Note

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Samioside, a New Phenylethanoid Glycoside with Free-Radical Scavenging and Antimicrobial Activities from *Phlomis samia*

Irene Kyriakopoulou, Prokopios Magiatis, Alexios-Leandros Skaltsounis,* Nektarios Aligiannis, and Catherine Harvala

Laboratory of Pharmacognosy, University of Athens, Panepistimiopolis, Zografou, GR-15771 Athens, Greece

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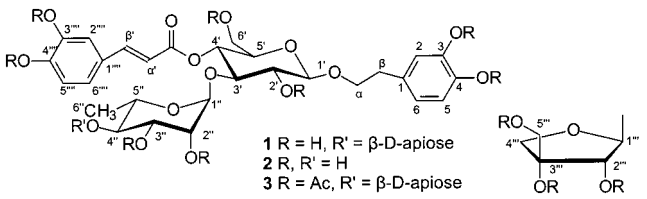
A new phenylethanoid glycoside, samioside, was isolated from the aerial parts of *Phlomis samia* and identified as 1-*O*-3,4-(dihydroxyphenyl)ethyl *β*-D-apiofuranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-4-*O*-caffeoyl-*β*-D-glucopyranoside (**1**). In addition, one known phenylethanoid glycoside and three known flavonoids were identified as acteoside (**2**), apigenin, chrysoeriol, and ermanin, respectively. The structure of **1** was elucidated on the basis of its spectroscopic data. Samioside (**1**) demonstrated scavenging properties toward the DPPH radical and antimicrobial activity against Gram-positive and -negative bacteria.

During a screening program for the discovery of antioxidant bioactive compounds from plants of the Greek flora, we detected free-radical-scavenging activity in a 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) assay by the methanolic extract of the aerial parts of *Phlomis samia* L. *Phlomis* is a genus of the family Lamiaceae (Labiatae) consisting of ca. 100 species (12 found in Europe) of herbs or shrubs, many of which are highly variable. *Phlomis samia* (first described from the Greek island of Samos) is a herb up to 100 cm, distinguished by its whorls of large dull-purple flowers, with the upper lip galeate.[1] This species grows in woods and bushy places of Greece, Turkey, and southern Yugoslavia.[1,2] It has not undergone any previous phytochemical study.

Investigation of the active methanolic extract has led to the isolation and structure elucidation of samioside (**1**), a new phenylethanoid glycoside. Moreover, acteoside (**2**) and the flavonoids apigenin, chrysoeriol, and ermanin were isolated.



Compound **1** was obtained as an amorphous yellowish powder. The positive-ion FABMS showed a sodiated molecular ion peak at *m*/*z* 779 $[M + Na]^+$ and a fragment ion at *m*/*z* 460 indicative of the cleavage of a pentose sugar and a desoxyhexose sugar. The molecular formula of **1** was determined by HRFABMS as $C_{34}H_{44}O_{19}$ (*m*/*z* $[M + H]^+$ 757.2549, calcd 757.2555). The ^{1}H NMR spectrum of **1** exhibited characteristic signals belonging to *E*-caffeoyl (three aromatic protons as an ABX system and two *trans*-olefinic protons) and 3,4-dihydroxyphenylethanol (three aromatic protons as an ABX system and two coupled methylenes) moieties, revealing a close structural similarity to acteoside (verbascoside) (**2**). Moreover, three doublets of anomeric protons at *δ* 5.26 (*J* = 1.2 Hz), 5.19 (*J* = 2.5 Hz), and 4.36 (*J* = 7.8 Hz) indicated its trisaccharide nature. The three anomeric protons were, respectively, assigned to α-rhamnose, *β*-apiose, and *β*-glucose. The above considerations were confirmed by the ^{13}C NMR spectrum of **1**, where three anomeric carbons at *δ* 111.4, 104.1, and 102.1 were observed. The highly deshielded carbon at *δ* 111.4 indicated a *β*-orientation of the anomeric center of apiose.[3] The DEPT experiment revealed a quaternary carbon at 80.5 ppm and a methyl at 18.7 ppm, which were assigned to C-3 of apiose and C-6 of rhamnose, respectively. In the same experiment there were observed five methylenes, corresponding to C-4 and C-5 of apiose, C-6 of glucose, and C-7 and C-8 of the aglycon. All the other signals in the ^{1}H NMR and ^{13}C NMR spectra of **1** were assigned after careful analysis of the 2D NMR data.

