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New compounds from the aerial parts of *Calligonum mongolicum*

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ABSTRACT

Previously undescribed compounds, (*R*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside (**1**) and (*E*)-5-(4-hydroxyphenyl)pent-2-enoic acid (**2**), together with 20 known compounds (**3-22**) were isolated from the aerial parts of *Calligonum mongolicum*. Compound **3** was obtained from a natural source for the first time, and all of the compounds (**1-22**) were reported for the first time in this plant. The structural elucidation of compounds **1** and **2** were performed mainly by HRFABMS, HREIMS, ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, and HMBC spectroscopy. Some of the enzyme inhibitory activities of the isolated compounds were estimated, and catechin (**7**) showed ten times higher phenoloxidase inhibitory activity (IC_{50} 9.1 μM) than epicatechin (**8**) (IC_{50} 148.3 μM). Compounds **7** and **8** have a common molecular structure, except for their stereochemistry, and this result was supported by a reproducibility test using pure guaranteed authentic samples.

1. Introduction

The genus *Calligonum* belongs to the family Polygonaceae, with about 80 species distributed in Northern Africa, Southern Europe, and Western Asia (Okasaka et al., 2004). *Calligonum mongolicum* is a shrub plant distributed in Middle Asia, including Mongolia, and is used for firewood and prevents aeolian erosion. It is an important fodder of livestock, especially camels (Jigjidsuren and Johnson, 2003). This plant is traditionally used as a hemostatic, nasal hemorrhagic, and for the relief of menstruation (Jigjidsuren and Johnson, 2003). Previous phytochemical investigations on several species of this genus led to the isolation of flavonoids (Ahmed et al., 2016), steroids (Samejo et al., 2013a), stilbenes (Okasaka et al., 2004), butanolides (Yawer et al., 2007), and terpenoids (Samejo et al., 2013b); some of which exhibited significant pharmacological activities, including cytotoxicity, anti-oxidative, anti-lipoxygenase, and antibacteria (Okasaka et al., 2004; Ahmed et al., 2016; Yawer et al., 2007). However, to the best of our knowledge, there is no previous report on the chemical constituents of a single species of *C. mongolicum*.

As part of our continuing investigation of the phytochemical and bioactive compounds of Polygonaceae plants, we have succeeded in the isolation of twenty-two (**1-22**) compounds from the aerial parts of this plant. Among these isolated compounds, **1** and **2** were previously undescribed compounds, and **3** was obtained from a natural source for the first time. Furthermore, all compounds (**1-22**) were isolated from this species for the first time. The inhibitory activities against cholinesterase, tyrosinase, and insect phenoloxidase of these isolated compounds were estimated to clarify the biological effects of the constituents on enzymes.

2. Results and discussion

The acetone-water (4:1) extract of aerial parts of *C. mongolicum* was suspended in water and partitioned with diethyl ether to obtain water and diethylether extracts. The water extract was subjected to the column using HP-20 resin. From each obtained fraction, compounds **1**, **4**, **7**–**9**, **13**, **14**, **17**, and **18** were purified using preparative high-performance liquid chromatography (HPLC). The diethyl ether extract was subjected to silica gel column chromatography, and then compounds **2, 3, 5**, **6**, **10**–**12**, **15**, **16**, **19**, and **20**–**22** were isolated using HPLC. The chemical structures of the isolated compounds were elucidated using spectroscopic data, including ^{1}H and ^{13}C NMR, HRFABMS, HREIMS, and specific rotation. Particularly, compounds **3**–**22** were identified by comparison of their spectral data with literature reported previously, (2*R*, 4a*S*, 8a*S*)-4a-hydroxy-2-methyl-3,4,4a,8a-tetrahydrobenzo-1 (2 H)-pyran-7 (8)-one (**3**) (Barradas et al., 2009), rhododendrin (**4**) (Kim et al., 2011), (*R*)-(−)-rhododendrol (**5**) (Kim et al., 2011), 4-(2-oxobutyl) phenol (**6**) (Bunce and Reeves, 1989), catechin (**7**) (Galotta et al., 2008),

