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A NEW ERGOSTANOL SAPONIN FROM *DIOSCOREA DELTOIDEA* WALL VAR. *ORBICULATA*

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From the fresh rhizomes of *Dioscorea deltoidea* Wall var. *orbiculata*, a novel ergostanol saponin, orbiculatoside **A** (**1**), was isolated and identified as 3-*O*-β-D-glucopyranosyl-ergost-5-ene-3β, 26-diol-26-*O*-β-D-glucopyranosyl(1 → 3)-[β-D-glucopyranosyl(1 → 2)-β-D-glucupyranosyl(1 → 6)]-β-D-glucopyranoside by various NMR techniques in combination with chemical methods. The new saponin showed strong activity against *Pyricularia oryzae*, with a MMDC (minimum morphological deformation concentration) value of 28.04 μmol/l and was cytotoxic to cancer cell line K562, HCT-15, A549, HT1080, and A2780a *in vitro*.

Keywords: *Dioscorea deltoidea* Wall var. *orbiculata*; Orbiculatoside **A**; *Pyricularia oryzae*; Cytotoxic

INTRODUCTION

We have used *Pyricularia oryzae* bioassay method, established by Kobayashi [1], to screen antineoplastic and antifungal natural products from traditional Chinese medicines (TCMs) [2–5]. Among more than 300 species of TCMs, *Dioscorea* family showed strong activity against *P. oryzae*. In this report, we describe the structure determination of a novel ergostanol saponin, orbiculatoside **A** (**1**) (Fig. 1), isolated from the ethanol extract of *Dioscorea deltoidea* Wall var. *orbiculata*.

RESULTS AND DISCUSSION

Orbiculatoside **A** (**1**), a white powder, was positive to Libermann-Burchard and Molish tests. The IR spectrum of **1** lacked the characteristic bands of a spirostane ring, only exhibited hydroxylic absorption (a broad peak at 3300 cm^{-1}) and a double bond at 1625 cm^{-1}. Its HRFABMS (negative) showed $[M - H]^-$ peak at 1225.6295 corresponding to the molecular formula of $C_{58}H_{98}O_{27}$. And the FABMS (negative) gave a molecular anion peak at *m*/*z* 1226

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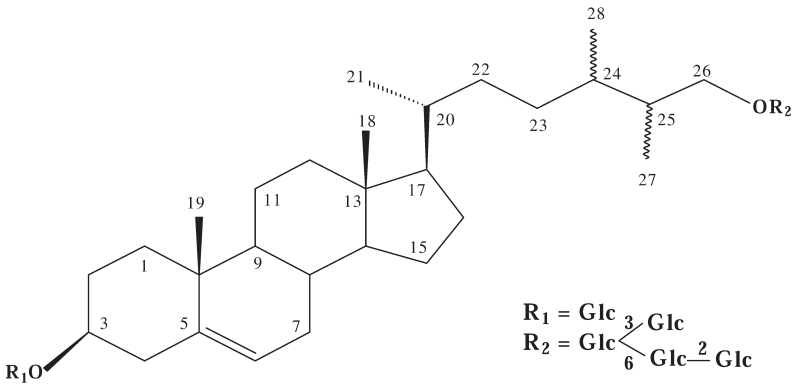

FIGURE 1 The structure of compound **1**.

$[M]^-$, together with five other major fragments at *m/z* 1063 $[M - H\text{-}162]^-$, 901 $[M - H\text{-}162 \times 2]^-$, 739 $[M - H\text{-}162 \times 3]^-$, 561 $[M - OH - 162 \times 4]^-$, and 399 $[M - OH - 162 \times 5]^-$, corresponding to the sequential losses of hexose units, respectively. After complete acid hydrolysis of **1**, the hydrolysate was trimethylsilylated and GC retention time (685 s) of the derived sugars were compared with those of the authentic samples prepared by the same manner, indicating all the sugars were D-glucose (685 s).

