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VERNONIOSIDES D AND E, TWO NOVEL SAPONINS FROM *VERNONIA AMYGDALINA*

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ABSTRACT.—Two new stigmastane-type steroid glycosides, vernoniosides D [**1**] and E [**2**], have been isolated from the leaves of *Vernonia amygdalina*, along with the known vernonioside A_3. Vernonioside D [**1**] was the most abundant glycoside obtained. The chemical structures of **1** and **2** were elucidated using a combination of spectroscopic techniques.

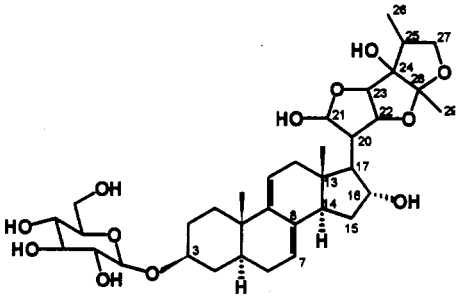
Vernonia amygdalina Del. (Compositae) is a small tree that grows throughout tropical Africa. The leaves are used as a vegetable and spice in West Africa, particularly Nigeria. The leaves are also extracted with alcohol, or with H_2O (hot or cold), as medicinals that have extensive therapeutic applications including antimalarial, antihelminthic, and antianorexic activities, as well as in gynecological applications (1). In southern Africa, an apparently sick chimpanzee is reported to have chewed and extracted juice from tissues of this plant to regain its health (2).

Several secondary metabolites have been isolated from the leaves of *V. amygdalina*, including antimalarial and insect antifeedant sesquiterpene lactones (1,3,4), bitter and non-bitter stigmastane-type glycosides (5–10), and flavones (11).

In a continuation of our investigation into the bioactive constituents of *Vernonia amygdalina*, we describe herein the isolation and chemical characterization of two new stigmastane-type steroidal glycosides, as well as vernonioside A_3 that was reported previously by Jisaka *et al.* (7).

The three compounds isolated from the MeOH extract of *V. amygdalina*, comprised the glycosides, **1**, **2**, and vernonioside A_3. Glucose was the only sugar identified by tlc after enzymatic hydrolysis of each glycoside. All three substances were shown to contain a hexose unit by fabms, which was identified as β-D-glucopyranoside by ^{1}H- and ^{13}C-nmr data (Table 1).

Nmr data led to the identification of the aglycone moiety of these compounds as a C-29 stigmastane-type sterol. These



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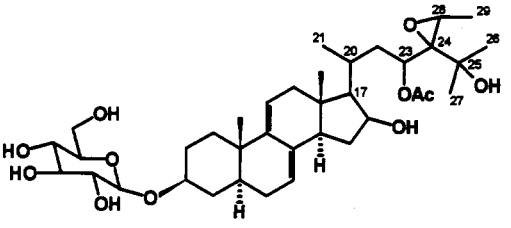

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TABLE 1. ^{1}H- and ^{13}C-Nmr Data for Compounds **1** and **2**.

Position	Compound		
	1[a]	**1**	**2**
	δ_H (J_{HH} in Hz)	δ_C	δ_C
1	α 1.05 β 1.45	35.00	30.97
2	α 1.95 β 1.60	30.55	30.55
3	α 3.75	78.41	78.86
4	α 2.00 β 1.51	35.94	35.91
5	1.30	40.13	39.90
6	α and β 1.88	30.98	30.98
7	5.44, br s	121.92	122.14
8	—	136.83	136.91
9	—	145.29	145.18
10	—	37.10	34.72
11	5.52 br d, J=6	119.00	119.22
12	α 1.42 β 2.33	40.70	40.46
13	—	44.51	43.61
14	2.53	49.55	50.06
15	1.80–2.20	36.00	37.07
16	4.25, br m	77.40	70.03
17	1.84	59.33	56.96
18	0.59, s	13.89	14.24
19	0.91, s	19.83	19.85
20	2.36, br m	53.56	33.00
21	5.31 d, J=6	104.96	12.98
22	4.90[c]	85.17	47.80
23	4.40[b]	86.01	80.88
24	—	88.28	71.00
25	2.12, br m	41.70	63.93
26	1.03, d, J=6	10.39	14.25
27	3.50, dd, J=8.9, 7 4.03, dd, J=8.5, 6.1	73.69	26.85
28	—	118.23	71.00
29	1.36, s	21.43	28.35
OCOMe	2.05		21.51 172.83
1′	4.40[b]	102.12	102.35
2′	3.22	75.13	75.13
3′	3.48	77.86	77.86
4′	3.40	71.67	71.67
5′	3.36	78.09	78.09
6′	3.87 3.70	62.80	62.80

