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Further Isolation of Antioxidative (+)-1-Hydroxypinoresinol-1-*O*-*β*-D-glucoside from the Rhizome of *Salvia miltiorrhiza* that Acts on Peroxynitrite, Total ROS and 1,1-Diphenyl-2-picrylhydrazyl Radical

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A furanofuranoid lignan glycoside, with radical scavenging on peroxynitrite, total reactive oxygen species (ROS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, was isolated from the rhizome of *Salvia miltiorrhiza* and characterized as (+)-1-hydroxypinoresinol-1-*O*-*β*-D-glucoside based on spectroscopic evidence. The compound exhibited peroxynitrite, total ROS and DPPH radical scavenging activities with IC_{50} values of 3.23 ± 0.04, 2.26 ± 0.07 and 32.3 ± 0.13 μM, respectively. Penicillamine, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and L-ascorbic acid, acting as positive controls, showed radical scavenging activities with IC_{50} values of 6.72 ± 0.25, 1.43 ± 0.04 and 11.4 ± 0.07 μM, respectively.

Key words: *Salvia miltiorrhiza*, Lignan, Antioxidant, (+)-1-Hydroxypinoresinol-1-*O*-*β*-D-glucoside, Peroxynitrite, Total ROS, 1,1-Diphenyl-2-picrylhydrazyl radical

INTRODUCTION

Tanshen, the rhizome of *Salvia miltiorrhiza* Bunge (Lamiaceae), has been used in traditional Chinese medicine to treat coronary heart diseases, particularly angina pectoris and myocardial infarction (Tang & Eisenbrand, 1992). In a previous study, we reported the antioxidative effect of *S. miltiorrhiza* and new components isolated from the BuOH and EtOAc soluble fractions of this plant, such as 3-(3', 4'-dihydroxyphenyl)-(2*R*)-lactamide and salviamiltamide (Kang *et al.*, 1997; Choi *et al.*, 2001). From further study on the active principles from the BuOH soluble fraction of *S. miltiorrhiza*, we isolated a furanofuranoid lignan glycoside, (+)-1-hydroxypinoresinol-1-*O*-*β*-D-glucoside, which differs from depsides, major antioxidants of *Salvia* species, i.e. rosmarinic acid, lithospermic acid and salvianolic acids. The lignan was evaluated for its scavenging ability on authentic peroxynitrite, total reactive oxygen species (ROS) and 1,1-diphenyl-2-picrylhydrayl (DPPH) radicals.

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MATERIALS AND METHODS

General experimental procedures

^{1}H-, ^{13}C-NMR and DEPT spectra were measured by JNM ECP-400 spectrometer. Chemical shifts were referenced to the respective residual solvent peaks (δ_H 3.0 and δ_C 49.0 for MeOH-d_4). HMQC, and HMBC spectra were recorded on a JNM ECP-400 spectrometer using pulsed field gradients. FAB-MS spectrum was measured on a JMS HX110A/HX110A Tandem mass spectrometer (JEOL). Optical rotations were recorded on a Perkin-Elmer polarimeter 341. Column chromatography was carried out using silica gel (Merck, 70-230 mesh), Sephadex LH-20, Amberlite IR-120 Plus (Sigma) and MCI gel (Mitsubishi Chemical Co. Japan, 75~150 μ). Thin layer chromatography (TLC) was performed on the precoated Merck Kieselgel 60 F_{254} plates (0.25 mm), and 50% H_2SO_4 was used as spray reagent. 3-Morpholinosydnonimine (SIN-1), and DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), L-ascorbic acid and DPPH were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were from Molecular Probes

(Eugene, Oregon, USA) and Cayman Chemical Co. (Ann Arbor, MI, USA), respectively. All the solvents for column chromatography were of a reagent grade from commercial sources.

