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Verticilloside, a New Daucosteryl Derivative from the Seeds of *Malva verticillata.*

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Abstract – A new daucosteryl derivative, verticilloside (**1**), was isolated from the seeds of *Malva verticillata* L. (Malvaceae). The structure was determined to be 3-*O*-[β-D-(6'-linoleoyl)glucopyranosyl]-β-sitosterol based on spectroscopic analyses (^{1}H and ^{13}C-NMR, DEPT, COSY, HMQC, and HMBC) and chemical reactions.
Key words – *Malva verticillata*, daucosteryl derivative, verticilloside, spectroscopic analysis

Introduction

The seed of *Malva verticillata* L. (Malvaceae) is an oriental crude drug used as diuretic, laxative, and galactopoietic (Jeong *et al*., 2011). The isolation of pure neural polysaccharides from the seeds of this plant has been mainly investigated as the principal ingredients (Gonda *et al*., 1990; Tomoda *et al*., 1992). However, the study on phytochemistry and bioactivity of the compounds isolated from *M. verticillata* has not been reported so far. As a part of ongoing research to characterize the chemical components of *M. verticillata*, seeds were extracted and processed, resulting in the isolation of a new daucosteryl derivative (**1**) and five known compounds (**2-6**) (Fig. 1). The current study deals with the isolation and structure elucidation of 3-O-[ß-D-(6'-linoleoyl) glucopyranosyl]-ß-sitosterol, named as verticilloside (**1**).

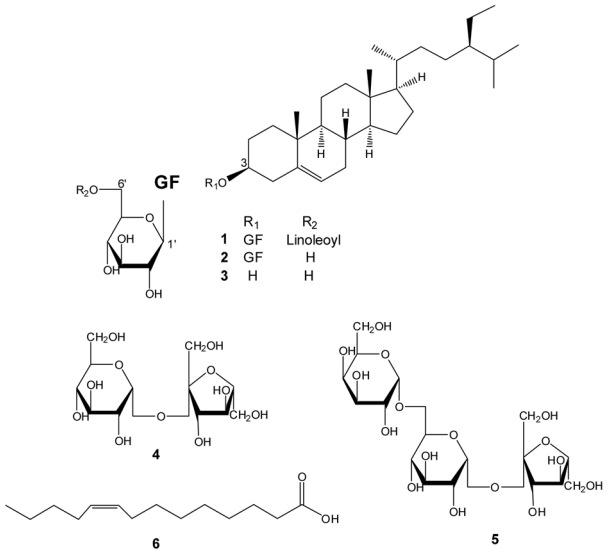

Fig. 1. Structures of components (**1 - 6**) isolated from the seeds of *M. verticillata.*

Materials and Methods

General procedures – Optical rotations were obtained using a DIP-360 digital polarimeter (Jasco, Easton, MD). NMR spectra were recorded on JNM-ECA 600 NMR spectrometers (JEOL Ltd., Japan). High resolution-electrospray ionization (HR-ESI)-MS was carried out on a JMS-T100TD spectrometer (Tokyo, Japan). ESI-MS spectra were recorded on a LCMS-2010EV spectrometer (Shimadzu, Japan). GC (Shimadzu-2010, Tokyo, Japan) using a DB-05 capillary column (0.5 mm i.d. × 30 m) [column temperature: 210 ºC; detector temperature: 300 ºC; injector temperature: 270 ºC; He gas flow rate: 30 mL/min (splitting ratio: 1/20)] was used for sugar determination. Column chromatography was performed on silica gel (70 - 230 and 230 - 400 mesh, Merck) and YMC RP-18 resins (30 - 50 μm, Fuji Silysia Chemical Ltd., Aichi, Japan). TLC was performed on Kiesel gel 60 F_{254} (1.05715; Merck, Darmstadt, Germany) or RP-18 F_{254s} (Merck) plates. Spots were visualized by spraying with 10 % aqueous H_2SO_4 solution, followed by heating.