[a]Assignments confirmed by COSY, HETCOR, and COLOC experiments.
[b]Overlapped signals.
[c]Under D_2O signal.

three glycosides also showed similar patterns in their uv spectra, having three absorption maxima at 234, 241, and 251 nm, indicating the presence of a chromophoric conjugated $\Delta^{7,9(11)}$ diene function in the aglycone (7,12). This chromophore has been shown to be a characteristic structural feature of stigmastane-

type saponins in *V. amygdalina*, and together with their particular side-chain, may have some biogenetic and chemotaxonomic significance.

The lsims of compound **1** showed clearly quasi-molecular ions at *m/z* 663 $[M-H]^-$ and 665 $[M+H]^+$, in the negative- and positive-ion modes, respectively. The eims spectral data of its aglycone, obtained by enzymatic hydrolysis, indicated a peak at *m/z* 488, considered to be a $[M-CH_3]^+$ ion, calcd for $C_{29}H_{42}O_7$ (*m/z* 502). These ms data, together with ^{13}C-nmr and DEPT ^{13}C-nmr analysis, showed the molecular formula of compound **1** to be $C_{35}H_{52}O_{12}$. Additionally, the eims spectrum of the aglycone showed a base peak at *m/z* 268, consistent with the composition $[M-\text{side-chain}-H_2O]^+$. This indicated that the steroid-cyclic system of compound **1** had a mol wt of 286 daltons $[C_{19}H_{26}O_2]$, identical to that of a series of vernoniosides reported previously (7). It was ascribable to a $\Delta^{7,9(11)}$ steroidal nucleus with two hydroxyl groups at C-3β and C-16α by ^{13}C-nmr spectroscopy, as the data for C-1 to C-19 were very similar to those reported for vernonioside A_2 (6). In fact, the chemical shifts of C-2 (30.55 ppm), C-3 (78.41 ppm), and C-4 (35.94 ppm) revealed the presence of a β-oriented glucosyl moiety at C-3 by comparison with the same signals of the aglycone. Chemical shifts of C-16 (77.40 ppm), C-15 (36.00 ppm), and C-17 (59.33 ppm), which were somewhat different from the analogous signals of vernonioside A_1, but close to those of vernonioside A_2 (7), suggested that the second hydroxyl group was at C-16 and was α-orientated in compound **1**, as in vernonioside A_2.

Having established this information, the presence of an unusually oxygenated side-chain with the composition $C_{10}H_{15}O_5$ was evident, whose structure was established by the extensive use of 1D and 2D nmr techniques. A combination of COSY and HETCOR experiments showed the following connectivities: C-23–C-22–C-20–C-17–C-16–C-15–C-14, and C-20–C-21. The presence of an unusual $HC(CH_2O)$-CH_3 group, instead of the terminal isopropyl group typical of other vernoniosides, was shown by the ^1H-nmr coupling of C-25 (CH) with both C-26 (Me) and C-27 (CH_2O-) in the COSY spectrum.

The chemical shifts of C-21 (104.96 ppm) and H-21 (5.31 ppm) suggested that C-21 is involved in a hemiacetal linkage with C-23. Nmr data of C-20 (^1H, δ 2.3 t, ^{13}C, δ 53.56), C-22 (^1H, δ 4.90, ^{13}C, δ 84.85) and C-23 (^1H, δ 4.40, ^{13}C, δ 86.01) demonstrated that they constituted one angular methine and two hydroxymethines, respectively. A quaternary ^{13}C-nmr signal at 118.23 ppm together with a second Me signal (^{13}C, 21.43 ppm, ^1H, 1.36, s) proved the formation of a ketal at C-28 and the complete cyclization of the side-chain of **1**. Therefore, the remaining quaternary C signal at 88.28 ppm was assigned to the hydroxylated C-24 position.

