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Isolation and NMR Assignment of a Pennogenin Glycoside from *Dioscorea bulbifera* L. var *sativa*

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Abstract – A steroidal saponin, 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 3)]-β-D-glucopyranosylpennogenin (**1,** spiroconazole A) was isolated from the tubers of *Dioscorea bulbifera* L. var *sativa* and ^{1}H-and ^{13}C-NMR assignment was completed using HMBC correlation. In addition, four phenolic substances, 2,7-dihydroxy-4-methoxyphenanthrene (**2**), quercetin (**3**), quercetin-3-*O*-β-D-glucopyranoside (**4**), and quercetin-3-*O*-β-D-galactopyranoside (**5**) were also isolated.
Keywords – *Dioscorea bulbifera* L. var *sativa*, steroidal saponin, phenanthrene, flavonoid, HMBC

Introduction

The Dioscoreaceae, distributed throughout the tropics and some temperate regions, constitute a family consisting mainly of tropical climbers. *Dioscorea bulbifera* L. var *sativa* grows wild and its bitter tuber is used by tribal people of Bangladesh for treatment of leprosy and tumors (Murray *et al.*, 1984). Earlier chemical investigation on the tubers afforded two norclerodane diterpenoids (Murray *et al.*, 1984). Numerous species of the genus *Dioscorea* are characterized by the rich occurrence of steroidal saponins often consisting of diosgenin as aglycone (Mahato *et al.*, 1981; Dong *et al.*, 2001; Sautour *et al.*, 2006), but initial attempts to isolate saponins from *D. bulbifera* has failed (Komori, 1997). In our continuous search for new and/or biologically active saponins from Cameroonian medicinal plants (Naheed *et al.*, 2002; Tapondjou *et al.*, 2002; Tapondjou *et al.*, 2003; Tapondjou *et al.*, 2005), we have investigated the tubers of *Dioscorea bulbifera* L. var *sativa*. This paper deals with the isolation and NMR assignment of a steroidal saponin (**1,** spiroconazole A) and other four phenolic compounds **2-5**. Full NMR assignment of compound **1** has not been reported before.

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Experimental

General procedure – ^{1}H-NMR spectra were recorded in deuterated solvents (CD_3OD and pyridine-d_5) on a Brucker AMX-400 Spectrometer at 400 MHz while ^{13}C-NMR spectra were recorded in the same solvents and the same apparatus at 100 MHz. All chemical shifts (δ) are given in ppm units with reference to tetramethylsilane (TMS) as an internal standard and the coupling constants (J) are in Hz. ESI-MS were taken on an Agilent 1100 ESI/LCMSD/Trap. IR spectra were measured as a film on a KBr pellet using a FTIR-8400S Shimadzu spectrometer. Melting points were determined using the Gallenkamp Melting Point Apparatus. Optical rotations were measured on a Perkin Elmer 241 polarimeter and column chromatography was performed using silica gel 60 Merck (0.040-0.063 mm) and sephadex LH-20. TLC was carried out on precoated Kieselgel 60 F_{254} (Merck) plates which were first viewed with an ultraviolet lamp MULTIBAND UV-254/365 nm for fluorescent spots and thereafter developed by spraying with 50% H_2SO_4 and heating for 10 mins at 110 °C.

Plant material – The tubers of *Dioscorea bulbifera* L. var *sativa* were collected in Bafou village near Dschang (West province of Cameroon) in March 2005. Specimens documenting the collection are deposited in the Cameroon National Herbarium in Yaoundé (Ref: 22211/SRF/CAM).

Extraction and isolation – The air dried tubers of *Dioscorea bulbifera* L. var *sativa* (1.8 kg) were pulverized and extracted four times (each time for 24 hours followed by heating for 20 minutes) with 80% MeOH. The aqueous methanolic extract was concentrated under reduced pressure to yield a dark residue (75 g), which was suspended in water (200 ml) and was successively partitioned with CH_2Cl_2 and *n*-butanol. The *n*-butanol soluble portion (29 g) was subjected to silica gel column chromatography, eluting with EtOAc-MeOH with increasing polarity to afford six main fractions A-E. Fraction E (6 g) eluted by EtOAc-MeOH (1:1) mainly yielded compound **1** (25 mg), after multiple chromatographic steps over silica gel using the mixtures CH_2Cl_2-EtOAc-MeOH (5:5:1) and EtOAc-MeOH-H_2O (85:15:5) as eluents. Repeated silica gel column chromatography of fraction A (4.5 g) (EtOAc) using the mixture CH_2Cl_2-MeOH (98:2 or 95:5) as eluents yielded compounds **2** (10 mg) and **3** (15 mg). From fraction D (5 g), compounds **4** and **5** were obtained as an inseparable isomeric mixture (50 mg), after repeated silica gel column chromatography eluted with CH_2Cl_2-MeOH (85:15) and sephadex LH-20 column chromatography eluted with CH_2Cl_2-MeOH (1:1).

