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A NEW *ent*-KAURANE DITERPENE GLYCOSIDE FROM SEEDS OF *Pharbitis nil*

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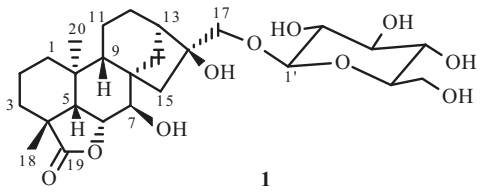
A new ent-*kaurane diterpene glycoside, pharboside H (**1**), and three known compounds, benzyl* β-*D-glucopyranoside (**2**), 1-(4′-*O*-glucopyranosyl-4′-hydroxy-3′,5′-dimethoxyphenyl)-2{2″,6″-dimethoxy-4″-[1-(*E*) propen-3-ol]-phenoxy}-propane-l,3-diol (**3**), and butyl caffeate (**4**), were isolated from the seeds of* Pharbitis nil. *The structure of the new compound **1** was elucidated by 1D and 2D data analysis and enzyme hydrolysis. All the isolated compounds **1–4** were reported from this source for the first time. Compounds **1–4** were tested for cytotoxicity against four human tumor cell lines* in vitro *using the sulforhodamine B bioassay.*

Keywords: *Pharbitis nil*, Convolvulaceae, *ent*-kaurane type diterpene, pharboside H.

Pharbitis nil (Convolvulaceae) is an annual climbing herb widely distributed throughout Korea, Japan, and China [1]. In China, it has been used as folk medicine for the treatment of a variety of digestive problems [2]. Previous phytochemical investigation reported resin glycosides, triterpenoid saponins, and anthocyanins in the flowers and seeds of *P. nil* [3–5]. Its extract showed antitumor and antifungal activities in some biological studies [6]. Our group has been interested in the phytochemicals of *P. nil* seeds and investigated the intriguing biological compounds from the MeOH extracts. As a result, we have reported the isolation of *ent*-kaurane type diterpenes, lignans, phenolic amides, and allogibberic acid, most of which exhibited cytotoxic activity and the ability to inhibit NO production [7–9]. Interestingly, *ent*-kaurane type diterpenes were identified from genus *Pharbitis* for the first time in our group. As part of our continuing effort to search for *ent*-kaurane type diterpenes from *P. nil* seeds, we investigated further its *n*-BuOH-soluble fraction. Repeated column chromatographic purification resulted in the isolation of one new *ent*-kaurane type diterpene glycoside (**1**), named pharboside H, along with three known compounds (**2–4**). The structure of new compound was determined on the basis of 1D and 2D NMR spectroscopic data and chemical means.



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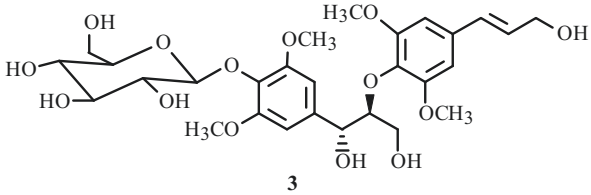

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TABLE 1. ^{1}H (700 MHz) and ^{13}C NMR (175 MHz) NMR Data of Compound **1** (CD_3OD, δ, ppm, J/Hz)

C atom	δ_H	δ_C	C atom	δ_H	δ_C
1	1.63 m, 1.12 m	38.7	15	2.24 (1H, d, J = 13.0)	46.4
2	1.60 m, 1.56 m	18.5		1.03 (1H, dd, J = 13.0, 3.0)	
3	2.05 m, 1.44 m	29.4	16	–	82.8
4	–	43.1	17	3.81 (1H, d, J = 10.0)	79.3
5	1.89 (1H, d, J = 6.0)	52.7		3.47 (1H, d, J = 10.0)	
6	4.67 (1H, t, J = 6.5)	85.4	18	1.30 s	26.0
7	4.20 (1H, d, J = 6.5)	72.5	19	–	185.1
8	–	47.0	20	0.88 s	21.1
9	1.55 m	57.5	1′	4.32 (1H, d, J = 7.5)	105.1
10	–	35.4	2′	3.25 m	75.3
11	1.42 m, 1.37 m	17.8	3′	3.39 m	78.1
12	2.08 m, 1.70 m	21.9	4′	3.33 m	71.7
13	2.11 m	39.3	5′	3.31 m	78.0
14	1.67 m, 1.57 m	34.7	6′	3.80 (1H, dd, J = 12.0, 3.5)	62.8
				3.68 (1H, dd, J = 12.0, 2.0)	

