Allergy-Preventive Phenolic Glycosides from *Populus sieboldii*

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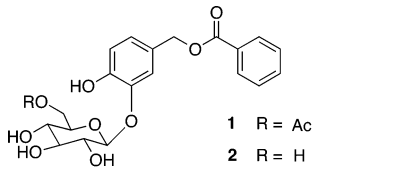
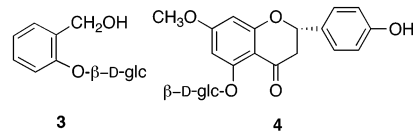
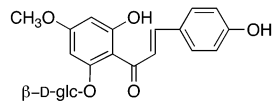
Allergy-preventive activity was demonstrated for an extract of the bark of *Populus sieboldii* in a continuing search for allergy-preventive substances from natural sources. By bioassay-directed fractionation of this plant bark, two new phenolic glycosides, siebolside A {2-hydroxy-5-[(benzoyloxy)methyl]phenyl (6′-*O*-acetyl) *β*-D-glucopyranoside} (**1**) and siebolside B {2-hydroxy-5-[(benzoyloxy)methyl]phenyl *β*-D-glucopyranoside} (**2**), were isolated, together with three known compounds, salicin (**3**), sakuranin (**4**), and neosakuranin (**5**). The structures of **1** and **2** were elucidated by spectroscopic methods. Compounds **1**−**5** all showed allergy-preventive effects.

Populus species have been used to treat rheumatism, hypertension, pain, and edema in traditional Chinese medicine. *Populus sieboldii* Miq. (Salicaceae) has been reported to inhibit platelet aggregation, and an active phenolic compound, pyrocatechol, has been isolated,[1] but no medicinal uses of this plant have been described. In a continuing search for allergy-preventive substances from natural sources, we found that ethyl acetate and 70% methanol extracts of the bark of *P. sieboldii* exhibited this type of activity. This paper describes the structure of two new phenolic glycosides, named siebolsides A (**1**) and B (**2**), from *P. sieboldii*, together with three known compounds (**3**−**5**), and the evaluation of the allergy-preventive effects of these five compounds.







Siebolside A (**1**) was obtained as colorless needles, and its HRFABMS showed a $[M + 1]^+$ ion at *m*/*z* 449.1446, which established that the molecular formula is $C_{22}H_{24}O_{10}$. The IR spectrum indicated the presence of a phenolic hydroxy group at 3320 cm^{-1} and two ester functions at 1725 and 1700 cm^{-1}. In the UV spectrum, λ_{max} values at 228 and 284 nm were observed, indicating the presence of aromatic groups. The ^1H NMR spectrum of **1** showed the presence of signals corresponding to an acetyl group at δ 2.02, and an anomeric proton of a sugar moiety appeared at δ 4.74 ($J = 7.4$ Hz). A typical ABX spin system at δ 6.72−7.06 was used to identify a 3,4-dihydroxybenzyl moiety. Another set of peaks appearing between δ 7.47 and 8.06 was attributed to aromatic protons of a benzoyl group moiety. The ^{13}C NMR spectrum of **1** showed 22 carbon signals in the molecule, of which two belonged to an acetyl group, six to a glucose unit, and the others to a monosubstituted benzoyl group and a trisubstituted benzyl group. These data suggested that compound **1** is the acetylated monoglucoside of 3,4-dihydroxyphenyl methyl benzoate.[2] This was confirmed by the EIMS, which showed fragment peaks at *m*/*z* 105, 122, and 138 (Figure S2). Although 3,4-dihydroxyphenyl methyl benzoate was synthesized in 1978, its spectroscopic data have not been reported.[2] Thus, all ^1H and ^{13}C NMR signal assignments of **1** were confirmed by the present study from the $^1H-^1H$ COSY, HMQC, and HMBC NMR spectra.

The position of the glucose linkage in **1** was established at the C-3 hydroxyl group of the 3,4-dihydroxybenzyl moiety by the HMBC NMR technique (Figure 1). The anomeric configuration was determined as *β* from the ^1H NMR ($J = 7.4$ Hz) and ^{13}C NMR (Glc-1 at δ 104.7) data. The position of the acetyl group was also established at the C-6″ position of the glucose by a correlation in the HMBC spectrum and the acetylation shift observed between C-5″ (Δ −2.7) and C-6″ (Δ 2.0) in the ^{13}C NMR spectrum. Thus, the structure of the new compound, siebolside A, was established as 2-hydroxy-5-[(benzoyloxy)methyl]phenyl (6′-*O*-acetyl) *β*-D-glucopyranoside (**1**).