Plant material

The rhizome of *S. miltiorrhiza* was obtained from the Chien Yuan Herbal Medicinal Co., Taipei, Taiwan in 1995 and authenticated by Prof. H. J. Chi of the Natural Products Research Institute, Seoul National University. A voucher specimen (No. 950921) has been deposited in the Herbarium of the Natural Products Research Institute.

Extraction and isolation

The powdered rhizome (2.9 kg) was refluxed with MeOH (3×5 L) for 3 hr. The extract (982 g) was suspended in water and partitioned with CH_2Cl_2 (40 g), *n*-BuOH (150 g), in sequence. The *n*-BuOH soluble fraction (150 g) was first subjected to Amberlite IR-120 Plus ion exchange column chromatography (500 mL) by eluting with water. The eluate (110 g) was applied to MCI-gel column chromatography by eluting with water containing increasing proportions of MeOH to give five subfractions, the second of which (30.1 g) underwent further silica gel column chromatography eluting with CH_2Cl_2 : MeOH (9 : 1) to yield a known compound 1 (27.5 mg).

(+)-1-Hydroxypinoresinol-1-*O*-β-D-glucoside (1): Amorphous powder, $[\alpha]_D^{20}$: -9.3° (c 0.009, MeOH). Positive FAB-MS *m/z* 559 $[M+Na]^+$, $536[M]^+$. ^{1}H-NMR (400 MHz, CD_3OD) : δ 7.11 (1H, d, *J* = 1.7 Hz, H-2'), 7.03 (1H, d, *J* = 1.8 Hz, H-2"), 6.88 (1H, dd, *J* = 1.8 & 7.9 Hz, H-6"), 6.86 (1H, dd, *J* = 1.8 & 7.8 Hz, H-6'), 6.80 (1H, d, *J* = 8.1 Hz, H-5"), 6.72 (1H, d, *J* = 8.1 Hz, H-5'), 4.69 (1H, s, H-2), 4.58 (1H, s, H-6), 4.49 (1H, t, *J* = 8.8 Hz, H-4), 4.39 (1H, d, *J* = 10.4, H-8), 4.33 (1H, d, *J* = 7.7 Hz, H-1'''), 3.93 (1H, d, *J* = 10.4 Hz, H-8), 3.88 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.80 (1H, dd, *J* = 5.9 & 9.1 Hz, H-4), 3.68 (1H, dd, *J* = 2.2 &12.[illegible] Hz, H-6'''), 3.51 (1H, dd, *J* = 5.8 & 12.0 Hz, H-6'''), 3.40 (1H, m, H-5), 3.14 (2H, dd, *J* = 2.5 & 6.8 Hz, H-3''', 4'''), 3.02 (1H, dt, *J* = 2.3, 7.3 & 7.5 Hz, H-2'''), 2.87 (1H, m, H-5"); ^{13}C-NMR (100 MHz, CD_3OD) : δ 150.1 (C-3"), 149.2 (C-3'), 148.3 (C-4"), 148.1 (C-4'), 133.9 (C-1"), 129.4 (C-1'), 123.2 (C-6'), 120.7 (C-6"), 117.1 (C-5"), 116.0 (C-5'), 114.9 (C-2'), 111.5 (C-2"), 100.9 (C-1'''), 100.0 (C-1), 90.8 (C-2), 87.6 (C-6), 79.1 (C-3'''), 78.8 (C-5'''), 75.6 (C-2'''), 74.[illegible] (C-8), 73.0 (C-4), 72.0 (C-4'''), 63.3 (C-6'''), 61.1 (C-5) 57.4 (OMe), 57.2 (OMe)

DPPH radical scavenging effect

The DPPH radical scavenging effect was evaluated as previously described by Blois (1958) with minor modifications. A methanolic sample solution of 160 μL at several concentrations and 40 μL of DPPH methanolic solution (1.5×10^{-4} M) were added to a 96-well microplate, in a total volume of 200 μL. After letting the reaction mixture stand at room temperature for 30 min, its absorbance was determined at 520 nm, in a microplate reader (VERSA max, USA).

