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Two New Chemical Constituents from the Rhizome of *Sparganium stoloniferum*

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Sparganium (Bur-reed) is a genus of flowering plants, which contains about 20 species in temperate regions of both the Northern and Southern Hemispheres. Three *Sparganium* species, *S. stoloniferum*, *S. angustifolium*, and *S. japonicum*, grow in Korea. *S. stoloniferum* is widely distributed in the wet valley areas, and has been used as an emmenagogue, a galactogogue, and an antispasmodic agent in Chinese folk medicine,[1,2] and also for the treatment of menstrual disorders and chronic hepatitis.[3] Previous phytochemical investigations on this plant reported the isolation of pyrrole carboxylic acid ester,[4] phenylpropanoid glycosides,[5-7] and two sucrose esters.[8] Aldose reductase inhibition,[9] *anti*-inflammatory, and *anti*-thrombotic[10] activities of an EtOH extract have also been reported. In our continuing study on the constituents of Korean medicinal plant sources, we have identified molecules from the rhizome of *S. stoloniferum.* Column chromatograpic purification of the MeOH extract of the rhizome of this source led to isolation of two new constituents (**1**-**2**), together with three known compounds (**3**-**5**). The structures of the new compounds (**1**-**2**) were determined through spectral analysis, and chemical means. The isolated compounds (**1**-**5**) were tested for cytotoxicity against four human tumor cells *in vitro* using a sulforhodamin B (SRB) bioassay.

Compound **1** was isolated as a colorless gum, $[\alpha]_D^{25}$ +4.0° (*c* 0.2, MeOH). The molecular formula $C_{11}H_{13}NO_6$ was determined by the HR-FAB MS *m/z* 255.0743 $[M]^+$ (calcd. 255.0743). Compound **1** displayed three proton signals at δ_H 7.02 (1H, m, H-5'), 6.92 (1H, m, H-3'), 6.22 (1H, m, H-4') in an ^{1}H-NMR spectrum and five carbon signals at δ_C 160.1, 124.3, 121.0, 116.5, and 109.8 in a ^{13}C-NMR spectrum, which were assignable to 1*H*-pyrrole-2-carboxylic acid.[11] The ^{1}H NMR spectrum also showed signals characteristic of 1,4-dimethyl malate group at δ_H 5.60 (1H, t, *J* = 7.0 Hz, H-2), 3.78 (3H, s, OCH_3-4), 3.73 (3H, s, OCH_3-1), and 3.02 (2H, m, H-3). The corresponding carbon resonances of these protons were observed at δ_C 170.4, 170.1, 68.2, 51.8, 51.3, and 35.5 in the HMQC spectrum. In addition, ^{1}H-^{1}H COSY correlations between the methine proton signal at δ_H 5.60 (t, *J* = 7.0 Hz, H-2), and the methylene proton signals at δ_H 3.02 (m, H-3) were observed. The HMBC correlations between the methoxy group at δ_H 3.78 (OCH_3-4) and the carbonyl carbon at δ_C 170.1 (C-4) and the other methoxy group at δ_H 3.73 (OCH_3-1) and carbonyl carbon at δ_C 170.4 (C-1) implied that two methoxy groups were present at C-1 and C-4. These data indicated the presence of a 1,4-dimethyl malate group.[12] The HMBC spectrum showed that the methine proton at 5.60 (1H, t, *J* = 7.0 Hz, H-2) correlated with the carbonyl carbon at δ_C 160.1 (C-6') (Fig. 2). Thus, compound **1** was

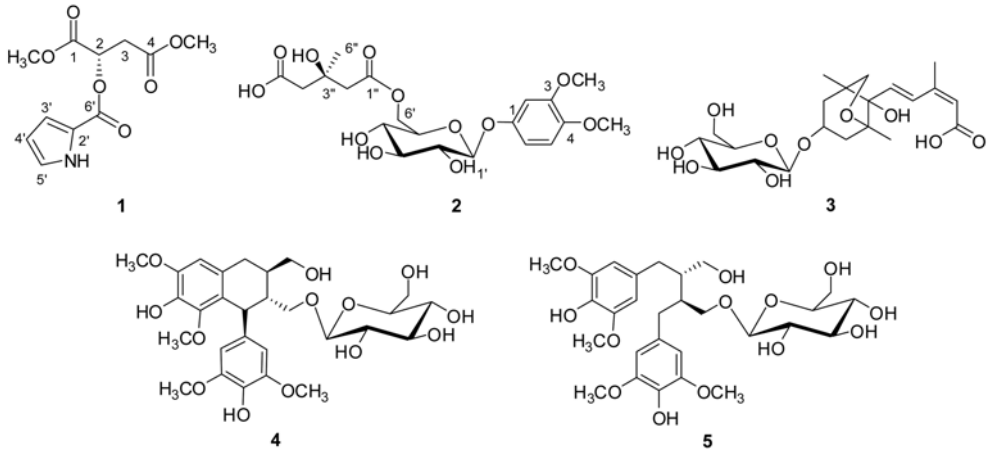

Figure 1. Chemical structures of compounds **1**-**5**.

