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Spirostane-type steroidal saponin from *Allium hookeri* roots with mushroom tyrosinase inhibitory activity

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Abstract *Allium hookeri* (Liliaceae) has been received the increasing attention as a bioactive resource due to its potent biological activities including anti-oxidant, anti-obesity, anti-microbial and lipid-regulating activities. The beneficial effects of *A. hookeri* are known contributed from the high content of organosulfur compounds in *A. hookeri*. Though a variety of articles demonstrated that *A. hookeri* contains 'saponin' as a bioactive constituent, the scientific evidence to prove it was limited. In the present study, we have attempted to identify saponin contained in *A. hookeri* through chromatographic isolation and NMR spectroscopic methods. As a result, a spirostane-type steroidal saponin (**1**) has been successfully isolated from the methanolic extract of *A. hookeri* roots. The structure of **1** was elucidated by extensive 1D and 2D spectroscopic methods including ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY; identified as (3β, 22*R*, 25*S*)-spirost-5-en-3yl *O*-6-deoxy-α-*L*-mannopyranosyl-(1→4)-*O*-6-deoxy-α-*L*-mannopyranosyl-(1→4)-O-[6-deoxy-α-*L*-mannopyranosyl-(1→2)]-β-*D*-glucopyranoside. **1** showed the significant inhibitory activity on mushroom tyrosinase with IC_{50} values of 248.7 μM while the inhibition on alpha-glucosidase was not significant.

Keywords *Allium hookeri* saponin constituent, alpha-glucosidase, mushroom tyrosinase, 1D and 2D NMR, spirosstanol oligoglycoside

Introduction

Allium hookeri Thwaites (Liliaceae) is evergreen and terrestrial perennial plant that is native to India, Sri Lanka, Myanmar, Bhutan.[1] *A. hookeri* is now widely cultivated in Asia and its fresh leaves and roots are consumed as vegetable. *A. hookeri* have been reported as showing diverse biological effects such as anti-oxidant, anti-inflammatory, anti-obesity, lipid-regulating, anti-microbial, anti-*Helicobacter pylori*. Regarding the beneficial effects of *A. hookeri* on lipid metabolism, *A. hookeri* extract attenuated the expression of GLUT-4 and adipogenesis in 3T3-L1 adipocytes.[2] In high-fat-diet-fed obese mice, the administration of *A. hookeri* for 4 weeks significantly suppressed body weight gain.[3] In high-fat-diet-fed hamster model, it was identified that glycerophospholipid metabolism was a significantly enriched pathway in *A. hookeri* extract-treated group.[4]
The unique taste of *A. hookeri* (like onion or garlic) comes from high level of sulfur compounds mainly

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contained in *A. hookeri*. The enzymatic hydrolysis of organosulfur results in the production of compounds with high volatility and strong odor.[5] Recent studies focused on the identification of bioactive organosulfur compounds that attribute to therapeutic benefits of *A. hookeri*.[6,7] One of the important issues on phytochemicals of *A. hookeri* is the presence of saponin. In Korea, it is generally known that 'Sam' of Samchae(the korean name of *A. hookeri*) means 'ginseng'. Though a variety of articles have demonstrated that high level of saponin is contained in *A. hookeri*, the scientific evidence to prove it was limited. The presence of saponin in *A. hookeri* was reported by solvent fractionation method.[8] The content of crude saponin was shown as 4.6 and 2.5 g/100g dry weight, in *A. hookeri* that is grown in Korea and Myanmar, respectively. However, there has been no report to identify saponin of *A. hookeri* through spectroscopic methods including NMR experiments.

Based on above, in the present study, we have attempted to identify saponin of *A. hookeri* through chromatographic isolation and NMR spectroscopic methods.

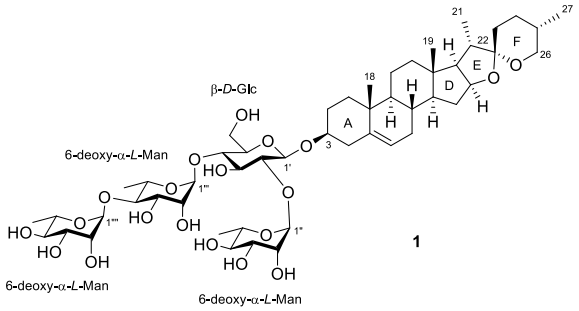

Figure 1. Structure of **1** isolated from *A. hookeri* roots

Experimental Methods

General Experimental - All NMR spectra were measured on a Varian VNMRS 500 spectrometer using $CDCl_3$ for compounds **1** and **2** and CD_3OD for compounds **3** and **4** as solvent. The ESI mass spectra were acquired using an ABSCIEX QTRAP 3200 instrument. HPLC was performed using a Varian Prostar system with a 355 refractive index (RI) detector or Agilent 1200 Chemstation with DAD detector. The separation was performed using the YMC ODS-A column. All solvents were distilled prior to use.

