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Isolation of steroidal saponins from the seeds of *Allium ramosum*

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***Abstract*—Protodioscin (1) and anguivioside A (2) were isolated from the seeds of *Allium ramosum* using combination of chromatographic techniques. Chemical structure of 1 and 2 were determined by extensive analysis of ^{1}D-NMR and ^{2}D-NMR spectral data, and comparison with those reported in the literature. This is the first report on the isolation of those compounds from *A. ramosum*.**

Keywords*—*Allium ramosum; steroidal saponin; isolation; protodioscin; anguivioside A.

I. INTRODUCTION

Allium genus has been documented over 700 species. Some of them such as *A. sativum* (garlic) and *A. cepa* (onion) have been used to be common vegetables for thousands of years [1, 2]. They also have been used in the traditional medicines for treatment of inflammation, cough, cardiovascular diseases [3, 4]. Phytochemical studies on *Allium* species, therefore, have been much attractive by medicinal chemists. Hundreds of sulfurs containing compounds have been reported from less polar fraction of *Allium* species which believed for anti-cancer, antibacterial, prevention of cardiovascular and as well as allergy and arthritis properties [5]. Additionally, polar fractions of *Allium* species are rich of steroidal saponins in term of spirostane-type and furostane-type steroidal saponins [6]. The saponins are considered responsible for numerous pharmacological properties not only for *Allium* species but also for many medicinal plants [6]. *A. ramosum* is a vegetable and culinary supplement of temperate countries. Up to date, chemical constituents of *A. ramosum* have not been reported. This paper, therefore, describes our primary results on the clarification of chemical constituents of *A. ramosum* which could be helpful for the explanation of the medicinal uses of this plant and its processed products.

II. MATERIALS AND METHODS

A. General experiment procedures

NMR spectra were recorded on a Bruker AVANCE III 500 MHz (Bruker BioSpin, Bremen, Germany). Column chromatography was carried out using silica gel (Merck, Whitehouse Station, NJ, USA), reversed phase C-18 gel (YMC Ltd., Kyoto, Japan), and diaion HP-20 resin as stationary phase. Semi-preparative HPLC was acquired on an Agilent 1260 Infinity II system including binary pump, autosampler, DAD detector, analytical fraction collector, and equipped with J'sphere ODS-H80 (20×250 mm, 4 µm). HPLC chromatogram was monitored at 205 nm. Thin layer chromatography was performed using pre-coated silica gel 60 F_{254} and RP-C18 F_{254S} plates (Merck, Darmstadt, Germany). Compounds were detected by spraying with aqueous solution of H_2SO_4 (5%, w/w) followed by heating on a hot plate.

B. Plant materials

The seeds of *Allium ramosum* L. were collected at Lam Dong province, Vietnam, in November 2019 and taxonomically determined by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, Hanoi, Vietnam. Voucher specimen (No. GTVT112019) is kept at the Faculty of Basic Science, University of Transport and Communications, Hanoi, Vietnam.

C. Extraction and isolation

The dried powdered *A. ramosum* seeds (5.0 kg) were extracted with methanol in an ultrasonic bath for three times (15 L MeOH and 30 minutes each time). After filtration, the solvent was removed under reduced pressure to give methanol residue. This residue (320 g) was suspended with water (3.0 L) and partitioned in turn with dichloromethane, ethyl acetate to give corresponding dichloromethane faction, ethyl acetate fraction, and water layer. The steroidal saponins are usually polar compounds. Thus, the water layer was chosen for purification. The water layer was loaded on a Diaion HP-20 column, washed with water, and then eluted stepwise with methanol/water (1:3, 1:1, 3:1, and 1:0, v:v) to give four fractions, WAR1-WAR4. Fraction WAR3 was subjected on a reversed phase C-18 column and eluted with gradient solvent system of acetone/water (1:2, 2:3, and 1:1, v:v) to give five fractions, WAR3A-WAR3E. Fraction WAR3B was subjected on a silica gel column and eluted with acetone/dichloromethane/water (4:1:0.5, v:v) to give four fractions, WAR3B1- WAR3B4. Fraction WAR3B4 purified by preparative HPLC using acetonitrile in water (27% acetonitrile) to give compound **2** (10 mg). Fraction WAR3C was also chromatographed on a silica gel column and eluted with acetone/ dichloromethane/water (4:1:0.5, v:v) to give six fractions, WAR3C1- WAR3C6. Fraction WAR3C1 was purified by preparative HPLC using acetonitrile in water (31% acetonitrile) to give compound **1** (6 mg).