Spiroconazole A (1) – White powder, m.p. 303.2°C; $[\alpha]_D^{21}$ -9.9 (c 0.045, DMSO); ESIMS (negative mode): *m/z* 883 $[M-H]^-$ ($C_{45}H_{71}O_{17}$), 737 $[M-H-146]^-$, 591 $[M-H-2\times146]^-$; IR ν_{max}^{KBr} (cm^{-1}): 3435, 3006, 2918, 1660, 1437, 1406, 1029, 955, 706; ^{1}H-NMR (400 MHz, pyridine-d_5) and ^{13}C-NMR (100 MHz, pyridine-d_5) : Table 1.

Acid hydrolysis of compound 1 – Compound **1** was hydrolyzed in 5% H_2SO_4 under reflux for 3 h. After neutralization with NH_4OH followed by extraction with $CHCl_3$, the aqueous layer was evaporated in vacuo to give a residue. The resilting resudue was applied to a TLC plate and developed with EtOAc-MeOH-H_2O-AcOH (13:6:3:3). The R_f values of the product were identical to those of D-glucose and L-rhamnose shown by co-TLC.

2,7-Dihydroxy-4-methoxyphenanthrene (2) – Yellow oil, ESIMS (Positive mode): *m/z* 241 $[M+H]^+$ ($C_{15}H_{13}O_3$); ^{1}H-NMR (400 MHz, CD_3OD): δ 4.08 (3 H, s, OMe), 6.76 (1H, d, J = 2.4 Hz, H-3), 6.82 (1H, d, J = 2.4 Hz, H-1), 7.08 (1 H, dd, J = 2.8 and 9.2 Hz, H-6), 7.15 (1H, d, J = 2.8 Hz, H-8), 7.47 (1H, d, J = 8.9 Hz, H-9), 7.51 (1 H, d, J = 8.9 Hz, H-10), 9.34 (1 H, d, J = 9.2 Hz, H-5); ^{13}C-

Table 1. ^{1}H-NMR (400MHz) and ^{13}C-NMR (100MHz) data of compound **1** (J in Hz)

position	δ_H	δ_C	position	δ_H	δ_C
1	0.95 (1 H, o) 1.73 (1 H, o)	37.8	23	1.60 (2 H, o)	32.7
2	1.90 (1 H, o) 2.11 (1 H, o)	30.3	24	1.60 (2 H, o)	29.1
3	3.90 (1 H, m)	78.1	25	1.60 (1 H, o)	30.7
4	2.73 (2 H, m)	38.9	26	3.52 (2 H, nd)	67.0
5	–	141.0	27	0.70 (3 H, d, J = 5.7)	17.6
6	5.28 (1 H, brs)	122.2	Glc 1'	4.90 (1 H, d, J = 7.6)	100.1
7	1.92 (1 H, m)	32.7	2'	4.06 (1 H, o)	78.7
8	1.73 (1 H, o)	32.4	3'	4.19 (1 H, t, J = 9.0)	87.4
9	0.98 (1 H, o)	50.5	4'	4.56 (1 H, o)	72.8
10	–	37.4	5'	3.83 (1 H, m)	78.3
11	1.55 (1 H, o)	21.2	6'	4.35 (1 H, o) 4.51 (1 H, o)	62.5
12	nd	37.4	Rha I 1"	5.87 (1 H, brs)	102.9
13	–	45.5	2"	4.89 (1 H, o)	72.9
14	2.11 (1 H, o)	53.3	3"	4.53 (1 H, o)	73.1
15	1.55 (1 H, o) 2.25 (1 H, o)	32.1	4"	4.35 (1 H, o)	74.1
16	4.51 (1 H, o)	90.3	5" 6"	4.06 (1 H, m) 1.77 (3 H, d, J = 6.2)	70.2 19.0
17	–	90.5	Rha II 1'''	5.77 (1 H, brs)	104.1
18	0.98 (3 H, s)	17.5	2'''	4.82 (1 H, o)	72.8
19	1.09 (3 H, s)	19.7	3'''	4.53 (1 H, o)	73.1
20	2.30 (1 H, s)	45.1	4'''	4.35 (1 H, o)	73.8
21	1.26 (3 H, d, J = 7.2)	10.1	5'''	4.06 (1 H, m)	70.9
22	–	110.2	6'''	1.67 (3 H, d, J = 6.2)	18.7