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epicatechin (**8**) (Davis et al., 1996), dihydrokaempferol (**9**) (Lee et al., 2003), dihydroquercetin (**10**) (Keihlmann and Slade, 2003), kaempferol (**11**) (Chang et al., 2000), quercetin (**12**) (Chang et al., 2000), kaempferol 3-*O*-β-D-glucuronopyranoside (**13**) (Dini et al., 2004), quercetin 3-β-*O*-glucuronopyranoside (**14**) (Castillo-Muñoz et al., 2009), *N*-*trans*-feruloyltyramine (**15**) (Kim et al., 2005), *N*-*cis*-feruloyltyramine (**16**) (Fukuda et al., 1983), 2-(4-hydroxyphenyl)ethyl--β-D-glucopyranoside (**17**) (Shi et al., 2011), isopentyl β-D-glucopyranoside (**18**) (Kurashima et al., 2004), *p*-hydroxy-*trans*-cinnamic acid (**19**) (Satake et al., 1980), *p*-hydroxybenzoic acid (**20**) (Chang et al., 2000), protocatechuic acid (**21**) (Zhang et al., 1998), and gallic acid (**22**) (Gottlieb et al., 1991), as shown in Fig. S18. Compound **1** and **2** were obtained as new compounds. Therefore, the structure determination procedures of them are described below.

Compound **1** (Fig. 1) was formulated as $C_{23}H_{29}O_{11}$ (*m/z*, 481.1709 $[M+H]^+$; calcd for $C_{23}H_{29}O_{11}$, 481.1710) by HRFABMS. In the ^{1}H NMR spectrum of **1**, a singlet proton at δ 7.11 (2H, s, H-2‴ and 6‴) and a set of *o*-coupling doublet methine protons at δ 6.91 (2H, d, J =8.5 Hz, H-2′, H-6′) and 6.59 (2H, d, J =8.5 Hz, H-3′, H-5′) were observed in its aromatic field. The singlet proton and its correlated carbons in the HMQC and HMBC spectra (δ 121.6, C-1‴; 110.3, C-2‴ and 6‴; 146.6, C-3‴ and 5‴; 139.9, C-4‴; 168.5, C-7‴) showed the presence of a galloyl moiety. The *o*-coupling doublet methine protons and remaining aromatic carbon resonances (δ 134.7, C-1′; 130.4, C-2′ and 6′; 116.0, C-3′ and 5′; 156.1, C-4′) indicated the presence of a *p*-substituted benzene ring. The lower field shifted C-4′ carbon suggested it was oxygenated. The ^{13}C NMR spectrum of **1** showed 10 carbon resonances in the aliphatic field, including 1 methyl, 3 methylene, and 6 methine carbons. Among them, the oxygenated five methine and one methylene carbons (δ 102.6, C-1″; 75.2, C-2″; 78.2, C-3″; 71.9, C-4″; 75.4, C-5″; 64.9, C-6″) indicated the presence of a 6-acylated glucopyranosyl moiety (Shikishima et al., 2001). This glucosyl moiety was verified as D-glucose by sugar analysis using HPLC after acid hydrolysis of **1**. The HMBC long-range correlations from H-6″ to C-7‴ showed that the acyl group was the galloyl group (Fig. 2). The ^{1}H-^{1}H COSY spectrum of **1** showed a 2-oxygenated butyl moiety (δ 1.17, 3H, d, J =6.0 Hz, H-1; 3.80, 1H, m, H-2; 1.78, 1H, m, H-3; 1.65, 1H, m, H-3; 2.52, 2H, m, H-4) (Fig. 2). The HMBC long-range correlation between H-1″ (δ 4.34, 1H, d, J =7.5 Hz) and C-2 indicated that the D-glucopyranosyl moiety was connected to C-2. The coupling constant (J = 7.5 Hz) of the anomeric proton in the glucosyl moiety indicated the β-orientation of the sugar moiety. The HMBC spectrum (Fig. 2) showed long-range correlations of H-4 with C-1', C-2', and C-6', which established that the *p*-substituted benzene ring was connected to C-4. From these data, the chemical structure of **1** was determined to be 4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside. Although this molecular structure is the same as that of (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside (Shikishima et al., 2001), the NMR data was not identical to that of the compound. The glycosidation shifts rule for (2*R*)- and (2*S*)-pentanol (Seo et al., 1978) was attempted for **1**, comparing it with (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside and 4-(4-hydroxyphenyl)-2-butanol (Shikishima et al., 2001); the (2*R*)-configuration of **1** was suggested (Fig. S). Moreover, the 2*R*-configuration rhododendron (**4**) (Kim et al., 2011) and (*R*)-(−)-rhododendrol (**5**) (Kim et al., 2011) were isolated together with **1**. Thus, compound **1** was identified as (*R*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside.