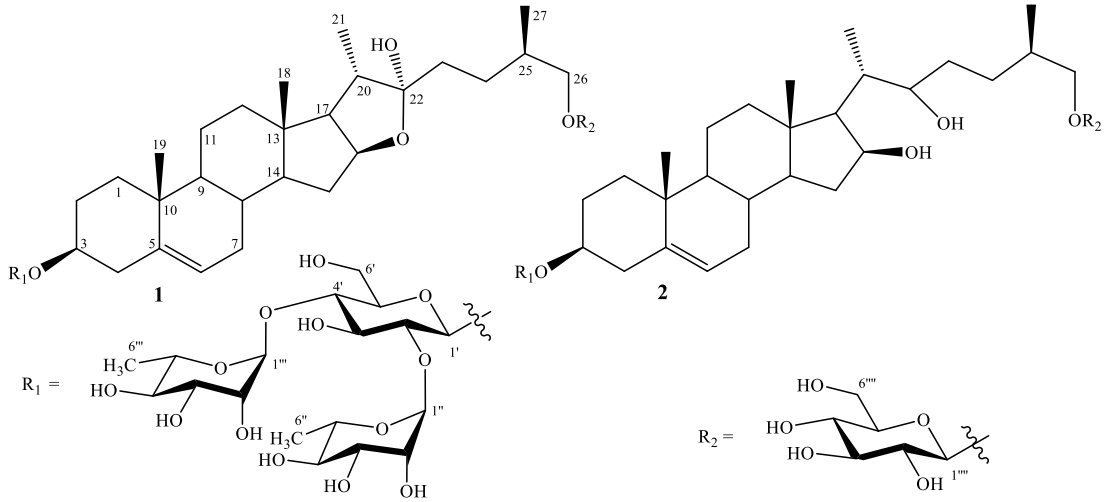

Fig. 1. *Chemical structure of compounds **1** and **2** isolated from Allium ramosum*

- Protodioscin (**1**): Molecular formula: $C_{51}H_{84}O_{22}$; White amorphous powder; $[\alpha]_D^{28}$: -54.5 (*c* = 0.1, MeOH); ^{1}H-NMR and ^{13}C-NMR data are given in the Table 1.

- Anguivioside A (**2**): Molecular formula: $C_{51}H_{86}O_{22}$; White amorphous powder; $[\alpha]_D^{28}$: -71.6 (*c* = 0.1, MeOH); ^{1}H-NMR and ^{13}C-NMR data are given in the Table 1.

III. Results and Discussion

The seeds of *A. ramosum* was ultrasonically extracted with methanol and then partitioned with dichloromethane and ethyl aceate to eliminate and weak polar compounds. The water soluble fraction usually expected rich of saponins which were fractionated and purified using combination of chromatographic methods to give compounds **1** and **2** (Fig. 1).