The data were measured in pyridine-d_5 with reference to TMS; o: overlapped; n.d: not determinated

NMR (100 MHz, CD_3OD): δ 54.9 (OMe), 99.2 (C-3), 104.6 (C-1), 111.3 (C-8), 115.0 (C-4a), 116.2 (C-6), 124.5 (C-5a), 126.9 (C-10), 127.4(C-9), 129.1 (C-5), 133.7 (C-8a), 134.8 (C-1a), 154.3 (C-7), 155.2 (C-2), 159.6 (C-4).

Quercetin (3) – Yellow amorphous powder; ESIMS (Positive mode): *m/z* 303 $[M + H]^+$ ($C_{15}H_{11}O_7$), 247 $[M + H - 2\ CO]^+$; ^{1}H-NMR (400 MHz, DMSO): δ 6.19 (1 H, brs, H-6), 6.41 (1 H, brs, H-8), 6.89 (1 H, d, *J* = 8.5 Hz, H-5'), 7.54 (1 H, dd, *J* = 2.2 and 8.5 Hz, H-6'), 7.68 (1 H, d, *J* = 2.2 Hz, H-2'), 12.49 (1 H, s, OH-5); ^{13}C-NMR (100 MHz, DMSO): δ 94.1 (C-8), 99.0 (C-6), 103.8 (C-4a), 115.8 (C-2'), 116.4 (C-5'), 120.8 (C-6'), 122.7 (C-1'), 136.5 (C-3), 145.8 (C-3'), 147.6 (C-4'), 148.4 (C-2), 156.9 (C-8a), 161.5 (C-5), 164.6 (C-7), 176.6 (C-4).

Quercetin-3-*O*-β-D-glucopyranoside (4) – Yellow amorphous powder; ESIMS (Positive mode): *m/z* 465 $[M + H]^+$ ($C_{21}H_{21}O_{12}$), 303 $[M + H - Glc]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ 5.24 (1 H, d, *J* = 7.5 Hz, H-1''), 6.22 (1 H, d, *J* = 2.1 Hz, H-6), 6.41 (1 H, d, *J* = 2.1 Hz, H-8), 6.90 (1 H, d, *J* = 8.5 Hz, H-5'), 7.60 (1 H, dd, *J* = 2.2 and 8.5 Hz, H-6'), 7.72 (1 H, d, *J* = 2.2 Hz, H-2'); ^{13}C-NMR (100 MHz, CD_3OD): δ 60.8 (C-6''), 70.1 (C-4''), 74.6 (C-2''), 77.0 (C-5''), 77.3 (C-3''), 93.8 (C-8), 98.9 (C-6), 103.2 (C-1''), 104.5 (C-4a), 115.1 (C-2'), 116.5 (C-5'), 121.8 (C-1'), 122.0 (C-6'), 134.5 (C-3), 144.8 (C-3'), 148.8 (C-4'), 157.4 (C-2), 158.0 (C-8a), 161.9 (C-5), 165.0 (C-7), 178.5 (C-4).

Quercetin-3-*O*-β-D-galactopyranoside (5) – Yellow amorphous powder; ESIMS (Positive mode): *m/z* 465 $[M + H]^+$ ($C_{21}H_{21}O_{12}$), 303 $[M + H - Glc]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ 5.15 (1 H, d, *J* = 7.8 Hz, H-1''), 6.22 (1 H, d, *J* = 2.1 Hz, H-6), 6.41 (1 H, d, *J* = 2.1 Hz, H-8), 6.90 (1 H, d, *J* = 8.5 Hz, H-5'), 7.60 (1 H, dd, *J* = 2.2 and 8.5 Hz, H-6'), 7.85 (1 H, d, *J* = 2.2 Hz, H-2'); ^{13}C-NMR (100 MHz, CD_3OD): δ 61.9 (C-6''), 68.9 (C-4''), 72.1 (C-2''), 74.0 (C-3''), 76.1 (C-5''), 93.8 (C-8), 98.9 (C-6), 104.2 (C-1''), 104.6 (C-4a), 115.1 (C-2'), 116.8 (C-5'), 121.9 (C-1'), 122.2 (C-6'), 134.7 (C-3), 144.9 (C-3'), 148.9 (C-4'), 157.8 (C-2), 158.0 (C-8a), 161.9 (C-5), 165.0 (C-7), 178.5 (C-4).