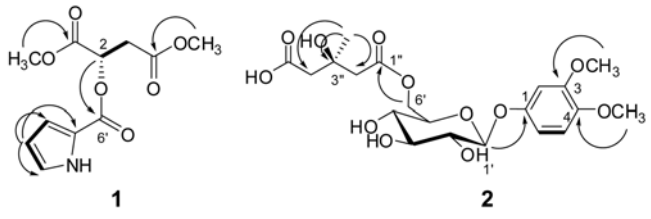

Figure 2. Key ^{1}H-^{1}H COSY (▬) and HMBC (⟶) correlations of **1-2**.

deduced as 1,4-dimethyl-2-(1*H*-pyrrole-2'-carbonyloxy)-malate. Alkaline hydrolysis (0.1 M KOH) afforded 1,4-dimethyl malate (**1a**), which was identified by the comparison of its optical rotation value, ^{1}H-NMR and MS spectra.[12] The 1,4-dimethyl malate with *S* configuration at C-2 was reported to show a positive optical rotation ($[\alpha]_D$ +20.4, $CHCl_3$).[13] The optical rotation of **1a** exhibited a positive value ($[á]_D$ +27.5, $CHCl_3$), indicating that the absolute configuration at C-2 in **1a** was to be the *S* form. Thus, compound **1** was determined to be (2*S*) 1,4-dimethyl-2-(1*H*-pyrrole-2'-carbonyloxy)-malate.

Compound **2** was isolated as a pale yellow gum, $[\alpha]_D^{25}$ −14.0° (*c* 0.15, MeOH). The molecular formula $C_{11}H_{13}NO_6$ was determined by the HR-FAB MS *m/z* 460.1584 $[M]^+$ (calcd. 460.1581). The ^{1}H-NMR spectrum of **2** showed three aromatic protons at δ_H 6.95 (1H, d, *J* = 8.5 Hz, H-5), 6.46 (1H, d, *J* = 2.5 Hz, H-2), and 6.32 (1H, dd, *J* = 8.5, 2.5 Hz, H-6), two methoxy groups at δ_H 3.80 (3H, s, H-4), and 3.75 (3H, s, H-3). In the ^{13}C-NMR spectrum, 8 carbon signals appeared, including two methoxyl carbons at δ_C 55.6 and 55.4, and an aromatic carbon at δ_C 153.9, 151.0, 139.5. 120.0, 106.6, and 100.8. which were assignable to 1,3,4-trisubstitued aromatic ring structure.[14] Also, signals of the sugar unit appeared at δ_H = 4.68 (1H, d, *J* = 7.5 Hz, H-1'), 4.44 (1H, dd, *J* = 11.5, 2.0 Hz, H-6'a), 4.20 (1H, dd, *J* = 11.5, 6.5 Hz, H-6'b), 3.51 (1H, m, H-5'), 3.43 (1H, m, H-2'), 3.41 (1H, m, H-3'), and 3.38 (1H, m, H-4') in the ^{1}H-NMR spectrum and δ_C 103.1, 76.5, 74.2, 73.8, 70.4, and 63.4 in the ^{13}C-NMR spectrum, which suggested the presence of D-glucopyranose unit.[15] The coupling constant (*J* = 7.5 Hz) of the anomeric proton of D-glucose indicated to be in the *β*-form.[15] Additionally, ^{1}H, and ^{13}C-NMR spectra showed signals for a 3-hydroxy-3-methylglutaryl group (HMG)[16]; a tert-methyl at δ_H 1.29 (3H, s, H-6"), and δ_C 26.6 (C-6"), two methylenes at δ_H 2.57 (2H, s, H-2"), and δ_C 46.1 (C-2"); 2.50 (1H, d, *J* = 15.5 Hz, H-4"a), and 2.34 (1H, d, *J* = 15.5 Hz, H-4"b), δ_C 46.8 (C-4"), and three quaternary carbons at δ_C 178.5 (C-5"), 171.5 (C-1"), and 69.7 (C-3"). The glucose position was established by an HMBC experiment, in which a long-range correlation was observed between the δ_H 4.38 (H-1) of D-glucose and the δ_C 139.5 (C-1) of the 1,3,4-trisubstituted aromatic ring. Also the location of HMG group was determined by correlations between δ_H 4.44, 4.20 (H-6') of the D-glucose moiety and δ_C 171.5 (C-1"), in the HMBC spectrum (Fig. 2). Alkaline methanolysis (1% NaOMe in MeOH) of **2** afforded 3-hydroxy-3-methylglutarate (**2a**), which was identified by the comparison of its optical rotation value, as well as ^{1}H-NMR and MS spectra.[17] The glucose was identified with authentic samples (Aldrich Co.) using silica gel co-TLC ($CHCl_3$:MeOH;H_2O = 9:4:0.5, R_f 0.30), and optical rotation value {$[\alpha]_D^{25}$ +49.5, (*c* 0.02, H_2O)}. The 3-hydroxy-3-methylglutarate with *S* configuration at C-3" was reported to show a positive optical rotation ($[\alpha]_D$ +8.3, $CHCl_3$).[17] The optical rotation of **2a** exhibited a positive value ($[\alpha]_D$ +17.1, $CHCl_3$), indicating that the absolute configuration of the asymmetric carbon at C-3" of the HMG moiety was determined to be *S* form. Thus, the structure of **2** was determined to be 3,4-dimethoxyphenyl-1-*O*-*β*-D-[6'-*O*-[(3"*S*)-3"-hydroxy-3"-methyl-glutaryl]]-glucopyranoside.