The upfield shift of C-6 of glucose indicated that the OH group in this position was free. The significant deshielding of H-4 of glucose (4.92 ppm) and the HMBC cross-peak between this proton and the carbonyl carbon at 168.1 ppm confirmed that the caffeoyl unit was attached to position 4 of glucose, as in the structure of **2**. A ^3J correlation in the HMBC spectrum between the C-1 of rhamnose (102.1 ppm) and the H-3 of glucose (3.83 ppm) and between the C-1 of glucose (104.1 ppm) and H-8 of the aglycon showed that **1** is a derivative of acteoside with an additional apiosyl moiety.

Indeed, mild acid hydrolysis[4] of **1** afforded **2** and apiose. The specific rotation of apiose revealed that it was a D-sugar.[5] On the basis of the specific rotation of **2**, which was identical with that of natural acteoside (**2**),[6] the other two sugars of **1** were identified as D-glucose and L-rhamnose.

The C-1 signal of apiose was correlated with a proton at 3.39 ppm, which corresponded to the overlapped signals of H-2 of glucose and H-4 of rhamnose. In the HMQC spectrum these two protons were correlated with carbons at *δ* 76.4 and 80.1. Consequently, the site of attachment of apiose could not be determined easily. This problem was resolved by application of the HMQC-TOCSY experiment, where it was clear that the carbon at *δ* 80.1 (which was correlated with one of the two overlapped protons) belonged to the signal sequence of rhamnose and the carbon at *δ* 76.4 to the signal sequence of glucose. On the basis of the deshielding of C-4 of rhamnose, the apiose moiety should be attached at this position. Acetylation of **1** afforded the undecacetate **3**, which showed four aromatic and seven aliphatic acetyl signals in the ^{1}H NMR spectrum. The H-4 signal of rhamnose, identified by COSY and HMQC-

* To whom correspondence should be addressed. Tel: +30-1-7274598. Fax: +30-1-7274594. E-mail: skaltsounis@pharm.uoa.gr.

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Table 1. ^{1}H NMR (CD_3OD/TMS, 400 MHz, δ ppm, J in Hz) and ^{13}C NMR (CD_3OD/TMS, 50 MHz, δ ppm) Data of **1** and **3** ($CDCl_3$)

	^{13}C NMR		^{1}H NMR	
position	**1**	**3**	**1**	**3**[a]
aglycon				
1	131.4	137.5		
2	116.6	123.7	6.67 (d, J = 2)	7.00 (s)
3	146.1	137.5		
4	142.0	140.4		
5	117.1	122.8	6.65 (d, J = 8)	7.06 (s)
6	121.2	127.2	6.54 (dd, J = 8, 2)	7.06 (s)
α	72.5	69.8	4.03 (m); 3.73 (m)	4.13 (m), 3.63 (m)
β	36.5	35.5	2.77 (t, J = 7)	2.84
glucose				
1	104.1	100.8	4.36 (d, J = 7.8)	4.36 (d, J = 8)
2	76.4	71.7	3.39 (dd, J = 9, 7.8)	5.02 (dd, J = 9, 8)
3	80.0	81.7	3.83 (dd, J = 10, 9)	3.84 (t, J = 8)
4	70.4	70.3	4.92 (t, J = 10)	5.17 (t, J = 9)
5	75.9	72.0	3.52 (*m*)	3.60 (m)
6	62.3	62.4	3.61 (dd, J = 12, 5); 3.51 (dd, J = 12, 2)	4.15 (m)
rhamnose				
1	102.1	99.6	5.26 (d, J = 1.2)	4.72 (d, J = 1)
2	72.3	71.1	3.84 (dd, J = 3, 1.2)	5.01 (dd, J = 3, 1)
3	72.3	70.7	3.66 (dd, J = 10, 3)	5.05 (dd, J = 9, 3)
4	80.1	76.8	3.39 (t, J = 10)	3.55 (t, J = 9)
5	68.8	68.1	3.60 (dq, J = 10, 6)	3.70 (m)
6	18.7	17.9	1.10 (d, J = 6)	1.09 (d, J = 6)
apiose				
1	111.4	106.8	5.19 (d, J = 2.5)	5.10 (d, J = 1)
2	78.5	76.4	3.67 (d, J = 2.5)	5.17 (d, J = 1)
3	80.5	81.5		
4	74.8	72.6	3.60 (s)	4.15 (d, J = 12), 4.08 (d, J = 12)
5	65.7	62.7	3.34 (d, J = 10); 3.27 (d, J = 10)	4.66 (d, J = 12); 4.34 (d, J = 12)
caffeoyl				
1	127.6	132.7		
2	115.2	123.1	7.05 (d, J = 2)	7.35 (d, J = 2)
3	147.0	141.7		
4	150.1	142.5		
5	116.3	124.0	6.78 (d, J = 8)	7.21 (d, J = 8)
6	123.3	126.4	6.96 (dd, J = 8, 2)	7.39 (dd, J = 8.2)
α	114.6	118.4	6.23 (d, J = 16)	6.34 (d, J = 16)
β'	148.0	144.2	7.57 (d, J = 16)	7.61 (d, J = 16)
CO	168.1	164.8		