Plant material - *A. hookeri* was cultivated and harvested in Hadong, Korea. A voucher specimen was deposited in laboratory of pharmacognosy in Gyeongnam National University of Science and Technology (GNP-082).

Extraction and Isolation - The roots of *A. hookeri* were washed three times with five volume of distilled water and dried using freezing-dryer for 5 days. The dried roots *A. hookeri* (7.7 kg) were extracted three times with 80% methanol for 3 h each in an ultrasonic apparatus. Removal of the solvent in vacuo yielded a methanolic extract (5.3 kg). The methanolic extract was then suspended in distilled water and partitioned successively with n-hexane (47.3 g) CH_2Cl_2 (32.6 g), EtOAc (40.6 g), and n-BuOH (473.1 g). ^{1}H-NMR experiment was performed on each organic fractions to chase characteristic signals found in saponins. CH_2Cl_2 soluble fraction was subjected to column chromatography on a silica gel column using mixtures of CH_2Cl_2–MeOH of increasing polarity as eluents to give 34 fractions (M1~34). M30 was subjected to silica gel chromatography with a gradient elution CH_2Cl_2–MeOH-Water to give seventeen fractions (M30-1~17). Compound **1** (14.6 mg) was isolated from M30-11 through Sephadex LH-20 (MeOH). M28 fraction was applied to MPLC silica gel chromatography using mixtures of CH_2Cl_2–MeOH of increasing polarity as eluents to give sixteen fractions (M28-1~16). M28-6 was further subjected to MPLC silica gel chromatography with a gradient elution of CH_2Cl_2–MeOH to give fourteen fractions (M28-6-1~14). M28-6-5 was subjected to HPLC (HECTOR-M C_{18}, 5 μm 4.6 x 250 mm, 60% MeOH, 1.0 ml/min) to give fourteen fractions (M28-6-5-1~14).

Results and Discussion

The methanolic extract of *A. hookeri* roots was sus-

pended in water and successively fractionated with *n*-hexnae, CH_2Cl_2, EtOAc, *n*-BuOH. The CH_2Cl_2 fraction was subjected to repeated column chromatography to yield compound **1** (Figure 1). Compound **1** was obtained as white powders. The molecular formula were deduced as $C_{51}H_{82}O_{20}$ by the combination of the positive- and negative-ion ESI-MS at m/z 1037 $[M + Na]^+$ and 1013 $[M - H]^-$ and the ^{13}C NMR spectrum. The ^1H and ^{13}C NMR spectra of **1** measured in CD_3OD solvent featured the overlapped signals in the upfield and medium field regions, which is reminiscent of the typical signals for saponin compounds (Figure 5). The assignable proton signals in the ^1H NMR spectrum of **1** was composed of four doublet methyls, singlet methyls, and four well-separated oxymethine protons at δ_H 4.82, 5.16, 5.17 (1H each, all br s, H-1''', H-1'''', H-1'') and δ_H 4.49 (1H, d, *J* = 7.6 Hz, H-1') which correspond to the anomeric protons for carbohydrate moieties. The aglycon part of **1** was elucidated by the COSY cross peaks on the basis of the HMBC correlations of the four methyl protons with their neighboring carbons (Figure 2). Specifically the presence of spiroketal group was suggested by the H-20 / C-22, H-23 / C-22, H-26 / C-22 HMBC correlations and supported by the H-16 / H-26 ROESY cross peak. The determined structure of the aglycon of **1** resulted in the spirostanol skeleton. Following this, the configuration of rings A~E in the aglycon substructure was characterized by the ROESY correlations: H-1α / H-3, H-1β / Me-19, H-8 / Me-18, H-8 / Me-19, H-14 / H-16, H-16 / H-17, H-17-H / Me-21 and Me-18 / H-20.