Plant material – The seeds of *M. verticillata* were purchased at market of oriental herbs, Samsundang, Daejeon, Korea, in November 2010, and were taxonomically identified by one of us (Young Ho Kim). Voucher

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specimens (CNU10105) have been deposited at the herbarium in College of Pharmacy, Chungnam National University.

Extraction and isolation – The seeds of *M. verticilata* (2.3 kg) were ground and extracted with ethanol for 6 hrs under sonification (3 × 4 L). The macerate was concentrated *in vacuo* (53.0 g) and partitioned to afford a methylene chloride extract (27.0 g), ethyl acetate extract (2.7 g), and an aqueous extract (23.0 g). The methylene chloride (27.0 g) was fractionated by a silica gel column chromatography eluting with a gradient system of n-hexane-EtOAc-MeOH-H_2O (9.5 : 0.5 : 0 : 0, 9 : 1 : 0 : 0, 4 : 1 : 0 : 0, 1 : 1 : 0 : 0, 0 : 1 : 0 : 0, 0 : 9 : 1 : 0, 0 : 4 : 1 : 0, 0 : 1 : 1 : 0, 0 : 0 : 1 : 0, 0 : 0 : 9.5 : 0.5, v/v) to give ten fractions (Fr.1 - Fr.10). Fr. 4 (2.6 g) using silica gel columns with n-hexane-EtOAc (9.5:0.5, v/v) as an eluent gave **3** (200.0 mg). Fr. 7 (0.9 g) was purified by recrystallization from cold MeOH to afford **2** (350.0 mg). Fr. 5 (0.8 g) and Fr. 6 (0.6 g) were combined and chromatographed on silica gel column chromatography eluting a mixture of CH_2Cl_2-MeOH (9.6 : 0.4, v/v) to yield four fractions (F5A-F5D). F5B (494.5 mg) were purified by using silica gel column chromatography (n-hexane-EtOAc, 1 : 1, v/v) to obtain **1** (40.0 mg). Fr. 8 (1.6 g) was chromatographed on silica gel column chromatography eluting a mixture of $CHCl_3$-MeOH-H_2O (7 : 3 : 0.3, v/v) to afford **4** (340.0 mg). Fr. 9 (1.6 g) were subjected to silica gel column chromatography with $CHCl_3$-MeOH-H_2O (7:3:0.3, v/v) to give **5** (130.0 mg) and **6** (220.0 mg).

Compound 1 – White amorphous powder, $[\alpha]_D^{20}$ −12.0 (c 0.2, $CHCl_3$); ESI-MS m/z 837 $[M - H]^-$; ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$), see Table 1.

Sugar analysis (Kim *et al*., 2010) – A solution of **1** (3.0 mg) in 1.0 M HCl (3.0 mL) was heated under reflux for 4 h. The reaction mixture was then concentrated to dryness under reduced pressure. The residue was extracted with EtOAc and H_2O (5 mL each, 3 times). Next, the sugar residue, obtained by concentration of the water layer, was dissolved in dry pyridine (0.1 mL) and L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. After heating the reaction mixture at 60 °C for 2 h, 0.1 mL of trimethylsilylimidazole was added. Heating at 60 °C was continued for another 2 h, and the reaction mixture was evaporated to give a dried product, which was then partitioned between hexane and H_2O. The hexane layer was analyzed by GC (Shimadzu-2010, Tokyo, Japan) using a DB-05 capillary column (0.5 mm ID × 30 m length; column temperature: 210 °C; detector temperature:

Table 1. ^{1}H (600 MHz) and ^{13}C (125 MHz) NMR data for compound 1 (δ values in $CDCl_3$).