Siebolside B (**2**) was isolated as colorless needles. It gave a HRFABMS $[M + 1]^+$ ion at *m*/*z* 407.1336, which was used to establish the molecular formula as $C_{20}H_{22}O_9$. Its IR spectrum was similar to that of siebolside A (**1**), but it did not show any ester absorption at 1725 cm^{-1}. The ^1H NMR and ^{13}C NMR spectra of **2** (Table 1) were also similar to the data obtained for **1**, except for the absence of the signal of the acetyl group and the acetylation shift in the ^{13}C NMR spectrum. Thus, the structure of the new compound siebolside B was established as 2-hydroxy-5-[(benzoyloxy)methyl]phenyl *β*-D-glucopyranoside) (**2**).

Three compounds of known structure, **3**−**5**, were identified as salicin,[3] sakuranin,[4,5] and neosakuranin,[4] respectively, by comparison of their physical and spectroscopic data with reported literature values. These substances were isolated for the first time from *P. sieboldii*.

The allergy-preventive effects of the AcOEt and 70% MeOH extracts of *P. sieboldii* bark and the isolated compounds were measured with a previously reported in vivo assay method,[6] which uses the blood flow decrease in the tail artery of mice subjected to hen egg-white lysozyme (HEL) sensitization as a monitor. This method can be used to search for preventive agents against allergy involving NO from inducible NO synthase, COX-1, 2, and PGI_2.[6]

Both the *P. sieboldii* ethyl acetate and 70% methanol extracts (200 mg/kg, po) significantly inhibited the blood flow decrease

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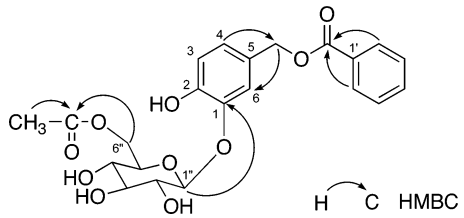

Figure 1. HMBC NMR correlations of siebolside A (**1**).

Table 1. ^{1}H NMR and ^{13}C NMR Spectroscopic Data of Siebolsides A (**1**) and B (**2**)[a]

	siebolside A (**1**)		siebolside B (**2**)	
position	δ_C	δ_H	δ_C	δ_H
1	149.8		149.9	
2	154.4		154.3	
3	119.9	7.06 d (8.8)	119.4	7.11 d (8.7)
4	116.6	6.72 dd (2.8, 8.8)	116.3	6.72 dd (3.2, 8.7)
5	129.4		128.8	
6	116.3	6.88 d (2.8)	116.3	6.86 d (3.2)
7	63.2	5.43 d (13.0)	63.2	5.44 d (13.3)
		5.46 d (13.0)		5.51 d (13.3)
1′	131.6		131.6	
2′,6′	130.7	8.06 dd (1.4, 6.9)	130.6	8.06 dd (1.4, 6.9)
3′,5′	129.7	7.47 brt	129.6	7.47 brt
4′	134.4	7.60 m	134.3	7.59 m
C═O	168.1		168.1	
1″	104.7	4.74 d (7.4)	104.4	4.78 d (9.2)
2″	75.1		75.0	
3″	78.0	3.34−3.58 (m)	78.2	3.13−3.45 (m)
4″	71.7		71.5	
5″	75.4		78.1	
6″	64.7	4.37 dd (2.3, 12.0)	62.7	3.87 dd (1.9, 11.9)
		4.23 dd (5.6, 12.0)		3.68 dd (5.1, 11.9)
*C*OMe	172.8			
CO*CH*$_3$	20.7	2.02 s		

[a] Data were recorded in CD_3OD at 500 MHz (^{1}H) or 125 MHz (^{13}C NMR). Values in parentheses are coupling constants in Hz (500 MHz).