Known compounds were identified as dihydrophaseic acid 3-*O*-*β*-D-glucopyranoside (**3**),[18] (+)-lyoniresinol 3*α*-*O*-*β*-D-glucopyranoside (**4**),[19] and (+)-5,5'-dimethoxy secoisolariciresinol 3*α*-*O*-*β*-D-glucopyranoside (**5**)[20] by comparison of physicochemical and spectroscopic data with previously reported literature values. Compounds **3-5** were isolated for the first time from this plant.

The cytotoxicities of compounds (**1-5**) were evaluated against the A549, SK-OV-3, SK-MEL-2, and HCT15 human cancer cell lines *in vitro* using the Sulforhodamine B (SRB) bioassay.[21] All the compounds showed little cytotoxicity against any tested cell line (IC_{50} > 100 μM).

Experimental Section

Plant Materials. *Sparganium stoloniferum* Buch.-Hamil. was purchased in Yeongchenon, Korea, in September, 2008, and the plant was identified by one of the authors (K.R.L.). A voucher specimen (SKKU 2008-19) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. The dried and chopped rhizomes of *S. stoloniferum* (5 kg) were extracted at room temperature with 80% MeOH and evaporated under reduced pressure to give a residue (280 g), which was dissolved in water (800 mL) and solvent-partitioned, resulting in *n*-hexane (17 g), CH_2Cl_2 (3 g), EtOAc (4 g), and *n*-BuOH (30 g). The EtOAc fraction (4 g) was separated over a silica gel column with a solvent system ($CHCl_3$:MeOH:H_2O = 25:3:0.1 – 100% MeOH) to give nine fractions (E1-E9). Fraction E1 (10 mg) was separated on a RP-C_{18} silica gel column with 100% MeOH and purified with a RP-C_{18} prep HPLC (95% MeOH) to yield compound **1** (4 mg, R_t = 13 min). The *n*-BuOH fraction (30 g) was separated over a silica gel column with a solvent system of ($CHCl_3$:MeOH:H_2O = 14:3.7:0.1 – 100% MeOH) to give nine fractions (B1-B10). Fraction B4 (400 mg) was separated over a RP-C_{18} Lobar A®-column with a solvent system of 40% MeOH to give two subfractions (B41-B42). Subfraction B41 (15 mg) was purified with a RP-C_{18} prep HPLC (50% MeOH) to yield compound **5** (5 mg, R_t = 15 min). Fraction B7 (590 mg) was subjected to Sephadex LH-20 column chromatography eluted with 100% MeOH as to give seven subfractions (B71-B77). Subfraction B72 (34 mg) was purified with a silica gel

prep HPLC (CH_3Cl:MeOH = 2:1) to yield compound **3** (4 mg, R_t = 16 min). Subfraction B76 (30 mg) was purified with a RP-C_{18} prep HPLC (50% MeOH) to yield compound **2** (5 mg, R_t = 13 min). Subfraction B77 (19 mg) was purified with a RP-C_{18} prep HPLC (50% MeOH) to yield compound **4** (5 mg, R_t = 17 min).

(2*S*) 1,4-Dimethyl-2-*O*-(1*H*-pyrrole-2'-carbonyloxy)-malate (1). Colorless gum, $[\alpha]_D^{25}$ +4.0° (*c* 0.2 in MeOH); FAB-MS *m/z*: 255 $[M]^+$; HR-FAB-MS *m/z*: 255.0743 $[M+]^+$ (calculated for $C_{11}H_{13}NO_6$, 255.0743); ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.02 (1H, m, H-5'), 6.92 (1H, m, H-3'), 6.22 (1H, m, H-4'), 5.60 (1H, t, *J* = 7.0 Hz, H-2), 3.78 (3H, s, OCH_3-4), 3.73 (3H, s, OCH_3-1), 3.02 (2H, m, H-3); ^{13}C-NMR (CD_3OD, 125 MHz): δ 170.4 (C-1), 170.1 (C-4), 160.1 (C-6'), 124.3 (C-5'), 121.0 (C-2'), 116.5 (C-3'), 109.8 (C-4'), 68.2 (C-2), 51.8 (OCH_3-4), 51.3 (OCH_3-1), 35.5 (C-3).