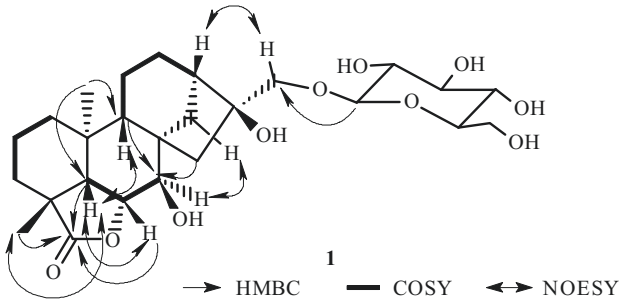

Fig. 1. Key HMBC, ^{1}H–^{1}H COSY, and NOESY correlations of **1**.

Compound **1** was isolated as a colorless gum. The molecular formula was determined as $C_{26}H_{40}O_{10}$ from its positive-ion HR-QTOF-MS at *m/z* 513.2698 $[M + H]^+$ (calcd for $C_{26}H_{41}O_{10}$, 513.2700). The ^{1}H NMR spectrum (Table 1) showed the signals of two oxygenated methine protons at δ 4.67 (1H, t, J = 6.5 Hz, H-6) and 4.20 (1H, d, J = 6.5 Hz, H-7), oxygenated methylene protons at δ 3.81 (1H, d, J = 10.0 Hz, H-17α) and 3.47 (1H, d, J = 10.0 Hz, H-17β), and two tertiary methyl protons at δ 1.30 (3H, s, H-18) and 0.88 (3H, s, H-20). In the ^{13}C NMR (including DEPT) spectrum, 26 carbon signals, composed of two methyls, eight methylenes, five methines, four quaternary, one carbonyl, and six glucose carbons were observed. The spectroscopic data of **1** were similar to those of 7β,16β,17-trihydroxy-*ent*-kauran-6α,19-olide [7] except for the presence of an additional glucose group at δ 4.32 (1H, d, J = 7.5 Hz, H-1′), 3.80 (1H, dd, J = 12.0, 3.5 Hz, H-6′α), 3.68 (1H, dd, J = 12.0, 2.0 Hz, H-6′β), 3.39 (1H, m, H-3′), 3.33 (1H, m, H-4′), 3.31 (1H, m, H-5′), and 3.25 (1H, m, H-2′) in the ^{1}H NMR, and at δ 105.1, 78.1, 78.0, 75.3, 71.7, and 62.8 in the ^{13}C NMR [10]. The coupling constant (J = 7.5 Hz) of the anomeric proton was shown to be of the β-form [7]. The position of glucose was confirmed by an HMBC experiment, in which long-range correlations were observed between H-1′ (δ 4.32) and C-17 (δ 79.3) (Fig. 1). The relative configuration of aglycone was confirmed to be the same as 7β,16β,17-trihydroxy-*ent*-kauran-6α,19-olide from the NOESY cross-peaks of H-5/H-6, H-18/H-6, H-5/H-9, H-7/H-14, and H-13/H-17 (Fig. 1) [7]. Enzyme hydrolysis of **1** yielded the aglycone and β-D-glucose. The former was identified by comparison of the ^{1}H NMR data with the previous reference [7], and the latter from the co-TLC system and specific rotation value [10]. Thus, the structure of **1** was determined to be 7β,16β,17-trihydroxy-*ent*-kauran-6α,19-olide 17-β-D-glucopyranoside and named pharboside H. To the best of our knowledge, this is the first report of the presence of an *ent*-kaurane type diterpene glycoside with a γ-lactone-ring in a natural product.

The other known compounds were identified as benzyl β-D-glucopyranoside (**2**) [11], 1-(4′-*O*-glucopyranosyl-4′-hydroxy-3′,5′-dimethoxyphenyl)-2{2″,6″-dimethoxy-4″-[1-(*E*)propen-3-ol]-phenoxy}-propane-1,3-diol (**3**) [12], and butyl caffeate (**4**) [13] by comparison of their spectroscopic data with those in the literature. All the isolated known compounds (**2**–**4**) were reported from this source for the first time.

The isolated compounds **1–4** were tested for their cytotoxicity against four human tumor cells, A549 (non-small cell lung carcinoma), SK-OV-3 (ovarian cancer ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) using the SRB assay [14]. Among the isolates **1–4**, the new one (**1**) showed the most potent cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT15 cell lines (IC_{50} 21.74, 5.92, 15.31, and 17.27 мМ, respectively), but the other compounds showed little cytotoxicity (IC_{50} > 30 mM).