Compound **1** was isolated as a white amorphous powder. The ^{1}H-NMR spectrum of **1** contained signals assigning for two tertiary methyl groups [δ_H 0.85 and 1.08 (each, 3H, s)], four secondary methyl group [δ_H 0.97, 1.02, 1.24, and 1.27 (each 3H, d, *J* = 6.0 Hz)], four anomeric protons [δ_H 4.52 (1H, d, J = 7.5 Hz), 4.26 (1H, d, *J* = 7.5 Hz), 4.85 (1H, d, *J* = 1.5 Hz), and 5.24 (1H, d, *J* = 1.5 Hz)], and an olefinic proton [δ_H 5.40 (1H, br d, J = 4.5 Hz)]. The ^{13}C-NMR spectrum of **1** observed signals of 51 carbons which were categorized by HSQC into four non-protonated carbons, 29 methines, 12 methylenes, and six methyl groups. Above NMR data suggested that **1** to be a steroidal saponins containing four sugar units. The appearance of two olefinic carbons (δ_C 122.6 and 141.9) indicating **1** contained one C=C double bond. Moreover, the HMBC correlations (Fig. 2) between H_3-19 (δ_H 1.08) and C-1 (δ_C 38.6)/ C-5 (δ_C 141.9)/ C-9 (δ_C 51.8)/ C-10 (δ_C 38.0) suggested the location of C=C double bond at C-5/C-6. The HMBC correlations between H_3-18 (δ_H 0.85) and C-12 (δ_C 41.0)/ C-13 (δ_C 41.8)/ C-14 (δ_C 57.7)/ C-17 (δ_C 64.0), a hemiketal carbon signal (δ_C 112.6) and HMBC correlations between H_3-21 (δ_H 1.02) and C-17 (δ_C 64.0)/ C-20 (δ_C 40.8)/ C-22 (δ_C 112.6) demonstrated for a furostane-type steroidal skeleton. Continuously, locations of sugar moieties were also clarified by HMBC analysis. The HMBC correlations between anomer proton Glc H-1′ (δ_H 4.52) and C-3 (δ_C 79.3), Glc H-1‴ (δ_H 4.26) and C-26 (δ_C 76.0) indicated that **1** contains two sugar sequences locating at C-3 and C-26. Position of sugar moiety at C-26 was also supported by HMBC correlations between H_3-27 (δ_H 0.97) and C-24 (δ_C 28.7)/ C-25 (δ_C 35.0)/ C-26 (δ_C 76.0). Furthermore, signals of six carbinol groups (δ_C 104.6, 75.2, 78.2, 71.7, 78.0, 62.8) were characterized for the presence of an *O*-glucopyranosyl group at C-26. The different between proton chemical shifts of oxygenated methylene group C-26 ($\Delta\delta_{Ha\text{-}26/Hb\text{-}26}$ = 0.25) indicated for the *β*-orientation of secondary methyl group C-27 (25*R*-configuration) as previously reviewed literature [7]. The two anomeric protons (δ_H 4.85 and 5.24) with small coupling constant values (*J* = 1.5 Hz) together with the two secondary methyl groups (δ_H 1.24 and 1.27) suggested for the presence of the two rhamnopyranosyl groups. The HMBC correlations between Rha H-1″ (δ_H 5.24) and Glc C-2′ (δ_C 79.3), Rha H-1‴ (δ_H 4.85) and Glc C-4′ (δ_C 80.1) indicated the second sugar moiety to be a 2′,4′-di-*O*-rhamnopyranosylglucopyranose trisaccharide. The small *J* coupling constant values of rhamnopyranosyl anomeric protons (*J* = 1.5 Hz) and large *J* coupling constant values of glucopyranosyl anomeric protons (*J* = 7.5 Hz) indicated for the *O*-*α*-L-rhamnopyranosyl and O-*β*-D-glucopyranosyl linkages, respectively. Thus, compound **1** was determined to be 26-O-*β*-D-gluco pyranosyl furost-5-ene-3,22,26-triol 3-*O*-(2′,4′-di-*O*-*α*-rhamnopyranosyl)-*β*-D-glucopyranoside. This compound was also named to be protodioscin and previously isolated from several *Allium* species such as *A. schoenoprasum* [8], *A. fistulosum* [9], and *A. tuberosum* [10]. However, this is the first report on the isolation of protodioscin from *A. ramosum*.

Compound **2** was isolated as a white amorphous powder. The ^{1}H-NMR spectrum of **2** contained signals assigning for two tertiary methyl groups [δ_H 0.94, 1.06 (each, 3H, s)], four secondary methyl group [δ_H 0.96, 0.98, 1.26, and 1.28 (each, 3H, d, *J* = 6.0 Hz)], four anomeric protons [δ_H 4.26 (1H, d, *J* = 7.5 Hz), 4.51