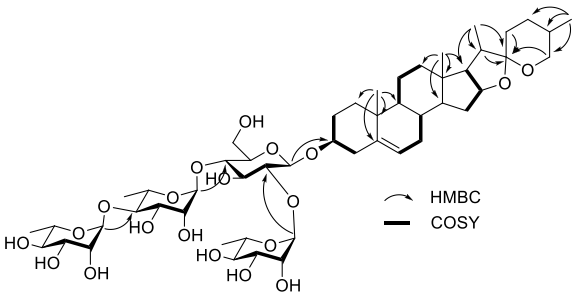

Figure 2. Key COSY and HMBC correlations of **1**

Furthermore, the ROE correlations of H-19 / H-23α, H-24α / H-20, H-16 / H-26α, Me-27 / H-26α and Me-27 / H-26β allowed us to establish the configuration of ring F as 22*R* and 25*S* (Figure 3). This result is nearly consistent with the ^{13}C chemical shift patterns of 22*S* and 22*R*-spirostanol oligoglycosides demonstrated by Yoshikawa et al., (2007).[9] The corresponding signals of 23- and 24-carbons (δC-23: 31.4~31.8, δC-24: 29.3~32.7) in 22*R*-*O*-spirostanol oligoglycosides were observed at downfield compared with those of corresponding 22*S*-*O*-spirostanol oligoglycosides (δC-23: 27.0~28.4, δC-24: 28.1~28.6). On the other hands, the signals of the 26-carbon (δC-26: 47.8~66.9) in 22*R*-*O*-spirostanol oligoglycosides were observed at relatively upfield compared with those of corresponding 22*S*-*O*-spirostanol oligoglycosides (δC-26: 50.2~69.7).

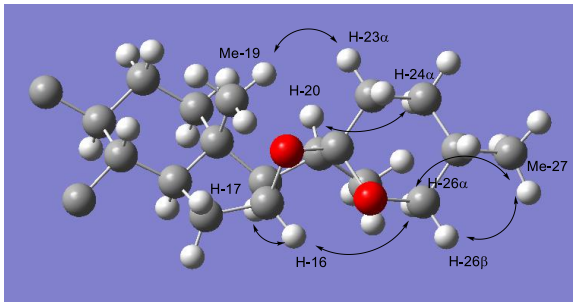

Figure 3. Key ROESY correlations for ring F in **1**.

On the other hand, the carbohydrates in **1** were determined as one β-*D*-glucose and three 6-deoxy-α-*L*-mannoses by the coupling constants measurement and ROESY correlations. The three 6-deoxy-hexoses showed commonly the same ROESY correlations as given in Figure 4, indicating 6-deoxy-mannose units. The other one sugar was revealed as β-glucose unit by the strong coupling between protons and the H-1' / H-5', H-1' / H-3', H-2' / H-4' ROESY peaks.

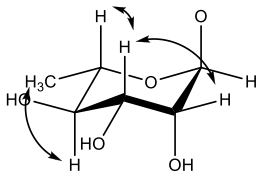

Figure 4. ROESY correlations for 6-deoxy-hexose in **1**

Most of the naturally occurring sugars is *D* form, while 6-deoxy-mannose occurs as *L* form in nature. With this aspect, the small coupling values of H-2", H-3"' and H-4"" led to designate the three common sugars as 6-deoxy-α-L-mannose. In the HMBC spectrum, the long-range correlations between the protons and carbons of aglycon and carbohydrate units were observed: H-1' / C-3, H-1" / C-2', H-1"' / C-4', H-1"" / C-4"' (Figure 2). The constructed compound **1** proved to be one of corresponding 22*R*-*O*-spirostanol oligoglycosides. Accordingly **1** was determined to be (3β, 22*R*, 25*S*)-spirost-5-en-3yl *O*-6-deoxy-α-*L*-mannopyranosyl-(1→4)-*O*-6-deoxy-α-*L*-mannopyranosyl-(1→4)-O-[6-deoxy-α-*L*-mannopyranosyl-(1→2)]-β-*D*-glucopyranoside.

In the previous study, anti-adipogenic and antidiabetic activities of *A. hookeri* have been assessed. In 3T3-L1 adipocytes, the treatment of the water extract of *A. hookeri* modulated the expression of adipokines associated with insulin resistance and sensitivity. Also, the extract of *A. hookeri* increased the expression of GLUT-4 which induces glucose uptake into adipocytes.[2] Regarding anti-obesity and hypolipidemic effects of *A. hookeri* in vivo, the effects of two different extracts of *A. hookeri* prepared by different drying methods (hot-air of low-temperature) were compared in high-fat diet induced obese mice.[10] Interestingly, although total amount of volatile sulfur compounds was found lower in hot-air dried extract of *A. hookeri* than in low-temperature dried extract, the hot-air dried one showed the potent activity to attenuate the weight gain and to decrease fat mass. These results suggested that another bioactive constituent exists besides volatile sulfur compounds that are responsible for anti-adipogenic or antidiabetic activities of *A. hookeri*.

