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PAF Antagonistic Activity of 2-Hydroxy-3-methoxybenzoic Acid Glucose Ester from *Gentiana scabra*

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In order to find out anti-platelet activating factor (PAF) from natural resources, Korean medicinal plants used for the treatments of peripheral circulation disorders were tested for their possible protective effects on PAF-induced anaphylactic shock. From the above screening, the methanol extract of *Gentiana scabra* showed a potent antagonistic activity against PAF. Water suspension of the extract was partitioned with CH_2Cl_2 and EtOAc, successively. The EtOAc fraction which showed the highest activity was chromatographed on silica gel to yield 6 fractions. From the fraction which showed higher PAF-antagonistic activity than the other fractions, compound **1** was isolated by recrystallization. On the basis of spectral data, compound **1** was identified as 2-hydroxy-3-methoxybenzoic acid glucose ester. The compound prevented the mice from the PAF-induced death at a dose of 300 μg/mouse.

Key words : Platelet activating factor (PAF), PAF-induced anaphylaxis, *Gentiana scabra,* 2-Hydroxy-3-methoxybenzoic acid glucose ester

INTRODUCTION

Platelet activating factor (PAF; 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine) is produced by a variety of cells involved in inflammatory reactions, including neutrophils, basophils, eosinophils, monocytes/macrophages, platelets, and endothelial cells (Camussi *et al.*, 1981, Mencia-Huerta, 1979) PAF has been known to be involved in a variety of pathologic conditions such as shock, airway-hyperreactivity, thrombosis, inflammation and cardiac anaphylaxis (Braquet *et al.*, 1987). Since PAF antagonists have greatly facilitated research in the field of PAF-mediated pathophysiological effects of PAF, and they have a numerous potential to be used as a chemotherapeutic, efforts have been made to find out or develop a new PAF antagonist. In the same context, many PAF antagonists have been developed from a variety of sources to inhibit the pathologic functions of PAF. In spite of the substantial advances that have been made in synthetic organic chemistry, antagonists derived from higher plants such as ginkgolide and kadsurenone remain as an integral part of modern therapeutics. To screen PAF antagonist, a few efficient bioassay system have been developed and used. In typical *in vitro* assays, biological screening could be performed in two ways: (1) by assaying the ability of a compound or extract to displace [^{3}H]PAF from platelet receptors (Taharoui *et al.*, 1988), (2) by measuring the ability of the compound to inhibit PAF-induced aggregation (Siess, 1989). An alternative to the *in vitro* assays could be the measuring the ability of the compound to inhibit PAF-induced mortality in mice. If lethal dose of PAF (1~2 mg or above/mouse) was injected into mice, it produces changes that are typical of the anaphylactic shock syndrome and the animals without PAF antagonist(s) treatment usually become to death 30 min after PAF injection (Ha *et al.*, 1990, Herbert *et al.*, 1991) Since *in vitro* assays require the use of a radioisotope or special equipment, the *in vivo* assay could be an alternative as a prescreening system. As a way to search for naturally occuring PAF antagonists, Korean medicinal plants being used for the treatments of peripheral circulation disorders were prescreened by using *in vivo* PAF-induced mortality assay. From the above screening, the methanol extract of Gentiana Scabrae Radix showed a potent activity. The present report describes the extraction and purification of the compound which protect mice from the PAF-induced anaphylactic shock.

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MATERIALS AND METHODS

General experimental procedures

^{1}H-NMR and ^{13}C-NMR spectra were run on a JEOL JNM-LA 300 spectrometer in dimethyl sulfoxide-d_6, at

300 MHz and 75 MHz, respectively, with TMS as an internal standard. FT-IR spectra were recorded on a Perkin-Elmer 1710 spectrophotometer. FABMS spectra were obtained on a VG 70-VSEQ mass spectrometer with direct inlet system using PEG600/glycerol as a matrix. TLC was carried out on silica gel precoated plates (Art. No. 5715, Merck)

Plant materials

All plant materials including Gentiana Scabrae Radix (*Gentiana scabra*) were purchased from Kyungdong herb market and were identified by Dr. Dae S. Han, Professor Emeritus, College of Pharmacy, Seoul National University. Voucher specimens documenting this purchase have been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Animals