[a] $COOCH_3$: δ 2.28, 2.27, 2.26, 2.25, 2.08, 2.07, 2.06, 2.04, 2.03, 2.01, 1.97.

TOCSY experiments, was not shifted downfield, thus confirming the site of attachment of apiose. Accordingly, compound **1** was assigned as 1-*O*-3,4-(dihydroxyphenyl)-ethyl-β-D-apiofuranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside, for which we propose the trivial name samioside. It is noteworthy that there are very few examples of phenylethanoid glycosides with a trisaccharide moiety containing apiose.[4] Additionally, the specific trisaccharide moiety of samioside (**1**) is reported herein for the first time in the structure of a natural product.

The free-radical-scavenging activities of the extracts of *P. samia* and the pure compounds isolated were evaluated with the DPPH assay. The scavenging effect corresponded to the intensity of quenching the DPPH radical, as previously described.[7,8] The *P. samia* dichloromethane extract and apigenin, chrysoeriol, and ermanin were inactive (with the percent reduction of the DPPH radical being less than 40% for each of these samples). On the other hand, for the methanolic extract and for compounds **1** and **2**, this value was more than 70%. The IC_{50} values for these three samples were determined and found to be 66.0, 18.0 (23.8 μM), and 30.5 μg/mL (48.9 μM), respectively. These results showed that samioside (**1**) was more active than acteoside (**2**)[9] and also more than the standard caffeic acid (66.7 μM), while it possessed similar activity to gallic acid (17.6 μM).

The antibacterial activity of samioside (**1**) was examined against two Gram-positive (*Staphylococus aureus* and *S. epidermidis*) and four Gram-negative bacteria (*Enterobacter cloaceae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) by the disk diffusion method (Table 2). Samioside (**1**) inhibited the growth of all the tested bacteria (zones of inhibition 12−16 and 10−15 mm for Gram-positive and Gram-negative, respectively). Its antibacterial activity was further detected using the agar dilution method, showing MIC values between 0.46 and 0.89 mg/mL (Table 2). These results are in good accordance with previous reports[10] on acteoside (**2**), which showed a similar antibacterial potency (MIC value 0.4 mg/mL against *S. aureus*).

Table 2. Antimicrobial Activity (Zones of Inhibition) by Disk Diffusion Method and MIC (mg/mL) of Samioside (**1**)

	zones of inhibition			MIC (mg/mL	
organism	**1**	amphotericin B[a]	netilmicin[a]	**1**	netilmicin[a]
S. aureus	12		22	0.46	4×10^{-3}
S. epidermidis	16		24	0.48	4×10^{-3}
E. cloacae	10		22	0.89	8×10^{-3}
E. coli	15		23	0.52	10×10^{-3}
K. pneumoniae	11		25	0.79	8×10^{-3}
P. aeruginosa	10		20	0.85	8.8×10^{-3}
C. albicans	8	20		NT	
C. glabrata	11	24		NT	
C. tropicalis	10	22		NT	

[a] Positive control substances.

Finally, the antifungal activity of samioside (**1**) was examined against three pathogenic fungi (*Candida albicans*, *C. glabrate*, and *C. tropicalis*) by the disk diffusion

method (Table 2), and it was found to exhibit moderate activity (zones of inhibition 8−11 mm).

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. UV spectra were recorded in spectroscopic grade MeOH on a Shimadzu-160A spectrophotometer. IR spectra were taken on a Perkin-Elmer Paragon 500 instrument. ^{1}H NMR spectra were measured on a Bruker DRX-400 (400 MHz) spectrometer and ^{13}C NMR on a Bruker AC-200 (50 MHz). Chemical shifts are given in δ values with TMS as an internal standard. Coupling constants (J) are given in Hz. The signals in the ^{1}H and ^{13}C spectra were assigned unambiguously using 2D NMR techniques: COSY, HMQC, HMQC-TOCSY, and HMBC. These 2D experiments were performed using standard Bruker microprograms. FABMS were obtained using a ZAB HF instrument, in glycerol matrix with NaCl as additive for the positive-ion mode. HRFABMS were obtained on a AEI MS-902 mass spectrometer. Column chromatography was conducted using Si flash gel 60 Merck (40−63 μm), with an overpressure of 300 mbar. Medium-pressure liquid chromatography (MPLC) was performed with a Büchi model 688 apparatus on columns containing Si gel 60 Merck (20−40 μm) or RP-18 Si gel 60 Merck (20−40 μm).