3,4-Dimethoxyphenyl-1-*O*-*β*-D-[6'-*O*-[(3"*S*) 3"-hydroxy-3"-methyl-glutaryl]]-glucopyranoside (2). Pale yellow gum, $[\alpha]_D^{25}$ −14.0° (*c* 0.15 in MeOH); FAB-MS *m/z*: 460 $[M]^+$; HR-FAB-MS *m/z*: 460.1584 $[M+Na]^+$ (calculated for $C_{20}H_{23}O_{12}$, 460.1584); ^{1}H-NMR (CD_3OD, 500 MHz): δ 6.95 (1H, d, *J* = 8.5 Hz, H-5), 6.46 (1H, d, *J* = 2.5 Hz, H-2), 6.32 (1H, dd, *J* = 8.5, 2.5 Hz, H-6), 4.68 (1H, d, *J* = 7.5 Hz, H-1'), 4.44 (1H, dd, *J* = 11.5, 2.0 Hz, H-6a), 4.20 (1H, dd, *J* = 11.5, 6.5 Hz, H-6b), 3.80 (3H, s, OCH_3-4), 3.75 (3H, s, OCH_3-3), 3.51 (1H, m, H-5'), 3.43 (1H, m, H-2'), 3.41 (1H, m, H-3'), 3.38 (1H, m, H-4'), 2.57 (2H, s, H-2"), 2.50 (1H, d, *J* = 15.5 Hz, H-4"a), 2.34 (1H, d, *J* = 15.5 Hz, H-4"b), 1.29 (3H, s, H-6"); ^{13}C-NMR (CD_3OD, 125 MHz): δ 178.5 (C-5"), 171.5 (C-1"), 153.9 (C-4), 151.0 (C-3), 139.5 (C-1), 120.0 (C-5), 106.6 (C-6), 103.1 (C-1'), 100.8 (C-2), 76.5 (C-3'), 74.2 (C-5'), 73.8 (C-2'), 70.4 (C-4'), 69.7 (C-3"), 63.4 (C-6'), 55.6 (OCH_3-4), 55.4 (OCH_3-3), 46.8 (C-4"), 46.1 (C-2"), 26.6 (C-4").

Alkaline Hydrolysis of Compound 1. Compound **1** (1.7 mg) was hydrolyzed with 0.1 M KOH (1 mL) at room temperature for 3 h. Then H_2O (3 mL) was added and the mixture was extracted with $CHCl_3$ three times, and the $CHCl_3$ extract was evaporated *in vacuo*. The $CHCl_3$ extract was purified over a silica gel Waters Sep-Pak Vac 6cc ($CHCl_3$:MeOH = 10:1) to give **1a**, which was identified by ^{1}H-NMR, MS and optical rotation.

1a: Colorless gum; FAB-MS *m/z*: 163 $[M+H]^+$; $[\alpha]_D^{25}$ +27.5° (*c* 0.08 in $CHCl_3$); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.85 (2H, m, H-3), 3.72 (3H, s, OCH_3-1), 3.81 (3H, s, OCH_3-4), 3.20 (1H, br s, OH), 4.51 (1H, m, H-2).

Alkaline Methanolysis of Compound 2. Compound **2** (2.0 mg) was treated with 1% NaOMe in MeOH (1 mL) at room temperature for 3 hr. The reaction mixture was neutralized through an Amberlite IR-120B column and chromatographed on Sephadex LH-20 with MeOH to give **2a**, which was identified by ^{1}H-NMR, MS and optical rotation.

2a: Colorless gum; FAB-MS *m/z*: 176 $[M]^+$; $[\alpha]_D^{25}$ +17.1° (*c* 0.06 in $CHCl_3$); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.73 (3H, s, OMe), 2.72 (1H, d, *J* = 16.0, H-4"a), 2.71 (1H, d, *J* = 16.0, H-2"a), 2.67 (1H, d, *J* = 16.0, H-4"b), 2.65 (1H, d, *J* = 2.0, H-2"b), 1.30 (3H, s, H-6").

A detailed description of the bioassays is available in the Supporting Information. The positive control, doxorubicin (purity ≥ 98%) was purchased from Sigma Corporation.

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Supporting Information. Spectral data of compounds **1** and **2**, general experimental procedures and bioassay protocols are available upon request from the correspondence author.

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