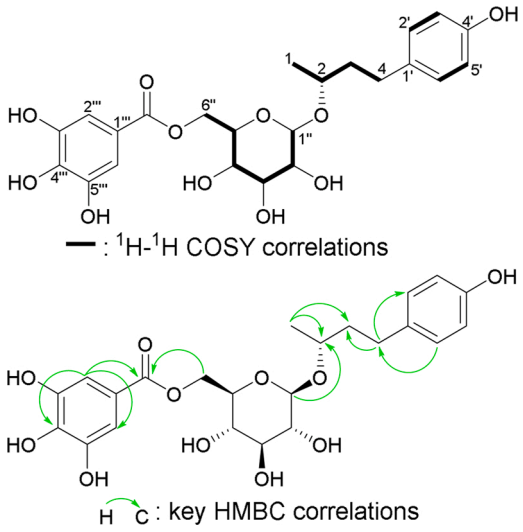

Fig. 2. ^{1}H-^{1}H COSY and key HMBC correlations of compound **1**.

Compound **2** was formulated as $C_{11}H_{12}O_3$ (*m/z* 192.0790 $[M]^+$; calcd for $C_{11}H_{12}O_3$, 192.0786) by HREIMS. The ^{1}H NMR spectrum of **2** exhibited 10 proton resonances. The ^{1}H–^{1}H COSY spectrum of **2** (Fig. 3) suggested the presence of a butyl aliphatic chain [δ 5.78 (1H, d, J =15.5 Hz, H-2); 6.91 (1H, dt, J = 16.0, 7.0 Hz, H-3); 2.46 (2H, m, H-4); 2.67 (2H, t, J =7.0 Hz, H-5)] and *p*-substituted benzene ring [δ 7.00 (2H, d, J =8.5 Hz, H-2′, 6′) and δ 6.69 (2H, d, J = 8.5, H-3′, 5′)]. The coupling constant of the two olefinic proton resonances (J =15.5 Hz) between H-2 and H-3 indicated their *E* configuration. In the ^{13}C NMR spectrum, one of the quaternary carbons at δ 171.0 (C-1) suggested the presence of the carboxylic acid moiety, and it was long-range coupled with H-2 and H-3 in its HMBC spectra (Fig. 3). Additionally, aromatic carbons at δ 133.2 (C-1′), 130.4 (C-2′ and 6′), 116.2 (C-3′ and 5′), and 156.6 (C-4′) were assigned to the 4-oxygenated benzene ring. These NMR data and the molecular formula determined by HRFABMS indicated that **2** had one

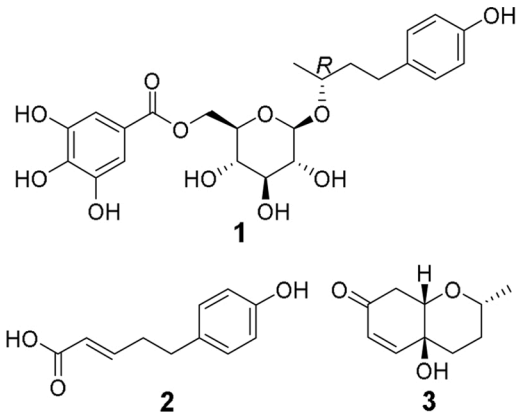

Fig. 1. Structures of compounds **1**–**3**.