To confirm the proposed structure of the side-chain of compound **1**, a COLOC nmr experiment was conducted, in which significant cross-peaks were seen between the ketal C-28 (118.23 ppm) and the chemical shifts of Me-29, H_2-27, and H-23; between the hemiacetal C-21 (104.96 ppm) and H-22; between C-17 (59.33 ppm) and Me-18; between the quaternary hydroxylated C-24 (88.28 ppm) and H-20, as well as between the carbinyl carbon C-22 (85.17 ppm) and H-23. The relative stereochemistry at the asymmetric carbon of the side-chain was ascertained by evaluating nOe effects. A nOe effect was recorded for both H-25 and H-21 by irradiation of Me-29. NOes were also recorded for H-22 by irradiating Me-26, as well as for both Me-19 and H-20 by irradiation of Me-18. Finally, these observations confirmed the correctness of the structure proposed for **1**. The trivial name vernonioside D is proposed for **1**. Although the occurrence of stigmastane-type glycosides which form a hemiacetal at C-21 or a ketal at C-28 (i.e.,

vernoniosides B_2 and B_1) has been reported in *Vernonia* species (7,13), vernonioside D is the first example of a glycoside with complete cyclization of the side-chain.

Compound **2** in the negative lsims spectrum revealed a molecular ion at *m/z* 677, together with ions at *m/z* 633 and 515, corresponding to the loss of an acetyl function and glucose from the parent ion, respectively. Thus, the molecular formula, deduced also by ^{13}C- and DEPT ^{13}C-nmr data was $C_{37}H_{58}O_{11}$. The eims spectrum of the aglycone showed a molecular ion at *m/z* 472 and some additional ions (*m/z* 268 and 311), indicating a steroid system similar to that found in **1**. The ion at *m/z* 472 was interpreted as being a quasi-molecular peak resulting from the loss of an acetyl function from the aglycone molecule. The presence of an acetyl group was confirmed by signals at 21.51 and 172.83 ppm in the ^{13}C-nmr spectrum of **2**. The nmr spectrum also demonstrated (Table 1) that **2** possesses the same carbon skeletal structure of the sterol framework from C-1 to C-19 of compound **1**. A difference was in the β-orientation of the OH group at C-16. In fact, C-16 and C-17 resonated at higher field whereas C-15 resonated at lower field than in **1** (Table 1). These observations are in good agreement with the data reported for vernoniosides A_1 and A_2 (6) and with some other steroids (14). Consequently, the acetyl group was located in the side-chain.

The ^1H-nmr spectrum of **2** showed the presence of two tertiary methyls (1.45 and 1.46 ppm) and two secondary methyl signals (δ 1.67, d, J=5.5 Hz and 1.16, d, J=6.0 Hz) as well as a proton (δ 5.28) geminal to an $OCOCH_3$ group. The tertiary methyls linked to a carbinol (C-25) were assigned to C-26 and C-27 of a *tert*-hydroxypropyl group. A Me resonating at δ 1.67, which was coupled to a proton at δ 3.12, was placed at C-28 (63.93 ppm) forming an epoxide with C-24 (71.0 ppm), and the remaining Me at δ 1.16 was assigned to C-21 of an open side-chain (combination of ^1H-^1H COSY and ^1H-^{13}C HETCOR).

A series of selective decoupling experiments starting from the Me-21 signal suggested the partial sequence H_3-21–H-20–H_2-22–H-23. The acetylated hydroxyl group was confirmed to be at C-23 by the relevant chemical shift in the ^{13}C-nmr spectrum (CH, 80.28 ppm), as well as by the chemical shift, the form of the signal, and the coupling pattern of H-23 in the ^1H-nmr spectrum (1H, δ 5.28, t). From all of these data the structure of compound **2** was confirmed, for which the trivial name vernonioside E has been given.

Vernonioside A_3, on enzymatic hydrolysis, produced an aglycone showing an eims molecular ion at *m/z* 468 $[M]^+$, calcd for $C_{29}H_{40}O_5$. The lsims spectrum indicated the mol wt of the glycoside as 630 daltons. These data were similar to the results reported previously for vernonioside A_3 (6). This identity was further confirmed by ^1H- and ^{13}C-nmr data, which were in good agreement with those reported in the literature (6).