TABLE I. ^{1}H- AND ^{13}C-NMR SPECTROSCOPIC DATA OF COMPOUNDS **1** AND **2** IN CD_3OD

No.	**1**		**2**	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
1	38.6	1.10 (m)/ 1.90 (m)	38.5	1.10 (m)/ 1.89 (m)
2	30.8	1.30 (m)/ 1.84 (m)	30.7	1.62 (m)/ 1.94 (m)
3	79.3	3.41 (m)	79.4	3.42 (m)
4	39.5	2.21 (dd, 12.0, 12.0)	39.5	2.31 (dd, 11.5, 11.5)
		2.48 (br d, 12.0)		2.47 (br d, 11.5)
5	141.9	-	141.9	-
6	122.6	5.40 (br d, 4.5)	122.7	5.40 (br d, 5.0)
7	33.2	1.56 (m)/ 2.01(m)	33.0	1.98 (m)/ 2.03 (m)
8	32.5	1.68 (m)	32.3	1.50 (m)
9	51.8	1.26 (m)	51.8	0.97 (m)
10	38.0	-	37.9	-
11	22.0	1.50 (m)/ 1.58 (m)	21.9	1.52 (m)/ 1.56 (m)
12	41.0	1.20 (m)/ 1.80 (m)	41.3	1.18 (m)/ 2.02 (m)
13	41.8	-	43.4	-
14	57.7	1.17 (m)	55.9	0.95 (m)
15	32.8	1.30 (m)/ 2.00 (m)	37.5	1.29 (m)/ 2.24 (m)
16	82.3	4.58 (m)	72.6	4.37 (m)
17	64.0	1.81 (m)	58.5	1.40 (dd, 7.0, 11.0)
18	16.8	0.85 (s)	13.5	0.94 (s)
19	19.8	1.08 (s)	19.8	1.06 (s)
20	40.8	2.13 (m)	36.4	2.10 (m)
21	15.9	1.02 (d, 6.0)	14.2	0.98 (d, 6.0)
22	112.6	-	75.8	3.75 (m)
23	37.0	1.70 (m)/ 1.75 (m)	32.9	1.56 (m)/ 1.58 (m)
24	28.7	1.30 (m)/ 1.70 (m)	31.7	1.15 (m)/ 1.72 (m)
25	35.0	1.75 (m)	34.8	1.80 (m)
26	76.0	3.40 (m)/ 3.65 (m)	76.2	3.44 (m)/ 3.71 (m)
27	17.4	0.97 (d, 6.0)	17.6	0.96 (d, 6.0)
3-O-Glc				
1′	100.5	4.52 (d, 7.5)	100.5	4.51 (d, 7.5)
2′	79.3	3.60 (m)	79.3	3.60 (m)
3′	78.1	3.60 (m)	77.9	3.58 (m)
4′	80.1	3.51 (m)	80.1	3.52 (m)
5′	76.6	3.33 (m)	76.6	3.33 (m)
6′	62.0	3.66 (dd, 5.0, 12.0)	62.0	3.64 (dd, 4.5, 12.0)
		3.80 (dd, 2.0, 12.0)		3.81 (br d, 2.0, 12.0)
2′-*O*-Rha				
1″	102.3	5.24 (d, 1.5)	102.3	5.22 (d, 1.5)
2″	72.5	3.84 (dd, 1.5, 3.0)	72.4	3.84 (dd, 1.5, 3.0)
3″	72.2	3.63 (m)	72.2	3.62 (m)
4″	73.8	3.41 (m)	73.7	3.41 (m)
5″	70.7	3.93 (m)	70.7	3.93 (m)
6″	17.9	1.27 (d, 6.0)	17.9	1.28 (d, 6.0)
4′-*O*-Rha				
1‴	103.0	4.85 (d, 1.5)	103.0	4.85 (d, 1.5)
2‴	72.2	3.93 (dd, 1.5, 3.0)	72.4	3.93 (dd, 1.5, 3.0)
3‴	72.4	3.66 (m)	72.2	3.66 (m)
4‴	74.0	3.41 (m)	73.9	3.41 (m)
5‴	69.8	4.12 (m)	69.8	4.14 (m)
6‴	17.9	1.24 (d, 6.0)	17.9	1.26 (d, 6.0)
26-O-Glc				
1⁗	104.6	4.26 (d, 7.5)	104.6	4.26 (d, 7.5)
2⁗	75.2	3.20 (dd, 7.5, 9.0)	75.2	3.19 (dd, 7.5, 9.0)
3⁗	78.2	3.37 (m)	78.1	3.35 (m)
4⁗	71.7	3.30 (m)	71.7	3.29 (m)
5⁗	78.0	3.29 (m)	78.0	3.28 (m)
6⁗	62.8	3.69 (dd, 5.0, 12.0)	62.8	3.68 (dd, 5.0, 12.0)
		3.88 (dd, 2.0, 12.0)		3.88 (dd, 2.0, 12.0)