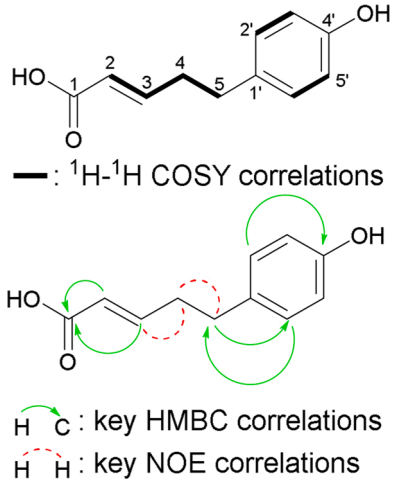

Fig. 3. ^{1}H-^{1}H COSY, key HMBC, and NOE correlations of compound **2**.

hydroxy and one carboxylic acid group. The HMBC correlations from H-5 to C-1′, C-2′, and C-6′ established that the 4-hydroxy phenyl moiety was connected to C-5. Hence, **2** was identified as 5-(4-hydroxyphenyl) 2-pentenoic acid.

To estimate the biological activities of the isolated compounds, acetylcholinesterase, tyrosinase, and insect phenoloxidase inhibitory activities were tested. Although electric eel acetylcholinesterase (0.8 mM) and mushroom tyrosinase (1 mM) inhibitor was not found, compounds **7** and **8** affected the enzymatic activities of phenoloxidase from *Acyrthosiphon pisum* (Table 1). Other tested compounds **1–6**, **13**, **15**, **17**, **19**, **20** did not show significant activity. Phenoloxidase is an important component in an insect's immune system, and it contributes to the elimination of phatogens by producing melanin (Stączek et al., 2020). Melanization involves oxidative steps. Catechin (**7**) from *C. mongolicum* (9.1 μM) showed a ten times stronger IC_{50} value compared with epicatechin (**8**) (148.3 μM). This result was confirmed by a repeatability test using guaranteed pure substances (+)-catechin hydrate (>97.0 %, Tokyo Chemical Industry, Tokyo, Japan): 18.6 μM and (−)-epicatechin, from green tea (>98.0 %, Fujifilm, Osaka, Japan): 195.8 μM. Because compounds **7** and **8** have a common molecular structure, it was expected that the stereochemistry of C-2 and C-3 was an important key structure for the inhibitory activity against this enzyme. According to previously reported literature data (Odonbayar et al., 2016), gallocatechin showed an inhibitory effect on phenoloxidase, and it was stronger than that of epigallocatechin. Although Odonbayar et al. (2016) suggested that the inhibitory activity of pyrogallol B-ring is stronger than that of catechol B-ring, catechin (**7**) with catechol B-ring showed stronger activity than gallocatechins in this study. Therefore, further studies are needed on structure-activity relationship of catechin derivatives. *N*-phenylthiourea was used as positive control. Although the activities of (+)-catechin and (-)-epicatechin were lower compared to positive control, the catechins are included in many plants. Therefore, the study of catechins as insect phenoloxidase inhibitors seems to be useful for the control of pests and may enable understanding of the interactions between insect immune systems and plant chemicals.

3. Experimental section

3.1. General experimental procedures

Specific rotation was taken on a JASCO P-2300 polarimeter (JASCO, Tokyo, Japan). NMR experiments were carried out using a JEOL JNM-AL400 FT-NMR spectrometer (JEOL, Tokyo, Japan) operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C, and chemical shifts were given as δ values with TMS as an internal standard at 25 °C (measured in methanol-d_4, chloroform-*d*, and pyridine-d_5). HMQC (optimized for $^{1}J_{C-H}$ =145 Hz) and HMBC (optimized for $^{n}J_{C-H}$ =8 Hz) pulse sequences with a pulsed field gradient. HRFABMS, and HREIMS data were processed using a JEOL JMS700 mass spectrometer (JEOL), with a glycerol matrix. Preparative and analytical HPLC was performed using a JASCO 2089 (JASCO) with UV detection at 210 nm, using the following columns: Ultra Pack ODS-SM-50C-M (Yamazen, Osaka, Japan, 37 × 100 mm), TSKgel ODS-120 T (Tosoh, Tokyo, Japan, 21.5 × 300 mm), Mightysil RP-18 GP (Kanto Chemical, Tokyo, Japan, 10 × 250 mm), Cosmosil $5C_{18}$ AR-II (Nacalai Tesque, Kyoto, Japan, 20 × 250 mm) and Develosil C_{30}-UG-5 (Nomura Chemical, Aichi, Japan, 20 × 250 mm).