The ^{1}H NMR spectrum of **1** showed five methyl signals at δ 0.66 (*s*, 3H, Me-18), 0.95 (*s*, 3H, Me-19), 0.96 (*d*, 3H, $J = 6.4$ Hz, Me-21), 0.92 (*d*, 3H, $J = 6.8$ Hz, Me-27), 0.87 (*d*, 3H, $J = 6.6$ Hz, Me-28), one olefinic proton at δ 5.39 (*br s*, 1H, H-6) and five anomeric proton signals at δ 5.05 (*d*, 1H, $J = 7.65$ Hz, H-1′), 4.95 (*d*, 1H, $J = 7.76$ Hz, H-1″), 5.19 (*d*, 1H, $J = 7.70$ Hz, H-1‴), 5.26 (*d*, 1H, $J = 7.65$ Hz,; H-1⁗), 5.35 (*d*, 1H, $J = 7.80$ Hz, H-1‴′). The *J* values of the anomeric protons suggested the five glucose units were all β-oriented. Complete analysis of its ^{13}C-NMR and DEPT spectra revealed that 28 resonance signals of aglycone moiety were assigned as 5 methyls (δ 19.9, 19.5, 14.8, 12.4, 12.1), 10 methylenes, 1 oxygenated methylene (δ 74.5), 7 methines, 1 oxygenated methine (δ 78.9), 2 quaternary (δ 37.3, 42.9), and 2 olefinic carbons (δ 141.4, 122.4). In the HMBC spectrum of **1**, carbon signals resonating at δ 37.9, 141.4, 50.8, and 37.3 were correlated with Me-19 (δ 0.95), indicating these signals to be C-1, C-5, C-9, and C-10, respectively. In the same way, according to the correlation with Me-18(δ 0.66), the signals resonating at δ 40.4, 42.9, 57.3, 56.7 were assigned to be C-12, C-13, C-14, and C-17, respectively. And from the correlations with Me-21 (δ 0.96), Me-28 (δ 0.87), and Me-27 (δ 0.92), C-22 (δ 34.4), C-23 (δ 32.0), C-24 (δ 34.5), C-25 (δ 38.1), and C-26 (δ 74.5) were accordingly assigned. In this way, the aglycone of **1** was identified to be ergost-5-ene-3β, 26-diol [6]. The NMR data of 1 are listed in Table I.

Combined analysis of the HMQC, HMBC, ^{1}H–^{1}H COSY spectra, and especially the HMQC-TOCSY spectrum, helped to clarify where the sugar residues were attached to aglycone and how they were linked to each other. In the HMBC spectrum, the $^3J_{C,H}$ correlations between C-3 (δ 78.9) and one anomeric proton (δ 5.05), and that between C-26 (δ 74.5) and another anomeric proton (δ 4.95), revealed the attaching positions of two sugar chains (Fig. 2). The HMQC-TOCSY spectrum revealed that three of five glucose residues were terminal sugars, and two were substituted by others, one at C-6″ (δ70.1) and C-3″ (δ 88.2) positions, and the other at C-2⁗ (δ84.1) position. The $^3J_{C,H}$ correlations between the anomeric protons (δ 5.19, 5.26, 5.35), and corresponding carbons (δ 70.1, 88.2, 84.1) were observed clearly in the HMBC spectrum. The NMR assignments of the sugar chain attached

TABLE I ^{1}H and ^{13}C NMR* data for compound **1** in C_5D_5N (δ values)