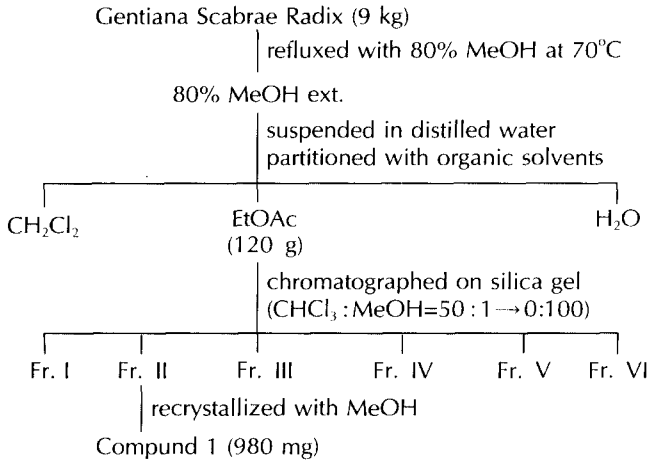
ICR mice were purchased from the Korean Research Institute of Chemistry and Technology (Taejon, Korea) and were housed throughout the experiments in a laminar-flow cabinet and maintained on standard laboratory chow *ad libitum*. All mice were used at 8 to 9 weeks of age.

Reagents

PAF (1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine) was purchased from Sigma Chemical Co. (St Louis, USA). The PAF antagonist BN 50739, a ginkgolide-derived synthetic compound, was kindly provided by Dr. P. Braquet (Institut Henri Beaufour, Le Plessis Robinson, France). BN 50739 in dimethyl sulfoxide (50 mg/ml) was stored -20°C until used and was diluted in RPMI 1640 (GIBCO, Grand Island, USA) when injected.

Extraction and isolation

Dried plant materials were extracted with 80% MeOH by using a reflux apparatus that yielded extract upon removal of the solvent *in vacuo*. The extract was suspended in distilled water and partitioned with CH_2Cl_2 and EtOAc, successively. Each solvent fraction was subjected to biological assay after complete dryness. For the isolation of active compound, dried Gentiana Scabrae Radix (9 kg) were extracted and partitioned as described above. The EtOAc fraction which showed the best activity was chromatographed on a silica gel column (2.2 kg, 230~400 mesh, column size 10× 110 cm) eluted with a stepwise gradient from $CHCl_3$-MeOH (50:1) to MeOH to give 190 fractions (1 L each). Fractions 50 through 60 were pooled and evaporated to dryness *in vacuo*, and the residue was recrystallized from MeOH to give a white powder.



Scheme 1. Procedure of activity-guided fractionation and isolation of active compound from Gentiana Scabrae Radix.

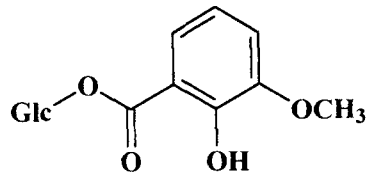


Fig. 1. Structure of compound **1** (2-hydroxy-3-methoxybenzoic acid glucose ester).

(Scheme 1)

Compound 1 (Fig. 1): white amorphous powder; mp 120~121°C; $C_{14}H_{18}O_9$; $[\alpha]_D^{25}$ -61.5° (c 0.01, H_2O); IR (KBr) ν 3350, 1680, 1620, 1260 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ; 3.86 (3H, s, methoxy), 4.84 (1H, d, J=6.8 Hz, glucosyl anomeric proton), 6.84 (1H. t, J= 8.04 Hz, H-5), 7.34, 7.39 (2H, H-4 and H-6) ^{13}C-NMR (75 MHz, DMSO-d_6) (see Table II); FABMS *m/z* 331 $[M+H]^+$, 169 $[M\text{-hexose}+H]^+$, Rf 0.55, developing solvent, EtOAc-MeOH-H_2O (77:15:8)

Biological activity

The PAF antagonistic activity of plant extracts and purified compounds was tested by injecting the samples intraperitoneally. The samples were injected 10 min before PAF (2 μg/mouse) injection. Injection of PAF into animals produces changes that are typical of the anaphylactic shock syndrome and the animals without PAF antagonist(s) treatment usually become to death 30 min after PAF injection. The PAF antagonistic activity was determined by counting the survived mice out of the mice which were injected PAF. The samples which prevented more than 50% of the mice from PAF-induced death were considered to have PAF-antagonistic activity.

RESULTS AND DISCUSSION

Among the sample extracts subjected to *in vivo* bio-

Table I. Screening of Korean medicinal plants being used for the treatment of peripheral circulation disorders

Sample	Fraction (mg/mouse)								
	H_2O			EtOAc			CH_2Cl_2		
	50	25	12.5	25	12	6	25	12	6
Platycodon grandiflorum	-[a]	-	-	-	-	-	-	-	-
Reynoutria elliptica	-	-	-	-	-	-	-	-	-
Rheum coreanum	-	-	-	-	-	-	+[b]	-	-
Uncaria rhynchophylla	-	-	-	-	-	-	-	-	-
Lonicera japonica	-	-	-	+	-	-	-	-	-
Albizzia julibrissin	-	-	-	-	-	-	-	-	-
Crataegus pinnatifida	-	-	-	-	-	-	-	-	-
Prunus mume	+	+	-	+	-	-	-	-	-
Eunoynus alatus	-	-	-	-	-	-	+	-	-
Gentiana scabra	-	-	-	+	+	+	+	-	-
Codonopsis pilosula	-	-	-	-	-	-	-	-	-
Sophora subprostrata	-	-	-	-	-	-	-	-	-
Lamium barbatum	-	-	-	-	-	-	-	-	-