Table 1
Insect phenoloxidase inhibitory activities for identified compounds from the aerial parts of *C. mongolicum*.

Compound	IC_{50} (μM)
7[a]	9.1 ± 0.3
catechin[b]	18.6 ± 0.6
8[a]	148.3 ± 2.6
epicatechin[c]	195.8 ± 4.8
N-phenylthiourea	0.053 ± 0.001

[a] Compounds were isolated from *C. mongolicum*.
[b] The compound was guaranteed by the Tokyo chemical industry.
[c] The compound was guaranteed by the Fujifilm.

3.2. Plant material

The aerial parts of *Calligonum mongolicum* were collected at Segs Tsagaan Bogd Mountain, Shine Jinst soum, Bayankhongor province, Mongolia, at 958 m above sea level, in July 2012 and identified by Prof. Ch. Sanchir, Institute of Botany, Mongolian Academy of Sciences. A voucher specimen (No.31.05.03.12A) was deposited at the Laboratory of Bioorganic Chemistry and Pharmacognosy, National University of Mongolia.

3.3. Extraction and isolation

The dried aerial parts (250 g) were extracted with acetone-water (4:1) (3 × 2.5 L). The extracts were combined and evaporated *in vacuo* at 50 °C. The evaporated extract (42 g) was suspended in water (0.5 L) and then partitioned with diethyl ether (3 × 0.5 L). The aqueous extract (37.8 g) was subjected to DIAION HP-20 column chromatography with a gradient eluent of water-methanol (1:0 to 0:1, v/v) to afford five fractions (1A-1E). Fraction 1C (4.0 g) was chromatographed over a reverse-phase ODS-SM-50C-M column eluted with water-methanol (gradient system from 4:1 to 3:2, v/v) to give subfractions 2A-2L. Subfraction 2C (82.5 mg) was separated by preparative HPLC to obtain compounds **7** (10.3 mg) and **17** (6.0 mg) [TSKgel ODS-120 T, CH_3CN–H_2O (3:17, v/v) containing 0.2 % TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (3:17, v/v) containing 0.2 % TFA]. Subfractions 2F-H (622.0 mg) were purified by preparative HPLC to isolate compounds **4** (113.4 mg), **8** (0.9 mg), **9** (4.5 mg), and **18** (1.9 mg) [TSKgel ODS-120 T, CH_3CN–H_2O (4:16, v/v) containing 0.2 % TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (6:14, v/v) containing 0.2 % TFA]. Fraction 1D (12.5 g) was loaded on a reverse-phase ODS-SM-50C-M column eluted with water-methanol (gradient system from 4:1 to 1:1, v/v) to give subfractions 3A-T. Subfractions 3J-K (172.4 mg) were separated by preparative HPLC to obtain compounds **1** (3.5 mg) and **13** (54.5 mg) [TSKgel ODS-120 T, CH_3CN–H_2O (1:4, v/v) containing 0.2 % TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (1:4, v/v) containing 0.2 % TFA] and subfractions 3H-I (169.9 mg) were subjected to preparative HPLC to isolate compound **14** (87.4 mg) [TSKgel ODS-120 T, CH_3CN–H_2O (1:4, v/v) containing 0.2 % TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (1:4, v/v) containing 0.2 % TFA].