No.	^{13}C	^{1}H	^{13}C		^{1}H
1	37.9	1.77 (*o*), 1.02 (*o*)†	3-*O*-Glc-1′	102.8	5.05 (*d*, *J* = 7.65)
2	30.6	2.19 (*d*, *J* = 11.55)‡, 1.78 (*o*)	2′	75.6	4.09 (*o*)
3	78.9	4.00(*o*)	3′	78.8	4.18 (*o*)
4	39.7	2.75 (*br d*), 2.52 (*t*)	4′	72.0	4.07 (*o*)
5	141.4		5′	78.6	3.97 (*o*)
6	122.4	5.39 (*br s*)	6′	63.1	4.54 (*o*), 4.35 (*o*)
7	32.6	1.92 (*o*), 1.57 (*o*)	26-*O*-Glc-1″	104.2	4.95 (*d*, *J* = 7.76)
8	32.5	1.37 (*o*)	2″	74.7	4.12 (*o*)
9	50.8	0.92 (*o*)	3″	88.2	4.35 (*o*)
10	37.3		4″	70.5	4.00 (*o*)
11	21.8	1.46 (*m*), 1.37 (*o*)	5″	76.8	4.31 (*o*)
12	40.4	1.99 (*o*), 1.13 (*m*)	6″	70.1	4.56 (*d*, *J* = 13.8), 4.30 (*o*)
13	42.9		Glc (1 → 3)-1‴	105.9	5.35 (*d*, *J* = 7.80)
14	57.3	0.97 (*o*)	2‴	76.0	4.06 (*o*)
15	25.0	1.57 (*m*), 1.06 (*o*)	3‴	78.8	4.17 (*o*)
16	28.9	1.82 (*o*), 1.26 (*o*)	4‴	71.8	4.16 (*o*)
17	56.7	1.07 (*o*)	5‴	78.8	3.85 (*o*)
18	12.4	0.66 (*s*)	6‴	62.9	4.52 (*o*), 4.20 (*o*)
19	19.9	0.95 (*s*)	Glc (1 → 6)-1⁗	103.4	5.19 (*d*, *J* = 7.70)
20	36.5	1.37 (*o*)	2⁗	84.1	4.09 (*o*)
21	19.5	0.96 (*d*, *J* = 6.4)	3⁗	78.3	4.28 (*o*)
22	34.4	1.78 (*o*), 1.07 (*o*)	4⁗	71.5	4.11 (*o*)
23	32.0	1.27 (*o*), 1.03 (*o*)	5⁗	78.4	3.87 (*o*)
24	34.5	1.78 (*o*)	6⁗	62.7	4.49 (*o*), 4.31 (*o*)
25	38.1	2.03 (*o*)	Glc (1 → 2)-1′′′′′	106.3	5.26 (*d*, *J* = 7.65)
26	74.5	3.68 (*t*, *J* = 11.4), 4.37 (*m*)	2′′′′′	76.7	4.08 (*o*)
27	12.1	0.92 (*d*, *J* = 6.8)	3′′′′′	78.8	4.16 (*o*)
28	14.8	0.87 (*d*, *J* = 6.6)	4′′′′′	71.7	4.36 (*o*)
			5′′′′′	78.6	3.96 (*o*)
			6′′′′′	62.9	4.57 (*o*), 4.29 (*o*)

* Recorded on a Bruker-500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) NMR spectrometer.
† Overlapped signals are indicated by "(*o*)".
‡ *J* values (in parentheses) are reported in Hz.

to C-26 position were identical to those of segetoside **K** [7], a new triterpenoid saponin from *Vaccaria segetalis*.

On the basis of the above analysis, compound **1** was consequently identified to be 3-*O*-β-D-glucopyranosyl-ergost-5-ene-3β, 26-diol-26-*O*-β-D-glucopyranosyl(1 → 3)-[β-D-glucopyranosyl(1 → 2)-β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranoside, and named as orbiculatoside **A**. It was the first isolation of an ergostanol saponin from *Discorea*, and also the first time to find that the ergost-5-ene-3β, 26-diol [6] linking complicated sugar chains.

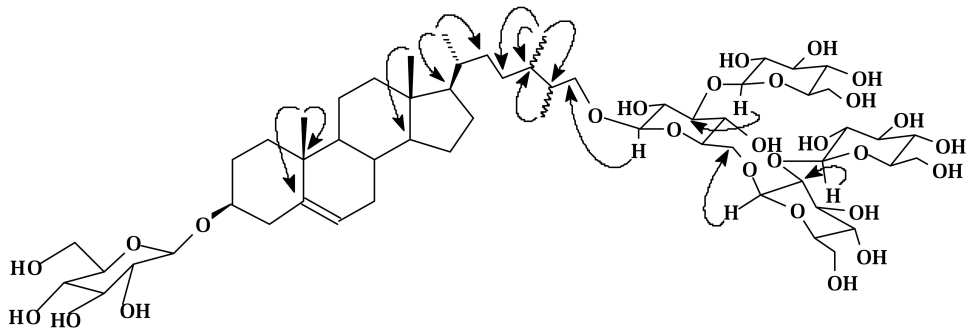

FIGURE 2 The main HMBC correlations for compound.

TABLE II Cytotoxicity data for compound **1***

Compound	*K562*	*A549*	*HCT15*	*HT1080*	*A2780*
1	44.31	–	127.60	54.27	540.87
cis-DDP†	69.33	1426.67	69.50	333.3	54.81

* Use the IC_{50} value (μmol/l) to evaluate the cytotoxicities of the samples.
† *cis*-DDP was used as the positive control.