Results and Discussion

Compound **1** was isolated as a white powder, m.p. 303.2 °C; $[\alpha]_D^{21}$ –9.9 (c 0.045, DMSO). Its ESIMS (negative mode) showed the pseudomolecular $[M - H]^-$ ion peak at *m/z* 883 (corresponding to the molecular formula $C_{45}H_{72}O_{17}$) and two important peaks at *m/z* 737 $[M - H - 146]^-$, 591 $[M - H - 2 \times 146]^-$, respectively corresponding to the loss of one and two deoxyhexopyranose moieties. The glycosidic nature of **1** was shown by strong IR absorptions at 3435 and 1029 cm^{-1}, which are characteristic of hydroxyl groups and glycosidic linkages, respectively (Dong *et al.*, 2001). On acid hydroysis, compound **1** yielded D-glucose and L-rhamose identified by comparison on TLC with authentic samples. The ^{13}C-NMR spectrum showed 45 signals (Table 1) among which 27 were assigned to the aglycone; the remaining 18 signals were indicative of the presence of three hexoses due to one D-glucose and two L-rhamnoses. The structure of the aglycone moiety was recognized to be pennogenin (3β,17α-dihydroxyspirost-5-ene) by ^{1}H$^-$ and ^{13}C-NMR spectral analysis (Table 1) using connectivities observed in COSY, HMQC, and HMBC and was in full agreement with the literature data (Mahato *et al.*, 1981; Hufford *et al.*, 1988). The salient features of this aglycone part were the ^{13}C-NMR signals at δ 141.0 (C-5) and 122.2 (C-6), characteristic of Δ^5-spirostene-type sapogenin (Agrawal *et al.*, 1985). Nohara *el al.* (1974) has established the structure of pennogenin as 25*R*-spirost-5-en-3β,17α-diol on the basis of chemical and spectroscopic evidence. Our NMR data of the aglycone moiety of compound **1** agreed pennogenin. The proton coupling constant between H-25 and H-26_{ax} (*J* = 10.6 Hz), and the ^{13}C-NMR shifts of the F-ring part gave an evidence for the C-25*R* configuration.

The sugar part of compound **1** consisted of one disubstituted glucose and two terminal rhamnose moieties (Dong *et al.*, 2001; Hufford *et al.*, 1988). This was indicated by the presence of three anomeric proton signals observed in the ^{1}H-NMR spectrum at δ 5.87 (1 H, brs, H-1''), 5.77 (1H, brs, H-1'''), and 4.90 (1 H, d, *J* = 7.6 Hz, H-1'), correlated with three anomeric carbons at δ 102.9 (C-1''), 104.1 (C-1'''), and 100.1 (C-1'), respectively, in the HMQC spectrum. Furthermore, the presence of two upfield signals of methyl protons at δ 1.67 (1 H, d, *J* = 6.2 Hz, CH_3-6''') and 1.77 (1 H, d, *J* = 6.2 Hz, CH_3-6'') in its ^{1}H-NMR spectrum confirmed that the two deoxyhexopyranose units were rhamnose moieties. The coupling constants observed for the three anomeric proton signals suggested that the linkages of the glucose and rhamnoses were of the β and α forms, respectively. The α-anomeric configurations for the rhamnoses were also confirmed by their C-5 ^{13}C-NMR data [δ 70.2 (C-5'') and 70.9 (C-5''')] (Dong *et al.*, 2001). The ring protons of each monosaccharide residue were assigned starting from the readily identifiable anomeric proton using COSY spectrum. The assignments of the protons of ^{13}C-NMR resonances for each sugar unit were corroborated by HMQC and HMBC spectra (Table 1). Evaluation of spin-spin couplings and chemical shifts then confirmed the