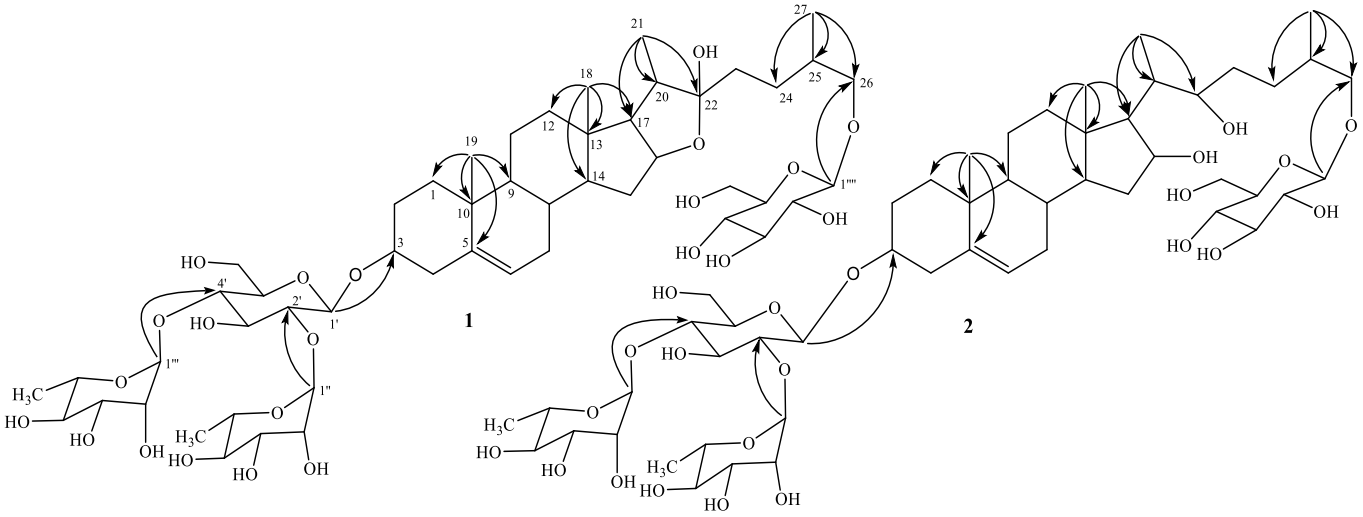

Fig. 2. *Key HMBC correlations (H→C) for compounds **1** and **2***

(1H, d, *J* = 7.5 Hz), 4.85 (1H, d, *J* = 1.5 Hz), and 5.22 (1H, d, *J* = 1.5 Hz)], and an olefinic proton [δ_H 5.40 (1H, br d, *J* = 5.0 Hz)]. The ^{13}C-NMR spectrum of **2** observed signals of 51 carbons which were categorized by HSQC into three non-protonated carbons, 30 methines, 12 methylenes, and six methyl groups. As mentioned above, the ^{1}H- and ^{13}C-NMR spectral data of **2** recognized close similarity with those of **1** except the presence of an oxygenated methine instead of a hemiketal carbon which suggested the opening of the furan ring at oxygen atom (Fig. 1). This deduction was then reconfirmed by HMBC analysis. The HMBC correlations between H_3-21 (δ_H 0.98) and C-17 (δ_C 58.5)/ C-20 (δ_C 36.4)/ C-22 (δ_C 75.8), and carbon chemical shift of C-22 (δ_C 75.8) indicated the presence of hydroxy group at C-22. The carbon chemical shift of C-16 (δ_C 72.6) was well agreed with the presence of hydroxy group at C-16. Due to lack of solid evidence, the stereochemistry of hydroxy group at C-22 was undetermined. Meanwhile the configuration of hydroxy group at C-16 was proposed to be β-configuration by the opening of E-ring of the furostane-type sterol. The HMBC correlations between Rha H-1″ (δ_H 5.22) and Glc C-2′ (δ_C 79.3), Rha H-1‴ (δ_H 4.85) and Glc C-4′ (δ_C 80.1), Glc H-1′ (δ_H 4.51) and C-3 (δ_C 79.4) indicated that the 2′,4′-di-*O*-rhamnopyranosyl glucopyranosyl group linked at C-3. The HMBC correlation between Glc H-1⁗ (δ_H 4.26) and C-26 (δ_C 76.2), H_3-27 (δ_H 0.96) and C-24 (δ_C 31.7)/ C-25 (δ_C 34.8)/ C-26 (δ_C 76.2) suggested the presence of *O*-glucopyranosyl group at C-26. Similar with **1**, the different of proton chemical shifts of oxymethylene group C-26 ($\Delta\delta_{Ha\text{-}26/Hb\text{-}26}$ = 0.27) indicated for the *β*-orientation of secondary methyl group C-26 (25*R* configuration) [7]. Consequently, compound **2** was determined to be 26-*O*-*β*-D-gluco pyranosyl cholest-5-ene-3,16,22,26-tetraol 3-*O*-(2′,4′-di-*O*-*α*-rhamnopyranosyl)-*β*-D-glucopyranoside and previously named as anguivioside A [11]. The opening in the E-ring of furostane-type steroidal saponins as in the compound **2** is unusual and rarely found in the nature. To the best of our knowledge, up to date, this compound has been previously isolated from three species including *Solanum anguivi*, *S. nigrum*, and *Cestrum newellii* [11, 12].

In summary, two steroidal saponins, protodioscin (**1**) and anguivioside A (**2**) were isolated from the seeds of *Allium ramosum*. This is the first report on the chemical constituents of *A. ramosum*. Additionally, chemical structure of compound **2** is unusual which rarely found from natural sources.

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