mr. Nana in the Cameroon National Herbarium, Yaounde (Cameroon) where a voucher specimen (No 11133 SRF/Cam) is deposited.

3.3. Extraction and isolation

The air-dried and powder roots of *V. guineensis* (5 kg) were extracted with CH_2Cl_2–MeOH (1:1, 3 × 15 L) at room temperature for 72 h to yield a dark brown extract (409 g) after evaporation under reduced pressure. 100 g of this extract were subjected to silica gel column chromatography and eluted with a gradient system of CH_2Cl_2–MeOH, to afford 46 fractions of 600 mL each. These fractions were combined on the basis of their TLC profiles into four major fractions A–D (A: 1–10; B: 11–18; C: 19–28; D: 29–46). Fraction A (7 g) contained mostly fats and mixture of phytosterols. Vernoguinoside (**2**, 28 mg), stigmasterol 3-*O*-β-D-glucoside (**3**, 110 mg) and sitosterol 3-*O*-β-D-glucoside (**4**, 30 mg) crystallized from fraction B (5 g). Fraction C (42 g) was chromatographed on a silica gel column, eluting with a mixture of AcOEt–MeOH–H_2O (95:5:2) to afford 23 fractions of 400 mL each. These fractions were combined on the basis of their TLC profiles into five major subfractions C_1–C_5 (C_1: 1–6; C_2: 7–9; C_3: 10–14; C_4: 15–20; C_5: 20–23). Subfraction C_2 was further purified by Column

chromatography on silica gel using the mixture AcOEt–MeOH–H_2O (90:2:1) followed by purification on a Sephadex LH-20 column using CH_2Cl_2–MeOH (1:1) as eluent to afford vernoguinoside A (**1**, 17 mg).

3.4. Vernoguinoside A (*1*)

Yellow powder; IR ν_{max} (KBr) 3454, 3404, 1772, 1454, 1375, 1170 cm^{-1}; $[\alpha]_D^{20}$ −1.5 (c. 0.731, MeOH); ^1H and ^{13}C NMR data: see Table 1; FABMS: *m*/*z* 687 ([M+Na]$^+$, 60), 664 (100), 617 (60), 560 (70); HRFABMS: *m*/*z* 687.3354 (Calcd for $C_{35}H_{52}O_{12}Na$: 687.3356).

3.5. Acid hydrolysis of *1*

Compound **1** (4.1 mg) was dissolved in THF (1 mL) and 3 M hydrochloric acid (1 mL). After being stirred for 12 h at 90 °C, the solution was evaporated. The residue was chromatographed over ODS (Cosmosil 140C18-OPN (Nacalai, Japan)) to obtain D-glucose (0.4 mg) ($[\alpha]_D^{20}$ +41.0 (c. 0.044, H_2O)).

3.6. Microorganisms

The microorganisms used in this study consisted of one Gram-positive (*Staphylococcus aureus* ATCC 25923) and one Gram-negative (*Salmonella typhi* ATCC 6539) bacteria; as well as two *Candida* species (*C. albicans* ATCC 2091 and *C. parapsilosis* ATCC 22019) all of which are reference strains obtained from the American Type Culture Collection. One strain of *Cryptococcus neoformans* IP 95026 and one clinical isolate of *S. flexneri* obtained from the Pasteur Institute (IP, Paris, France) were also included. The bacteria and yeast strains were grown at 35 °C and maintained on nutrient Agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) slants respectively.

3.7. Antimicrobial assay

The antimicrobial assays were performed using the broth micro dilution method as reported by Tamokou et al. (2009). The minimum inhibitory concentration (MIC) was the lowest concentration of the test substances that prevented visible growth of microorganisms. Minimum bactericidal concentrations (MBCs) or minimum fungicidal concentrations (MFCs) were determined by plating 5 μL from each negative well and from the positive growth control on Mueller Hinton Agar (for bacteria) and Sabouraud Dextrose Agar (for yeasts). MBCs or MFCs were defined as the lowest concentration yielding negative subcultures or only one colony. All experiments were performed in triplicate. Ciprofloxacin and nystatin at the concentration ranging from 0.97 to 62.50 μg/mL serve as positive controls for antibacterial and antifungal activities respectively.

Acknowledgements

Financial support from the University of Dschang is gratefully acknowledged. H.K.W. is grateful to The Matsumae International Foundation (MIF) for a four-month fellowship at the Graduate School of Pharmaceutical Sciences, Tohoku University, Japan.

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