Position	^{1}H	^{13}C
1	1.85 (m), 0.98 (m)	37.2
2	2.05 (m), 1.60 (m)	31.9
3	3.57 (m)	79.6
4	2.28 (m)	39.7
5		140.3
6	5.37 (m, overlapped)	122.2
7	1.46 (m)	31.5
8		31.8
9		50.1
10		36.6
11		21.0
12	1.13 (m, overlapped)	38.8
13		42.3
14		56.7
15	1.07 (m)	24.8
16	1.30 (m, overlapped)	29.1
17		56.0
18	0.68 (s, CH_3)	11.9
19	1.02 (s, CH_3)	19.3
20		36.1
21	0.91 (d, J = 6.6 Hz, CH_3)	18.7
22	1.13 (m, overlapped)	34.1
23	1.30 (m, overlapped)	28.2
24		45.8
25		29.1
26	0.83 (d, J = 7.1 Hz, CH_3)	19.7
27	0.81 (d, J = 7.1 Hz, CH_3)	18.9
28	1.44 (m)	23.0
29	0.85 (t, J = 7.1 Hz, CH_3)	11.8
1'	4.39 (d, J = 7.6 Hz)	101.2
2'	3.36-3.46 (m)	73.5
3'	3.36-3.46 (m)	76.0
4'	3.36-3.46 (m)	70.1
5'	3.36-3.46 (m)	75.4
6'	4.45 (dd, J = 12.2, 1.2 Hz) 4.29 (dd, J = 12.2, 4.4 Hz)	63.2
1"		174.8
2"	2.36 (t, J = 7.3 Hz)	34.2
3"	1.63 (m)	24.9
4"-7"	1.27 (br s)	29.5-29.7
8"	2.07 (m)	33.9
9", 13"	5.35 (m, overlapped)	130.1 or 130.3
11"	2.78 (t, J = 6.4 Hz)	37.2
10", 12"	5.39 (m, overlapped)	128.0 or 128.1
14"	2.07 (m)	33.9
15"-16"	1.27 (br s)	29.5-29.7
17"	1.31 (br s)	22.6
18"	0.88 (t, J = 6.2 Hz, CH_3)	14.0

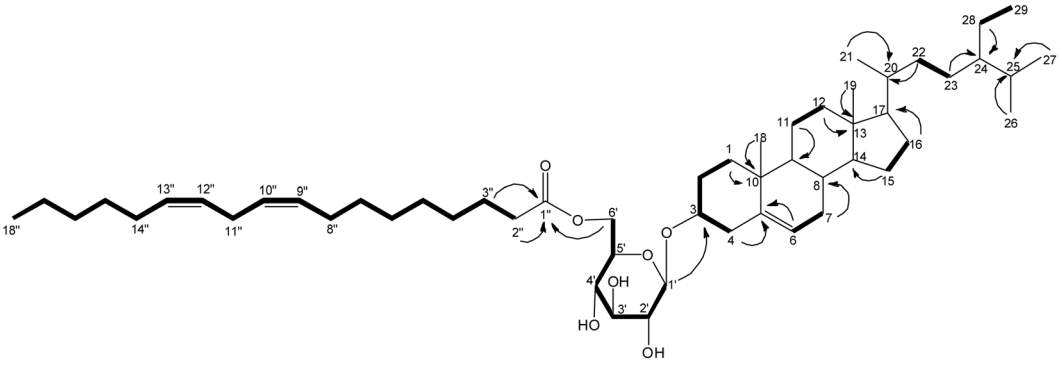

Fig. 2. Selected ^{1}H-^{1}H COSY (bold lines) and HMBC (HC) correlations of **1.**

300 ºC; injector temperature: 270 ºC; He gas flowrate: 30 mL/min). The peak of the hydrolysate was detected at 14.12 min for D-glucose. The retention times of the authentic samples (Sigma-Aldrich), after being treated in a similar manner, were 14.12 min (D-glucose), and 14.25 min (L-glucose).

Alkaline methanolysis – A solution of **1** (2.0 mg) in 5 % dry NaOMe–MeOH (2 mL) was stirred at room temperature for 5 h. The reaction mixture was neutralized with 2N HCl-MeOH and partitioned between MeOH and n-hexane. The n-hexane layer containing the fatty acid methyl esters was analyzed by the GC-MS procedure (General procedures). The peak of the hydrolysate was detected at 8.85 min for methyl linolenate.