The diethyl ether extract (3.7 g) that was subjected to silica gel column chromatography was eluted with n-hexane-acetone (gradient system from 1:0 to 0:1, v/v) and aqueous methanol (1:1 and 0:1, v/v) to produce 37 fractions (4A-4K1). Fractions 4M-P (150.8 mg) were applied to the column using HP-20 resin with a gradient eluent of water-methanol (1:4 and 0:5, v/v) to generate two subfractions (5A-5B). 5A (39.9 mg) was purified by HPLC on the $5C_{18}$-AR-II column with CH_3CN–H_2O (1:4, v/v) to yield compounds **3** (10.1 mg) and **6** (3.7 mg). Fractions 4S-T (118.2 mg), 4U (213.4 mg), and 4X (315.8 mg) were isolated using the same procedure as 4M-P to achieve compounds **2** (2.7 mg), **5** (119.0 mg), **10** (12.7 mg), **11** (3.3 mg), **12** (2.3 mg), **15** (27.7 mg), **16** (1.3 mg), **19** (2.6 mg), **20** (13.7 mg), **21** (9.6 mg), and **22** (20.9 mg).

3.3.1. *(R)-4-(4-hydroxyphenyl)-2-butanol 2-O-(6-O-galloyl)-β-D-glucopyranoside (1)*

Yellowish, amorphous solid; $[\alpha]^{22}_{D}$ –15.7° (*c* 0.05, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz): δ 7.11 (2H, s, H-2‴, 6‴), 6.91 (2H, d, *J* =8.5 Hz, H-2′, 6′), 6.59 (2H, d, *J* =8.5 Hz, H-3′, 5′), 4.50 (1H, dd, *J* = 12.5, 2.5 Hz, H-6″), 4.45 (1H, dd, *J* =12.5, 5.5 Hz, H-6″), 4.34 (1H, d, *J* =7.5 Hz, H-1″), 3.80 (1H, m, H-2), 3.53 (1H, m, H-5″), 3.45 (1H, t, *J* =9.0 Hz, H-4″), 3.39 (1H, t, *J* =9.0 Hz, H-3″), 3.22 (1H, dd, *J* = 7.5, 9.0 Hz, H-2″), 2.52 (1H, m, H-4), 1.78 (1H, m, H-3), 1.65 (1H, m, H-3), 1.17 (1H, d, *J*

=6.0 Hz, H-1); ^{13}C NMR (methanol-d_4, 100 MHz): δ 168.5 (C-7‴), 156.1 (C-4′), 146.6 (C-3‴, 5‴), 139.9 (C-4‴), 134.7 (C-1′), 130.4 (C-2′, 6′), 121.6 (C-1‴), 116.0 (C-3′, 5′), 110.3 (C-2‴, 6‴), 102.6 (C-1″), 78.2 (C-3″), 75.6 (C-2), 75.4 (C-5″), 75.2 (C-2″), 71.9 (C-4″), 64.9 (C-6″), 40.6 (C-3), 31.8 (C-4), 20.2 (C-1); HMBC (methanol-d_4, 400 MHz): from 1.17 (H-1) to 75.6 (C-2), and 40.6 (C-3); from 2.52 (H-4) to 40.6 (C-3), 134.7 (C-1′), and 130.4 (C-2′, 6′); from 6.91 (H-2′) to 31.8 (C-4), 130.4 (C-6′) and 156.1 (C-4′); from 6.59 (H-3′) to 134.7 (C-1′); from 4.34 (H-1′') to 75.6 (C-2); from 3.22 (H-2″) to 102.6 (C-1″); from 4.45 (H-6″) to 168.5 (C-7‴); from 7.11 (H-2‴) to 121.6 (C-1‴), 146.6 (C-3‴), 139.9 (C-4‴), 110.3 (C-6‴), and 168.5 (C-7‴); HRFABMS (positive) *m/z* 481.1709 $[M+H]^+$ (calcd for $C_{23}H_{29}O_{11}$, 481.171).