Plant Material. The plant material was collected on Mount Dirphys (Greece) in July 1998. A voucher specimen (Eb007) is deposited in the herbarium of the Laboratory of Pharmacognosy, University of Athens, Greece.

Extraction and Isolation. Air-dried and pulverized aerial parts of the plant (1.5 kg) were extracted with CH_2Cl_2 (3 × 6 L) and then with MeOH (3 × 6 L). A part of the methanolic extract (10 g) was evaporated to dryness and then fractionated by column chromatography over Si gel 60 Merck (40−63 μm), using a CH_2Cl_2−MeOH gradient. Fractions 24−26 were chromatographed by MPLC [Si gel 60 Merck (20−40 μm), CH_2Cl_2−MeOH gradient] to afford apigenin (24 mg, 0.0032%),[11,12] chrysoeriol (35 mg, 0.0047%),[13] and ermanin (15 mg, 0.002%).[14] Fractions 32−34 were chromatographed by MPLC [RP-18 Si gel 60 Merck (20−40 μm), H_2O−MeOH gradient] to afford acteoside (**2**) (500 mg, 0.07%) and samioside (**1**) (100 mg, 0.013%).

Samioside (1): yellowish powder; $[\alpha]^{25}_D$ −68.4° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 332 (3.09), 306 (sh) nm; IR ν_{max} 3299, 1695, 1599, 1517, 1445, 1258 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; FABMS *m/z* 779 $[M + Na]^+$, 460 [M-Rha-Api-H_2O]$^+$; HRFABMS *m/z* 757.2549 (calcd for $C_{34}H_{45}O_{19}$, 757.2555).

Acid Hydrolysis of 1. A solution of **1** (20 mg) in HCl (0.1 N) was refluxed for 20 min under an Ar atmosphere. On cooling, the reaction mixture was neutralized with an Amberlite IR-50 resin. The solvent was removed under reduced pressure, and the residue was chromatographed by MPLC [RP-18 Si gel 60 Merck (20−40 μm), H_2O−MeOH gradient] to afford D-apiose {$[\alpha]_D$ +8° (*c* 0.1, H_2O)} and acteoside (**2**) {$[\alpha]_D$ −70° (*c* 0.1, MeOH)}.

Acetylation of 1. Treatment of **1** (10 mg) with Ac_2O (1 mL) and pyridine (1 mL) at room temperature overnight gave an undecaacetate (**3**, 90%); ^{1}H and ^{13}C NMR, see Table 1; FABMS *m/z* 1241 $[M + Na]^+$.

DPPH Free-Radical-Scavenging Assay. In brief, 100 μL of sample solution in DMSO was added to 1.9 mL of 315 μM DPPH in ethanol and allowed to react for 30 min at 37 °C, with the optical density measured at 515 nm. For the blank, EtOH was used instead of the DPPH solution, and for the control, DMSO was used instead of the sample solution. The IC_{50} values, the amount of each sample required to scavenge 50% DPPH free radicals, were calculated from regression lines, where the abscissa represented the concentration of tested compound and the ordinate the average percent reduction of DPPH radical, from three separate tests.

Antimicrobial Activity. The in vitro antimicrobial activity of samioside (**1**) was determined by the disk diffusion method of Bauer-Kirby,[15] as described previously,[16] against two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228), and four Gram-negative, *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), and *Pseudomonas aeruginosa* (ATCC 27853), as well as against three pathogenic fungi, *Candida albicans* (ATCC10231), *C. glabrata* (ATCC 28838), and *C. tropicalis* (ATCC 13801). Netilmicin and amphotericin B were used as standard antibiotics for comparison for the tested bacteria and the fungi. The results were reported as the diameter of the zone of inhibition around each disk (in mm), with the evaluation of inhibition corresponding to <7 mm (−), 7−10 mm (+), 11−16 mm (++), and >16 mm (+++). The experiments were repeated three times, and the results were expressed as average values. The minimum inhibitory concentrations (MICs) were determined as described previously.[17] MICs were determined as the lowest concentrations preventing visible growth.

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