compared with the control group after day 6 or 7 of the HEL sensitization, as shown in Figure S1. Compounds **1** and **2** (20 mg/kg, po) also significantly inhibited the blood flow decrease in a similar manner (Figure 2A). However the inhibitory activity of **2** was more potent than that of the acetate **1**. Further evaluation of **1** and **2** was carried out on platelet aggregation, and the results are shown in Figure 3. Although both **1** and **2** inhibited platelet aggregation compared with the control group, the percent inhibition of platelet aggregation by **2** was higher than that of **1** in HEL-sensitized mice at day 9. These findings suggest that the inhibition of platelet aggregation is involved in the inhibition mechanism of blood flow decrease of **1** and **2**. Interestingly, structurally related analogues of these compounds, salireposide and trichocarpin, from other *Populus* species exhibit inhibitory activity of phosphodiesterase I and antiviral activity, respectively. [7,8]

Salicin (**3**) (20 mg/kg, po) also significantly inhibited the blood flow decrease compared with the control group after day 5 of the HEL sensitization (Figure 2B). Compound **3**, commonly found in *Populus* species, is reported to have an inhibitory effect on serine protease[9] and is useful for the treatment of skin irritation.[10] The present findings reveal an allergy-preventive activity for this compound for the first time. The flavonoid derivatives **4** and **5** (20 mg/kg, po) also significantly inhibited blood flow decrease compared with the control group after day 6 or 7 of HEL sensitization (Figure 2B). However, **5** inhibited platelet aggregation, while **4** did not, as shown in Figure 3. These results suggest that the inhibition mechanism of blood flow decrease differs between these compounds.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanagimoto micro-melting-point apparatus. IR spectra were recorded on a Shimadzu 435 spectrometer and the UV absorption spectra with a Shimadzu UV-160A spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with a JEOL JEM-GSX 500 spectrometer (TMS as internal reference). FABMS were performed on a JMS-700 double-focusing mass spectrometer having a kinetic energy equivalent to 6 kV at an ion-acceleration voltage of 3 kV. The mass marker was calibrated with perfluoroalkylphosphazine (ultra marker), and 3-nitrobenzyl alcohol (NBA) and glycerin (GLY) were used as the matrix.

Plant Material. Bark of *P. sieboldii* was collected from Mitahora, Gifu Prefecture, Japan, in May 2001 and verified by one of us (M.I.). A voucher specimen (GPU-2001-S-05) is kept at Gifu Pharmaceutical University.

Extraction and Isolation. The dried bark (1 kg) of *P. sieboldii* was extracted with AcOEt and then extracted with 70% MeOH at room temperature. Both layers were evaporated to yield an AcOEt extract (50 g) and 70% MeOH extract (45 g). The AcOEt extract was subjected to chromatography on a silica gel column using a $CHCl_3$−MeOH step gradient to give seven fractions (Ia−g). Fraction Ic (10.1 g), which was eluted with $CHCl_3$−MeOH (30:1), was chromatographed on a silica gel column, again using a $CHCl_3$−MeOH step gradient, to obtain four further fractions (IIa−d). Fraction IIb (1.62 g), eluted with $CHCl_3$−MeOH (30:1), was purified by gel filtration on Sephadex LH-20 using MeOH, followed by recrystallization from AcOEt−MeOH to obtain **1** (84.9 mg).

The 70% MeOH extract was chromatographed over silica gel using a step gradient of $CHCl_3$−MeOH to yield 10 fractions (IIIa−j). Fraction IIIc (2.9 g), eluted with $CHCl_3$−MeOH (15:1), was chromatographed over silica gel using a $CHCl_3$−MeOH step gradient to give four fractions (IVa−d), followed by *n*-hexane−AcOEt, which gave four fractions (Va−d). Fraction Vb (210 mg), eluted with *n*-hexane−AcOEt (1:5), was purified by gel filtration on Sephadex LH-20 using MeOH, to yield five fractions (VIa−e). Fractions VIa (83 mg) and VIe (28 mg) were recrystallized from $CHCl_3$−AcOEt−MeOH to obtain **2** (60 mg) and **5** (mp 110 °C, yellow needles, 20 mg), respectively. Fraction VIc (51 mg) was also recrystallized from AcOEt−MeOH to afford **4** (mp 224 °C, white needles, 28 mg).