Recently, the potential of *A. hookeri* as a bioactive material in functional cosmetics has been emerged. It has been demonstrated that the organic fraction prepared from the extract of hot-air-dried *A. hookeri* (leaf or roots) showed the significant inhibition on tyrosinase activity and melanin production. The inhibitory activity of *A. hookeri* fraction was more potent compared to the positive control, arbutin.[11] In mouse melanoma B16F10 cells, the extract of *A. hookeri* reduced the melanin secretion and the expression of tyrosinase enzyme. Also, the expressions of enzymes necessary for melanogenesis in B16F10 cells such as TRP1 and TRP2 were significantly attenuated by *A. hookeri* extract. On the basis of these results, we attempted to evaluate the activity of **1**, the firstly reported saponin from *A. hookeri*, on α-glucosidase and mushroom tyrosinase. In mushroom tyrosinase assay, **1** showed the significant inhibition on tyrosinase enzyme with an IC50 value of 248.7 μM. The activity of tyrosinase was inhibited by 59.21% at the concentration of 250 μM of **1**. On the other hand, the inhibitory activity of **1** on α-glucosidase was not significant. The weak inhibition (13.7%) on α-glucosidase activity was found at the concentration of 400 μM of **1**; the inhibition was not increased from the concentration 400 uM upwards. Kojic acid and acarbose were used as positive control for mushroom tyrosinase and α-glucosidase assay, respectively. From above results, spirostan-type steroidal saponin (**1**) that is firstly identified from *A. hookeri* roots is thought as an active constituent that contributes to whitening effect of *A. hookeri*.