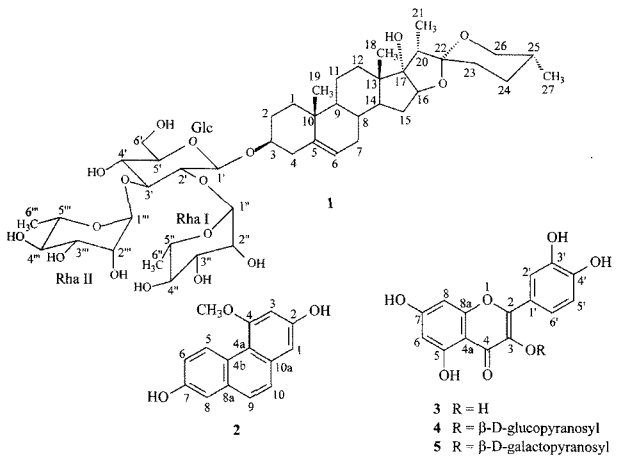

Fig. 1. Compounds **1-5** isolated from *Dioscorea bulbifera* L. var *sativa*.

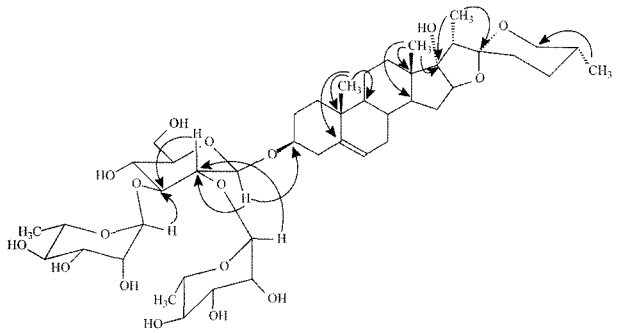

Fig. 2. Selected HMBC correlations for compound **1**.

identification of one 2,3-disubstituted β-glucopyranosyl and two terminal α-rhamnopyranosyl units. The rhamnopyranosyl residues were shown to be terminal units as suggested by the absence of any glycosylation shift for their carbon resonances (Agrawal, 1992). The 2,3-disubstitution of the glucopyranosyl moiety was shown by downfield chemical shifts at δ_C 78.7 (C-2') and 87.4 (C-3') in the ^{13}C-NMR spectrum. The sequences of the sugar chains were obtained by HMBC experiment. Cross-peak correlation observed in the HMBC spectrum between the anomeric proton at δ_H 4.90 (H-1') and the carbon at δ_C 78.1 (C-3) showed that the glucopyranosyl moiety is linked at C-3 of the aglycone. The correlation observed between the signals at δ_H 5.77 (H-1''') and the carbon at δ_C 87.4 (C-3') suggested the location of one rhamnopyranosyl unit at C-3'. Finally, the correlation observed between the signals at δ_H 5.87 (H-1'') and the carbon at δ_C 78.7 (C-2') showed that the second rhamnopyranosyl unit was located at C-2' of the glucose unit. Further correlations were also depicted in the HMBC spectrum (Fig. 2). The common D-configuration for glucose and L-configuration for rhamnoses were assumed to be those of the most commonly encountered analogues in the plant kingdom. Thus, the structure of compound **1** was elucidated as 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranosylpennogenin. This saponin has previously been isolated and characterised from *Draceana manii* and *D. arborea* and has also been shown to exhibit various biological activities like antileishimanial, antimalarial and molluscicidal (Okunji *et al.*, 1996).

Compound **2** was isolated as a yellow oil. It was identified as 2,7-dihydroxy-4-methoxyphenanthrene (flavanthrinin) by comparison of the ESIMS, ^{1}H-NMR, and ^{13}C-NMR data with those previously reported in the literature (Majumder *et al.*, 1999). Compound **3** was obtained as a yellow amorphous powder. Its structure was identified to be quercetin by comparison of the ESIMS, ^{1}H-NMR, and ^{13}C-NMR data with published data. This compound was previously characterized from the leaves of *Embelia schimperi* (Arot *et* Williams, 1997). Compounds **4** and **5** were obtained as an inseparable mixture of two isomers. They were identified as quercetin-3-*O*-β-D-glucopyranoside and quercetin-3-*O*-β-D-galactopyranoside using spectroscopic analysis (ESIMS, ^{1}H-NMR and ^{13}C-NMR) and by comparison with the literature data (El-Mousallamy *et al.*, 2000; Arot *et al.*, 1997). Compounds **II-5** have not been previously isolated from this plant.

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