Fraction IIIg (5.7 g), eluted with $CHCl_3$−MeOH (8:1) and subjected to chromatography on a silica gel column using a $CHCl_3$−MeOH step gradient, gave eight fractions (VIIIa−h). Fraction VIIIf (2.61 g), eluted with $CHCl_3$−MeOH (9:1), was purified by repeated recrystallization from $CHCl_3$−AcOEt−MeOH to give **3** (mp 201−207 °C, white needles, 715 mg).

Siebolside A (1): colorless needles (AcOEt−MeOH); mp 178−185 °C; UV (MeOH) λ_{max} (log ϵ) 204 (4.22), 228 (4.18), 284 (3.46) nm; IR (KBr) ν_{max} 3320 (OH), 2900 (CH), 1725, 1700 (Ac, ester), 1600, 1490 (C═C, Ar) cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; EIMS *m*/*z* 244 [M − glucose − Ac]$^+$ (28), 138 [$-OC_6H_3(OH)CH_2O-$]$^+$ (5), 122 [$-OC_6H_3(OH)CH_2-$]$^+$ or [$C_6H_5CO_2H$]$^+$ (100), 105 [C_6H_5CO]$^+$ (73), 77 [C_6H_5]$^+$ (22); FABMS (positive mode) *m*/*z* 449 [M + H]$^+$; HRFABMS *m*/*z* 449.1446 [M + H]$^+$ (calcd for $C_{22}H_{24}O_{10}$, 449.1448).

Siebolside B (2): colorless needles ($CHCl_3$−AcOEt−MeOH); mp 203−205 °C; UV (MeOH) λ_{max} (log ϵ) 204 (4.29), 228 (4.28), 284 (3.56) nm; IR (KBr) ν_{max} 3320 (OH), 2900 (CH), 1700 (C═O, ester), 1600, 1490 (C═C, Ar) cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; EIMS *m*/*z* 244 aglycone [M − glucose]$^+$ (46), 123 [$C_6H_3(OH)_2CH_2$]$^+$ (27), 122 [$-OC_6H_3(OH)CH_2-$]$^+$ or [$C_6H_5CO_2H$]$^+$ (100), 105 [C_6H_5CO]$^+$ (73), 77 [C_6H_5]$^+$ (12); FABMS (positive mode) *m*/*z* 407 [M + H]$^+$; HRFABMS *m*/*z* 407.1336 (calcd for $C_{20}H_{22}O_9$, 407.1342).

Animals. Male ddY mice (SPF grade), 5 weeks old, were obtained from Japan SLC (Shizuoka, Japan) and housed at 24 °C. Food and water were available ad libitum.

HEL Sensitization. Immunization with hen egg-white lysozyme (HEL) was performed as previously described[11] with slight modification. Male ddY mice of 5 weeks of age were sensitized subcutaneously with 50 μg of HEL in complete Freund's adjuvant (DIFCO) on day 0.

In Vivo Assay Method for Allergy-Preventive Substances. Subcutaneous blood flow in the mouse tail was monitored using a laser doppler blood flow meter of the noncontact type (OMEGA FLO-N1, Neuroscience Inc., Tokyo, Japan) as previously described.[6] Each mouse was prewarmed for 10 min at 37 °C prior to the experiment and placed on a holder in a measuring chamber kept at 37 °C throughout the measurement. The normal blood flow was measured for 10 min at 20