Vernonioside D [**1**] isolated in the present work was a major compound with a concentration of 0.45% (dry wt). As shown in our previous experiments described elsewhere (9), feeding mice with diets containing 25% *V. amygdalina* leaves, an alcoholic extract, a mixture of crude saponins, or vernonioside D for 14 days influenced animal performance. These treatments caused significant reduction in body weight gain and increased urine and fecal output. At necropsy, the liver weights and liver and plasma cholesterol levels were significantly reduced, and saponins were proved to be responsible for these effects. These experiments indicate that the chemistry and biological activity of *V. amygdalina* saponins should be more closely investigated and that their level of safe utilization should be definitively established.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Uv spectra were measured in MeOH using a Beckman DU-68 spectrophotometer. For nmr, Bruker WH-250 Spectroscopin and Bruker AMX-500 spectrometers equipped with a Bruker X-32 computer and UXNMR software package were used. Two-dimensional COSY nmr experiments were measured by employing the conventional pulse sequence. The COSY spectra were obtained using a data set (t1×t2) of 1024×1024 points for a spectral width of 1165 Hz (relaxation delay 1 sec). The data matrix was processed using an unshifted sine bell window function, following transformation to give a magnitude spectrum with symmetrization (digital resolution in both F_2 and F_1 dimensions 1.13 Hz/pt). The HETCOR nmr experiments were performed on a 512×1024 data matrix using CH coupling of 135 Hz and a relaxation delay of 1.5 sec. The data matrix was processed using a q sine window function. In the case of the COLOC experiment, delays were adjusted to an average CH coupling of 6 Hz; the spectral data were recorded in a 256×1024 data matrix. The nmr spectra were run in CD_3OD. Fabms were recorded using NBA as matrix, in both the negative- and positive-ion mode on a Finnigan-MAT 95 instrument. Eims were recorded with the same instrument operating at an ionization energy of 70 eV. Tlc was carried out on Si gel plates (Merck), using $CHCl_3$-CH_3OH-H_2O (65:14:1, S_1) for glycosides, and $CHCl_3$-CH_3OH (95:5, S_2) for aglycones; and on RP-18 using CH_3OH-H_2O (75:25, S_3) for both glycosides and aglycones. Spots were visualized by spraying the tlc plates with Liebermann-Burchard reagent (CH_3OH-$(CH_3CO)_2O$-H_2SO_4, 5:1:1).

PLANT MATERIAL.—The leaves of *Vernonia amygdalina* were harvested fresh from a horticultural garden in Ibadan, Nigeria, and were air-dried. The plant was botanically identified by Dr. J. Lowe of the Botany Department, University of Ibadan, Nigeria, and a voucher sample was deposited in the Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria.

EXTRACTION AND ISOLATION.—The dried and powdered leaves of *V. amygdalina* (600 g) were extracted exhaustively with 4 liters of MeOH and evaporated to near dryness. The dark-brown residue obtained was macerated with 30% hot MeOH and centrifuged at 3,000 g for 20 min, to obtain a clear orange-brown supernatant that was condensed *in vacuo* and then submitted to a C_{18} (25–40 μm, Merck) short-glass column (5 cm×5 cm i.d.). The column was washed with aqueous solvents containing increasing amounts of MeOH from 30% to 100%. Compound **1** was eluted with 50% MeOH and was further purified by reversed-phase (RP-18, Lichroprep, 15–25 μm, Baker) glass cc (42 cm×3.5 cm, Amicon) eluted with 50% MeOH. The semi-purified fraction was then purified by reversed-phase (C_{18}, Europrep 60–20; 15–25 μm, Knauer, steel column) and normal-phase (Si gel 60, particle size 15–25 μm, Merck, steel column) hplc using Type 64 pump (Knauer). In the reversed-phase purification, a solvent gradient of 40–70% MeOH was employed (Beckman Inc., Nyon, Switzerland). Compound **1** was purified using a Si gel column, eluting with $CHCl_3$-CH_3OH-H_2O (65:14:1). The pure fraction was taken to dryness under reduced pressure and precipitated from a CH_3OH-H_2O (1:3) mixture. The precipitate was lyophilized to afford a white amorphous powder (2.7 g); R_f 0.38 (S_1); uv λ max (MeOH) 234, 241, 251 nm; ^{1}H- and ^{13}C-nmr data, see Table 1; lsims (NBA) *m/z* (positive) 665 $[M+H]^+$, 503 $[M-glc+H]^+$, 467, 431, 267; (negative) 663 $[M-H]^-$, 633 $[M-2CH_3-H]^-$, 533; eims (aglycone) *m/z* 488 $[M-CH_3]^+$, 470 $[M-CH_3-H_2O]^+$, 452, 354, 268 $[M-side\text{-}chain-H_2O]^+$, 253.