Compound **1** showed strong activity against *P. oryzae* with a MMDC (minimum morphological deformation concentration) value of 28.04 μmol/l, and the cytotoxicity bioassay indicated that it was cytotoxic to several cancer cell lines. The bioassay results are listed in Table II.

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points were determined on a Yanaco MP-S_3 micromelting point apparatus, and were uncorrected. Optical rotations were measured on a HORIBA SEPA-300 polarimeter. IR spectra were recorded on Bio-Red FTS-135 spectrophotometer. ^1H and ^{13}C NMR, along with 2D NMR spectra were obtained on a Bruker DRX-500 (500 MHz for ^1H, 125 MHz for ^{13}C) NMR spectrometer, using TMS as an internal standard. FABMS spectra were taken on VG Autospec-3000 mass spectrometer. Column chromatography was carried out on silica gel H (200–300 mesh, Qindao Haiyang Chemical Factory), and precoated Kieselgel 60 F_{254} silica gel plates (0.2 mm, Merck) were used for analytical TLC. GC/MS analysis was run on a Fisons MD 800 GC/MS spectrometer, with HP AC-5 quartz capillary column (30 m × ϕ0.25 mm), quadrupole (230°C) detector, and column temperature was 180–240°C (rate 5°C min^{-1}), carrier gas was He (30 ml min^{-1}).

Plant Material

The fresh rhizomes of *D. deltoidea* Wall var. *orbiculata* were collected in Wenshan, Yunnan, China, and identified by Prof Heng Li. A voucher specimen (No. 0242227) is deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation

The fresh rhizomes of *D. deltoidea* Wall var. *orbiculata* (24.3 kg) were extracted repeatedly with 95% EtOH (101 × 3). The EtOH solutions were concentrated in vacuum to give a residue (1650 g), which was dissolved in water and further fractionated by Diaion HP-20 (H_2O, 70% EtOH) to give the total saponins (650 g), which was chromatographed on silica gel column, eluted with $CHCl_3$–MeOH mixtures of increasing polarity to yield six fractions. Fraction 6 (3.62 g) was further separated on silica gel column with $CHCl_3$–MeOH–H_2O (7:3:0.5), and RP-8 silica gel column (65%, 70% MeOH) to afford compound **1** (27 mg).

Orbiculatoside A (1)

White powder; mp 185–188°C (dec.); $[\alpha]_D^{20}$ −56.2 (*c* 0.003, C_5H_5N); Molecular formula $C_{58}H_{98}O_{27}$ (negative HRFABMS 1225.6295, calcd. for $C_{58}H_{97}O_{27}$, 1225.6217); ESIMS

(positive) *m/z*: 1227.1, 1064.9, 902.8, 740.6, 560.9; FABMS (negative) *m/z*: 1226, 1063, 901, 739, 561, 399; ^{1}H and ^{13}C NMR: Table I.

Acid Hydrolysis and GC/MS Analysis Of 1

A solution of compound **1** (3.5 mg) was treated with 2N HCl in dioxane-H_2O (1:1) (1 ml) at 100°C for 2 h. After cooling, the solution was concentrated by blowing with N_2, then the reaction mixture was trimethylsilated with trimethylchlorosilane (1 ml), stirred at 60°C for 5 min. After drying the solution with a stream of N_2, the residue was extracted with ether (1 ml). The ether layer was analyzed by GC using an HP AC-5 quartz capillary column (30 m × ϕ0.25 mm). The GC retention times of the derived sugars were compared with those of the authentic samples prepared by the same manner, indicating all the component sugars were D-glucose (685 s).

Cytotoxicity Assay

Cancer cells were cultured in PRMI-1640 medium supplemented with 5% fetal bovine serum. The cultures were incubated at 37°C in a 5% CO_2 humidified incubator and subcultured every 2 days to maintain them in a state of logarithmic growth. Then the cells were seeded into 96-well microtiter plates (2×10^4 cells per well). Compound **1** was dissolved in MeOH and added into the 96-well microtiter plates 24 h after seeding. The cells were incubated for 2 days in the presence of sample. For the evaluation of *in vitro* cytotoxicity, a microculture tetrazolium assay (MTT assay) was used. The anticancer drug *Cis*-dichlorodiamine platinum (*cis*-DDP) was used as the positive control.

Pyricularia Oryzae Bioassay

See the previous report of Kobayashi *et al.* [1]. The positive control employed rhizoxin, which exhibited activity with MMDC values of 0.008 μmol/l.

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