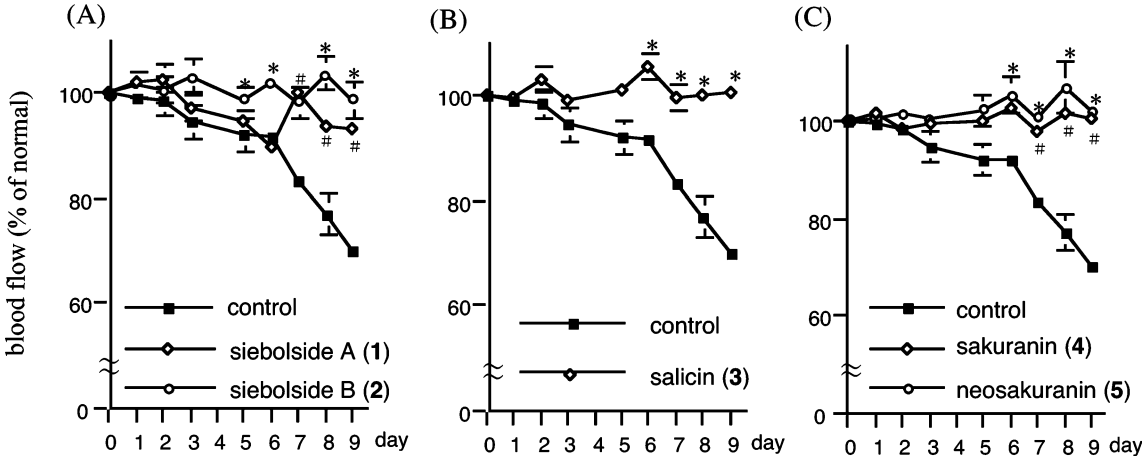

Figure 2. Allergy-preventive effects of isolated compounds **1**−**5**. ■: control (blood flow of HEL-sensitized mice); (A) ◇: pretreatment with siebolside A (**1**), ○: pretreatment with siebolside B (**2**); (B) ◇: pretreatment with salicin (**3**); (C) ◇: pretreatment with sakuranin (**4**), ○: pretreatment with neosakuranin (**5**), at 1 h prior to measurement at 0 (starting day), 3, 6, and 9 days from sensitization. Each value represents the mean ± SE ($n = 5$) (*p <0.05, #p <0.05 as compared with control group).

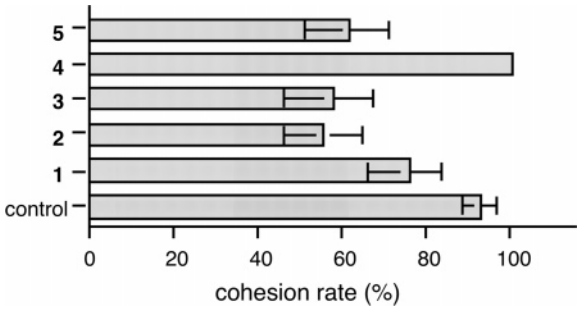

Figure 3. Platelet aggregation effects of compounds **1**−**5** and a control (blood flow of HEL-sensitized mice). Results are the means ± SE ($n = 5$).

min before the experiment. The blood flow of the sensitized mouse was measured for 10 min every day for 9 days from the sensitization (0 day) without anesthesia. The results were expressed as mean ± SE of the percent of the normal blood flow of each mouse.

All experiments were performed in accordance with the Guidelines for Animal Experiments of Mukogawa Women's University.

Allergy-Preventive Effects. Each of the extracts (200 mg/kg) and compounds (20 mg/kg) were administrated orally to HEL-sensitized mice at 0 (the start day), 3, 6, and 9 days. None of the reagents affected the blood flow. The measurements of blood flow were carried out every day for 9 days. The statistical calculations were determined in comparison with the HEL-sensitized mice (control group).

Platelet Aggregation. The platelet aggregation of whole blood induced by ADP (8 μM) was measured with a whole blood aggregometer (WBA) analyzer (MC Medical Co., Ltd. Tokyo, Japan) using a screen filtration pressure (SFP) method. The whole blood was obtained from each anesthetized mouse with diethyl ether on day 9. The samples were anticoagulated with 3.8% sodium citrate and then left to stand for 30 min at room temperature for stable aggregation. The results are expressed as mean ± SE of the cohesion rate for control and pretreatment with test compounds ($n = 5$).

Statistical Analysis. Two-way analysis of variance (ANOVA) was used to test for statistical differences. When significant differences (p <0.05) were identified, the data were further analyzed by Dunnett's multiple range test with Bonferroni inequality for significant differences between each test group and the control group. For the Bonferroni test, five points were used after day 4 of the HEL sensitization, because a significant difference was observed between the blood flow of nontreated and sensitized mice after day 4.

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Supporting Information Available: EIMS data of **1**, allergy-preventive effects of crude extracts of *Populus sieboldii*, and NMR data of compounds **1**−**5**. This information is available free of charge via the Internet at http://pubs.acs.org.

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