3.3.2. 5-(4-hydroxyphenyl) 2-pentenoic acid (2)

Yellowish, amorphous solid; ^{1}H NMR (methanol-d_4, 400 MHz): δ 7.00 (2H, d, J =8.5 Hz, H-2′, 6′), 6.91 (1H, dt, J = 16.0, 7.0 Hz, H-3), 6.69 (2H, d, J =8.5 Hz, H-3′, 5′), 5.78 (1H, d, J =15.5 Hz, H-2), 2.67 (1H, t, J =7.0 Hz, H-5), 2.46 (1H, m, H-4); ^{13}C NMR (methanol-d_4, 100 MHz): δ 171.0 (C-1), 156.6 (C-4′), 149.4 (C-3), 133.2 (C-1′), 130.4 (C-2′, C-6′), 123.8 (C-2), 116.2 (C-3′, C-5′), 35.4 (C-4), 34.7 (C-5); HMBC (methanol-d_4, 400 MHz): from 5.78 (H-2) to 171.0 (C-1), and 35.4 (C-4); from 6.91 (H-3) to 171.0 (C-1), and 35.4 (C-4); from 2.46 (H-4) to 123.8 (C-2), 149.4 (C-3), 34.7 (C-5) and 133.2 (C-1′); from 2.67 (H-5) to 149.4 (C-3), 35.4 (C-4), 133.2 (C-1′), and 130.4 (C-2′); from 7.0 (H-2′) to 34.7 (C-5), 130.4 (C-6′), 116.2 (C-3′), and 156.6 (C-4′); from 6.69 (H-3′) to 130.4 (C-2′), 116.2 (C-5′), and 156.6 (C-4′); HREIMS (positive) *m/z* 192.0790 $[M]^+$ (calcd for $C_{11}H_{12}O_3$, 192.0786).

3.4. Sugar identification

In accordance with a method described previously, hexose moiety identification was accomplished (Tanaka et al., 2007). Compound **1** (1.0 mg) was separately hydrolyzed with 7% HCl (1 mL) at 60 °C for 2 h, then the mixtures were subjected to aqueous sugar fractionation. The concentrated sugar fraction was stirred with L-cysteine methyl ester (3 mg) in pyridine (0.5 mL) at 60 °C, 1 h. Then *o*-tolyl isothiocyanate (3 μL) was added to the solution (60 °C, 1 h). The reaction mixtures were analyzed by HPLC (Siseido, Capcel Pak C_{18}, 4.6 × 250 mm; CH_3CN–H_2O (1:3, v/v) containing 0.2 % TFA, 1.0 mL/min; detection at 250 nm). The peaks of authentic L-glucose (*t*R =15.8 min) and D-glucose (*t*R =17.0 min) derivatives were used to identify the *O*-glucosidic moieties in **1** as D-glucose based on the corresponding retention times of 17.0 min.

3.5. A. pisum phenoloxidase assay

Insect phenoloxidase-containing crude enzyme solution from *A. pisum* was prepared, and tests were performed using the previously reported method (Odonbayar et al., 2016). Adults and the last instars of *A. pisum* (295 mg) were collected; H_2O (7.3 mL) was added, and they were frozen and stored at −20 °C for 24 h. After pestle homogenization and centrifugation (7000 rpm, 5 min), the solution was filtered (0.22 μm, 25 mm, hydrophilic nylon, Starlab Scientific, China). The phenoloxidase activity was assayed spectrophotometrically using 3,4-dihydroxy-L-phenylalanine (L-DOPA) as the substrate as described in the previous report (Odonbayar et al., 2016). The plate was incubated at 25 °C, and the absorbance was measured at 510 nm after 120 and 240 min. The percentage inhibition of insect phenoloxidase was calculated as follows: Inhibition percent (%) = [1 – (Sample $Abs_{510nm,240min}$ – Sample $Abs_{510nm,120min}$)/(Control $Abs_{510nm,240min}$ – Control $Abs_{510nm,120min}$)] x 100. Each sample (final concentration: 1,000, 500, 100, 50, 10 μM) was used for the assay, and *N*-phenylthiourea (final concentration: 1.0, 0.1, 0.01, 0.001 μM) was used as a positive control. The treatment was replicated three times for each concentration.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2020.12.002.

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