Results and Discussion

Six compounds (**1 - 6**) were isolated from the methylene chloride extract of the seeds of *M. verticillata* using a combination of silica gel and reverse C18 column chromatographies. Compounds **2 - 6** were identified by comparison of their physical and spectral data with literature values as daucosterol (**2**) (Voutquenne *et al*., 1999), β-sitosterol (**3**) (Kovganko *et al*., 1999), sucrose (**4**) (De Bruyn *et al*., 1991), raffinose (**5**) (Wu *et al*., 2006), and myristoleic acid (**6**) (Vlahov *et al*., 2009).

Compound **1**, $[\alpha]_D^{20}$ −12.0 (c 0.2, $CHCl_3$), was obtained as an amorphous white powder. The ESI-MS mass spectra of **1** showed a quasi-molecular ion peak at m/z 837 $[M-H]^-$, which was consistent with a molecular formula of $C_{53}H_{90}O_7$.

The ^{1}H and ^{13}C NMR data (Table 1) were identical to daucosterol (**2**) except for the presence of a unsaturated fatty acid. The ^{1}H NMR spectrum showed an olefinic proton signal at δ 5.37 (1H, m, overlapped, H-6), an oxymethine proton signal at δ 3.57 (1H, m, H-3), and six methyl signals as follows: Me-18 at δ 0.68 (s), Me-19 at δ 1.02 (s), Me-21 at δ 0.91 (d, J = 6.6 Hz), Me-26 at δ 0.83 (d, J = 7.1 Hz), Me-27 at δ 0.81 (d, J = 7.1 Hz) and Me-29 at δ 0.83 (t, J = 7.1 Hz). The possible peaks in ^{1}H-NMR for linoleic acid showed four olefinic protons [δ 5.35 (2H, m, overlapped, H-9" and H-13") and 5.39 (2H, m, overlapped, H-10" and H-12")], proton attached to the bis-allylic carbons [δ 2.78 (2H, t, J = 6.4 Hz, H-11")], protons attached to the allylic carbons [δ 2.07 (4H, m, H-8" and H-14")], and the terminal methyl group proton [δ 0.88 (3H, t, J = 6.2 Hz, H-18")]. Furthermore, methylene (CH_2) proton signals of saturated fatty acid showed at δ 1.27 (br s, H-4"-7" and H-15"-16"). The large coupling constant (J = 7.6 Hz) of the anomeric proton at δ 4.39 indicated the β configuration of sugar hexose unit.

The ^{13}C NMR spectrum showed the presence of 53 carbon atoms in the molecule. Out of these, six carbon signals were glycosidic group corresponding to a hexose moiety, 29 carbons signals for aglycone moiety and the remaining 18 carbon signals were due to the fatty acid residue. The spectrum showed an ester carbonyl signal at δ 174.8 (C-1"), four olefinic signals δ 128.0 or 128.1 (C-10" or 12"), 130.1 or 130.3 (C-9" or 13"), and two olefinic signals at δ 140.3 and 122.2 corresponding to the endocyclic double bond between C-5 and C-6 of sterols (Ahmed *et al*., 1992) It also showed deshielded oxymethine signal at δ 79.6 for C-3 and the anomeric carbon signal appeared at δ 101.2.

The structure was further confirmed by assignment of peaks in ^{1}H-^{1}H COSY, HMQC, and HMBC spectra. As shown in Fig. 2, selected HMBC correlations were observed between the following protons and carbon atoms: H-1' and C-3; H-6' and C-1"; H-2" and C-1"; H-3" and C-1". These data and comparison with those of literature (Sultana *et al*., 2007) suggested that compound **1** was a daucosterol with a linoleic acid ester bonded group. The sugar unit obtained from acid hydrolysis was identified as D-glucose by GC analysis. The α and β

configuration of the glucose unit was deduced from the coupling constant value of the anomeric proton as above. In addition, alkaline hydrolysis with NaOMe-MeOH of **1** yielded methyl linolenate, which was identified by GC-MS analysis. Based on the above evidences, compound **1** was determined to be 3-*O*-[β-D-(6'-linoleoyl) glucopyranosyl]-ß-sitosterol, which was named verticilloside (**1**).

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