Each fraction which was dissoved in DDW or EtOH was injected to ICR mouse intraperitoneally 10 min before PAF (2 μg/mouse) injection. Thirty min after PAF injection, the number of survived mice was counted. a) (-): No protection, b) (+): 50% or more protection of mice from death (Number of animals: 4-6)

Table II. ^{13}C-NMR spectral data of compound **1** (75 MHz, DMSO-d_6)

Carbon	Compound **1**	Reference[a]
1	114.5	114.8
2	145.9	146.2
3	149.8	150.1
4	122.8	123.0
5	118.7	119.0
6	120.8	121.0
1'	101.2	101.4
2'	73.2	73.5
3'	76.4	76.6
4'	69.7	69.9
5'	77.0	77.4
6'	60.7	60.9
COO	168.7	169.0
OMe	52.4	52.6

a) Kuo, S-H *et al.* (1996)

assay, the aqueous fraction of *Prunus mume* and EtOAC fraction of Gentiana Scabrae Radix protected mice from PAF-induced systemic anaphylaxis at the dose of 25 mg/mouse. (Table I) Since the EtOAC fraction of *G. scabra* was more potent than the aqueous fraction of *P. mume*, it was subjected to successive purification and isolation. The silica gel column chromatography of the EtOAc fraction with a stepwise gradient elution from $CHCl_3$-MeOH (50:1) to 100% MeOH gave 6 fractions. The residue of the evaporated second fraction was recrystallized in MeOH to give a white powder. The molecular formula of $C_{14}H_{18}O_9$ for compound **1** was determined by FABMS. In the positive FABMS, compound **1** exhibited significant fragment peaks at *m/z* 331 $[M+H]^+$, 169 $[M\text{-hexose}+H]^+$. The IR data of compound **1** indicated it to be an aryl substituted ester (1620, 1260 cm^{-1}), bearing a carbonyl (1680 cm^{-1}) and a hydroxyl (3350 cm^{-1}) functionality. The ^{1}H- and ^{13}C-NMR spectral data of compound **1** (Table II) indicated the presence of sugar, strongly suggesting the phenolic acid sugar ester nature of the molecule. The ^{1}H-NMR spectrum indicated the presence of a methoxyl signal at δ 3.86, a glucosyl anomeric proton at δ 4.84, three aromatic proton signals at δ 6.84, 7.34 and 7.39 and a phenolic hydroxyl signal at δ 10.0. The ^{13}C-NMR signals at δ 101.2 also suggested that the sugar in compound 1 was in the β-D-glucopyranose form. From the above evidence and comparison of chemical shift values with those of corresponding literature data, compound **1** was concluded to be a 2-hydroxy-3-methoxybenzoic acid glucose eater. Compound **1** has not been reported until Kuo *et al.* (1996) isolated it from *Gentiana formosana*. The PAF antagonistic activity of the samples was tested by observing their preventive effects on PAF-induced anaphylaxis in ICR mice. Usually 6 or more mice were used for the assay. The activity of the EtOAc fraction of the *G. scabra* was significant to protect all the mice from death at the dose of 6 mg/mouse. Purification of the EtOAc extract increased the activity. Fraction II of the EtOAc extract exerts its PAF antagonistic

Table III. PAF-induced mortality inhibitory activity of fractions and compound **1**

Fraction	Lethality		
	1.2 mg/mouse	0.6 mg/mouse	0.3 mg/mouse
Fr. I	5/6*	6/6	-
Fr. II	0/6	0/6	2/6
Fr. III	2/6	3/6	5/6
Fr. IV	4/6	5/6	6/6
Fr. V	6/6	-	-
Fr. VI	6/6	-	-
Compound 1	0/6	0/6	2/6

*Number of dead mice/total mice

activity as low as 0.3 mg/mouse. (Table III) Because the purified compound also has similar activity in same dose, it implies that the active component of the fraction could be the compound **1**. According to a report by Ha *et al.* (1990), a ginkgolide-derived synthetic PAF antagonist BN 50739 protected 50% of mice from PAF-induced systemic anaphylaxis at the dose of 60 μg/mouse. Even if the receptor binding assay and/or the platelet aggregation assay may provide more direct evidence regarding the antagonistic mechanism of the compound, the result implies that the potency of the compound **1** seems to be about one fifth of the BN 50739 in terms of its mortality relieving activity.

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