EXPERIMENTAL

General. TLC was performed using Merck precoated Silica gel F_{254} plates and RP-18 F_{254}s plates. Spots were detected on thin layer chromatography (TLC) under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v). Semipreparative HPLC was performed using a Gilson 306 pump (Gilson, Middleton, WI) with a Shodex refractive index detector (Shodex, New York, NY) and Econosil® RP-C_{18} 10 mm column (250 × 10 mm). Optical rotations were obtained on a JASCO P-1020 polarimeter (Jasco, Easton, MD). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. NMR spectra, including ^1H–^1H COSY, DEPT, HSQC, HMBC, NOESY experiments, were recorded on a Varian UNITY INOVA NMR spectrometer, with chemical shifts given in ppm (δ) using TMS as an internal standard. QTOF/MS was performed on a Waters Ultra-performance liquid chromatography–Xevo quadrupole-time of flight mass spectrometry (UPLC-qTOF-MS).

Plant Material. The seeds of *P. nil* were purchased at Kyungdong herbal market, Seoul, Korea, in July 2006, and were identified by one of the authors (K. R. Lee). A voucher specimen (SKKU 2006-7) has been deposited in the Herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. The dried seeds of *P. nil* (10 kg) were extracted with 50% EtOH (4 L, 3 times, on each of 3 days) at room temperature and filtered. The filtrate was evaporated *in vacuo* to obtain the EtOH extract (1.0 kg), which was suspended in distilled H_2O (7.2 L) and successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, to yield 4.7, 3.0, 13.1, and 270 g of dried organic extracts, respectively. The *n*-BuOH fraction (6 g) was chromatographed on a silica gel (230–400 mesh, 200 g) column and eluted with $CHCl_3$–MeOH–H_2O (7:3:0.5→1:1:1, gradient system) to yield three fractions (A–C). Fraction A (820 mg) was chromatographed further on an RP-C_{18} silica gel (230–400 mesh, 150 g) column and eluted with MeOH–H_2O (40→100% MeOH, gradient system) to obtain 19 subfractions (A1–A19). Subfraction A5 (17 mg) was purified by semipreparative reversed-phase HPLC using a solvent system of 20% MeCN to obtain **2** (3 mg) and **3** (3 mg). Subfraction A11 (20 mg) was purified by semipreparative reversed-phase HPLC using a solvent system of 25% MeCN to obtain **1** (6 mg). Subfraction A16 (40 mg) was purified by semipreparative reversed-phase HPLC using a solvent system of 45% MeCN to obtain **4** (19 mg).

Pharboside H (1). Colorless gum. $[\alpha]_D^{25}$ –5.3° (*c* 0.03; MeOH). IR (KBr, ν, cm^{-1}): 3374, 2947, 2835, 1748, 1651, 1451, 1021, 713. HR-QTOF-MS *m/z* 513.2698 $[M + H]^+$ (calcd for $C_{26}H_{41}O_{10}$, 513.2700). For ^1H NMR and ^{13}C NMR, see Table 1.

Enzyme Hydrolysis and Sugar Identification. Compound **1** (1.0 mg) in H_2O (3 mL) was hydrolyzed with crude hesperidinase (30 mg, from *Aspergillus niger*, Sigma-Aldrich) at 37°C for 48 h. The reaction mixture was extracted with an equal amount of $CHCl_3$ (3 times), and the individual $CHCl_3$ extract was evaporated under reduced pressure. The combined $CHCl_3$ layers from **1** was purified on silica gel [Waters Sep-Pak Vac 6 cc ($CHCl_3$–MeOH, 5:1)] to give 7β,16β,17-trihydroxy-*ent*-kauran-6α,19-olide (**5**), which was identified by comparing its ^1H NMR with that reported in the literature [9]. The aqueous phase of the hydrolysate of **1** were subjected separately to column chromatography over silica gel eluted with MeCN–H_2O (8:1) to yield D-glucose with positive specific rotation $[\alpha]_D^{25}$ +45.5° (*c* 0.04; H_2O). The D-glucose was analyzed by silica gel co-TLC and by comparison with an authentic sample [solvent system $CHCl_3$–MeOH–H_2O, 6:4:1; R_f value 0.3] [14].

Test for Cytotoxicity *in vitro*. SRB bioassays were used as cytotoxicity screening methods. Cytotoxicity assays for each compound were performed *in vitro* against four cultured human tumor cell lines at the Korea Research Institute of Chemical Technology. The cell lines used were A549 (non-small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer cells). Etoposide was used as the positive control.

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