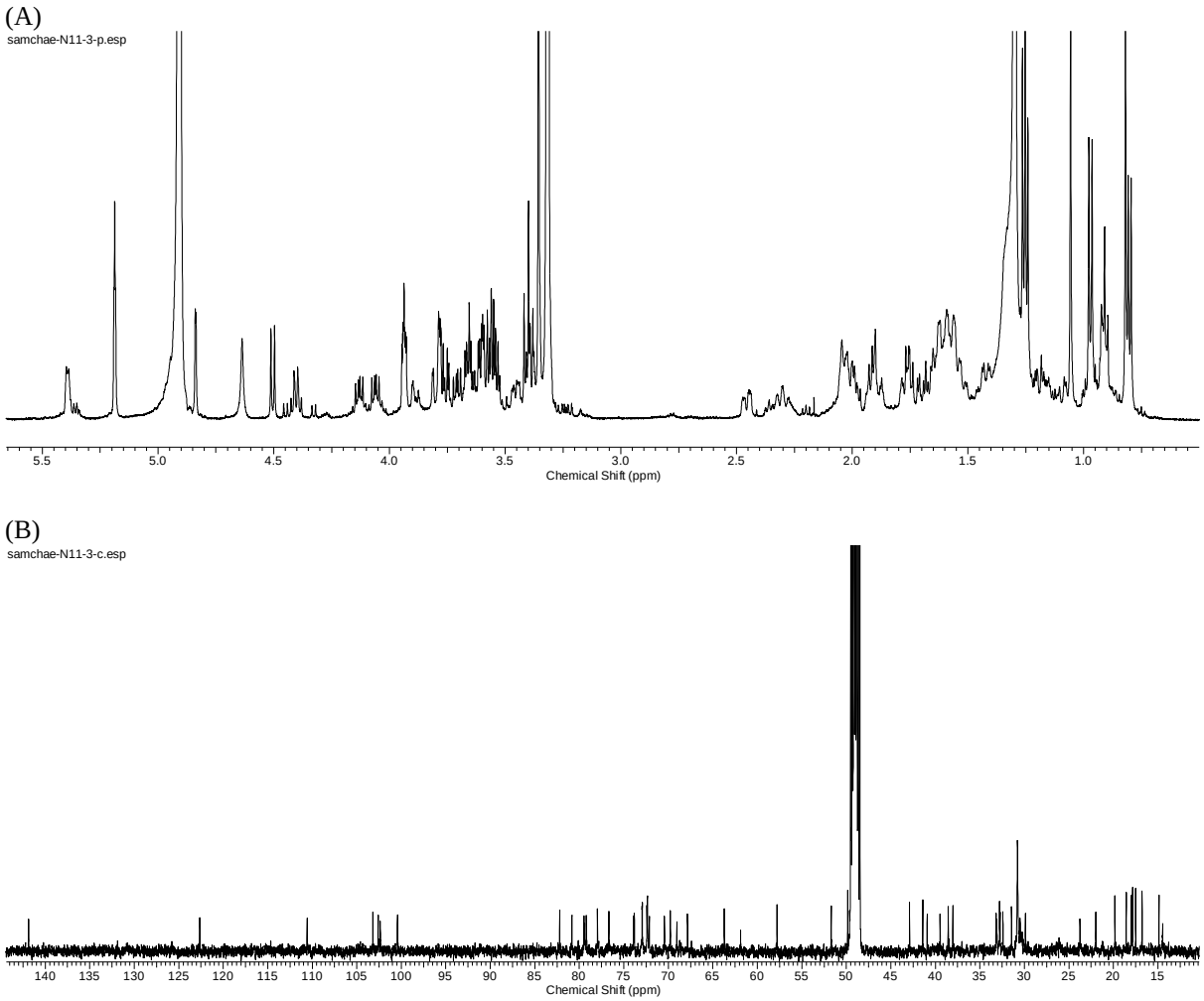

Figure 5. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of compound **1**

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** in $CDCl_3$ recorded at 500 MHz and 125 MHz

Aglycon			Carbohydrate units			
no	δ_H, mult, *J* Hz	δ_C	no		δ_H, mult, *J* Hz	δ_C
1	1.06, m	38.6, CH_2	β-*D*-Glc	1'	4.49, d, 7.8	100.4, CH
	1.86, m			2'	3.38, dd, 7.8, 8.8	79.5, CH
2	1.59, m	30.8, CH_2		3'	3.55, m	78.0, CH
	1.90, m			4'	3.53, m	79.4, CH
3	3.58, m	79.2, CH		5'	3.30, m	76.7, CH
4	2.29, t, 12.7	39.5, CH_2		6'	3.77, m; 3.64, m	61.9, CH_2
	2.43, dd, 12.7, 3.7					
5		141.9, C	6-deoxy-α-*L*-Man	1"	5.17, d, 1.6	102.4, CH
6	5.39, m	122.6, CH		2"	3.92, m	72.1, CH
7	1.54, dd, 12.5, 3.4	33.2, CH_2		3"	3.65, m	72.3, CH
	1.97, dd, 12.5, 5.8			4"	3.39, m	73.9, CH
8	1.65, m	32.8, CH		5"	4.11, dq, 9.4, 6.1	69.8, CH

9	0.95, m	51.7, CH		6"	1.25, d, 6.1	18.0, CH_3
10		38.0, C				
11	1.54, m	22.0, CH_2		1‴	4.82, d, 1.6	102.6, CH
12	1.19, m	40.9, CH_2		2‴	3.75, m	72.92, CH
	1.76, dd, 8.3, 6.5		6-deoxy-α-*L*-Man	3‴	3.75, m	72.94, CH
13		41.4, C		4‴	3.53, m	80.8, CH
14	1.13, m	57.8, CH		5‴	4.04, dq, 9.5, 6.4	69.0, CH
15	1.27, m	32.7, CH_2		6‴	1.30, d, 6.4	18.6, CH_3
	1.97, m					
16	4.39, m	82.2, CH		1⁗	5.16, d, 1.7	103.2, CH
17	1.72, m	63.7, CH		2⁗	3.94, m	72.4, CH
18	0.80, s	16.8, CH_3	6-deoxy-α-*L*-Man	3⁗	3.59, m	72.3, CH
19	1.04, s	19.8, CH_3		4⁗	3.38, m	73.8, CH
20	1.90, m	42.9, CH		5⁗	3.69, dq, 9.4, 6.2	70.4, CH
21	0.95, d, 6.8	14.9, CH_3		6⁗	1.26, d, 6.2	17.9, CH_3
22		110.7, C				
23	1.56, m	32.4, CH_2				
	1.69, t, 4.9					
24	1.40, m	29.9, CH_2				
	1.62, m					
25	1.59, m	31.4, CH				
26	3.31, m	67.9, CH_2				
	3.44, dd, 10.6, 4.0					
27	0.78, d, 6.4	17.5, CH_3				

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