Vernonioside A_3 was washed from the column with 65% MeOH. This was further purified by a combination of reversed- and normal-phase liquid chromatography as described for compound **1**, using a solvent gradient of 60–75% MeOH and isocratically in $CHCl_3$-CH_3OH-H_2O (65:15:1), respectively, as eluting solvents. The pure fraction was evaporated to dryness *in vacuo*, precipitated with a small volume of a CH_3OH-H_2O (1:3) mixture and lyophilized to afford a white amorphous powder (80 mg); R_f 0.38 (S_1), 0.40 (S_3); uv λ max (MeOH), 234, 241, 251 nm; lsims (NBA) *m/z* (positive) 629 $[M-H]^+$, 467 $[M-glc-H]^+$, 446 $[M-glc-H_2O-H]^+$, (negative) 629 $[M-H]^-$, 573 $[M-H-CH_3-C_3H_8]^-$, 531, 513, 411; eims *m/z* (aglycone) 468 $[M]^+$, 455, 354 $[M-CH_3-C_6H_{12}O]^+$, 337 $[M-CH_3-C_6H_{12}O-H_2O]^+$, 269 $[M-side\text{-}chain-H_2O]^+$ (calcd for $C_{29}H_{40}O_5$); the ^{13}C-nmr data were superimposable with those reported for vernonioside A_3 (7).

Compound **2** was eluted from the column with 70% MeOH. It was also subjected to reversed- and normal-phase liquid chromatographic purification using a solvent gradient of 65–80% MeOH, and $CHCl_3$-CH_3OH-H_2O (65:14:1) as eluting solvents, respectively. The purified fraction was taken to dryness, precipitated from a small volume of a CH_3OH-H_2O (3:1) mixture and lyophilized to afford a white amorphous powder (245 mg); R_f 0.35 (S_1), 0.42 (S_3); uv λ max (MeOH), 234, 241, 251 nm; lsims (negative) *m/z* 677 $[M-H]^-$, 633 $[M-H-COCH_3]^-$, 533, 515 $[M-H-glc]^-$, 477, 339, 325; eims *m/z* 470 $[M]^+$, 456 $[M-CH_3]^+$, 452 $[M-H_2O]^+$, 268 $[M-side\text{-}chain-H_2O]^+$ (calcd for $C_{29}H_{42}O_5$); ^{13}C-nmr data, see Table 1; ^{1}H nmr δ 1.03 (3H, s, Me-18), 0.68 (3H, s, Me-19), 1.10 (1H, m, H-20), 1.16 (3H, d,

J=6.0 Hz, Me-21), 1.45, 1.46 (3H each, s, Me-26 and Me-27), 1.67 (3H, d, J=5.5 Hz, Me-29), 2.00, 2.35 (1H each, m, H_2-22), 2.16 (3H, s, $COCH_3$), 3.12 (1H, q, J=5.5 Hz, H-28), 4.18 (1H, br m, H-16), 4.51 (1H, d, J=7.7 Hz, H-1), 5.28 (1H, t, J=6.8 Hz, H-23), 5.50 (1H, br s, H-7), 5.63 (1H, br d, J=6.0 Hz, H-11).

ENZYMATIC HYDROLYSIS.—The three glycosides (compounds **1**, **2**, and vernonioside A_3) were hydrolyzed with β-glucosidase (emulsin, Serva). Thus, a 20-mg sample of each glycoside was incubated with an equivalent amount of β-glucosidase at 32° for 7 days. Each hydrolysate was filtered through a sintered glass funnel and the filtrates were analyzed for sugars. The precipitates obtained were lyophilized and then extracted with hot $CHCl_3$. The $CHCl_3$ extracts were purified first by normal-phase cc using Si gel for flash chromatography (40 μm, J.T. Baker, B.V. Deventer, Holland) followed by normal-phase hplc using a Si gel steel column (Si 60, 15–25 μm, Merck). During the purification of the aglycone of compound **1**, the column was eluted with a $CHCl_3$-CH_3OH (99:1) mixture. The aglycone of compound **2** was purified using $CHCl_3$-CH_3OH (97:3) as the eluting solvent. Purification of the aglycone of vernonioside A_3 was carried out using $CHCl_3$-CH_3OH (98:2) as the eluting solvent. Each purified aglycone fraction was taken to dryness under reduced pressure and recrystallized from pure MeOH. The crystals were each filtered off from their mother solutions and dried at 60° to afford white crystalline solids.

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