Measurement of free radical generation (DCF Method)

The generation of ROS was assessed by using the ROS-sensitive fluorescence indicator 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes). Test samples (final concentration, 0.5 mg/mL) were added to kidney postmitochondrial fraction in 50 mM potassium phosphate buffer and incubated at 37°C for 5 min. Then the mixture was loaded with DCFH-DA (5 μg/mL) in potassium phosphate buffer. The fluorescence of DCF was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm for 30 min using a microplate fluorescence spectrophotometer FL 500 (Bio-Tek instruments, USA).

Measurement of $ONOO^-$ scavenging activity

$ONOO^-$ scavenging ability was measured by monitoring the oxidation of dihydrorhodamine 123 with a modified version of the method of Kooy *et al.* (1994). A stock solution of DHR 123 (5 mM) purged with nitrogen and was prepared in advance and stored at 80°C. A working solution of DHR 123 (final concentration, 5 μM) diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride with 100 μM (f.c.) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. The $ONOO^-$ scavenging ability based on the oxidation of DHR 123 was determined at room temperature by a microplate fluorescence spectrophotometer FL 500 (Bio-Tek instruments, USA) with excitation and emission wavelengths of 485 nm and 530 nm. The background and final fluorescent intensities were measured 5 min after treatment with or without SIN-1 (f.c. 10 μM) or authentic $ONOO^-$ (f.c. 10 μM) in 0.3 M sodium hydroxide. Oxidation of DHR 123 by decomposition of SIN-1 gradually increased whereas authentic $ONOO^-$ rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time. Penicillamine was used as a positive control.

RESULTS AND DISCUSSION

Column chromatography of the *n*-BuOH soluble fraction of the methanolic extract of *S. miltiorrhiza* yielded a known compound **1**. The structure of compound **1** was identified by comparison with published spectral data as (+)-1-hy-

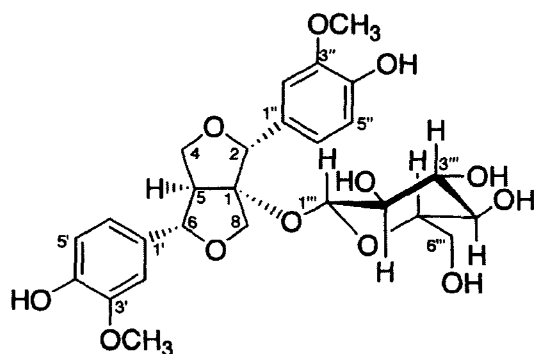

Table I. Scavenging activities of (+)-1-hydroxypinoresinol-1-*O*-β-D-glucoside isolated from *S. miltiorrhiza* on peroxynitrite, total ROS and DPPH radicals

	IC_{50} (μM)		
	Peroxynitrite	Total ROS	DPPH
(+)-1-Hydroxypinoresinol-1-O-β-D-glucoside	3.23 ± 0.04	2.26 ± 0.07	32.3 ± 0.13
Penicillamine	6.72 ± 0.25		
Trolox		1.43 ± 0.04	
L-Ascorbic acid			11.4 ± 0.07

Results are mean ± S.E. (n = 3)
Penicillamine, Trolox and L-ascorbic acid were used as positive controls for peroxynitrite, total ROS and DPPH radicals, respectively.

droxypinoresinol-1-*O*-β-D-glucoside (**1**) (Wang *et al.*, 1993; Wang *et al.*, 1998). The structure was further identified by detailed analysis of the ^{1}H- and ^{13}C-NMR spectra, aided by DEPT, HMQC and HMBC experiments. In the HMBC spectrum of **1**, the signal of glucose anomeric proton at δ 4.33 was correlated with that of C-1 at δ 100.0, suggested that the glucose was connected with hydroxypinoresinol at C-1. On the basis of these results, the structure of **1** was established as (+)-1-hydroxypinoresinol-1-*O*-β-D-glucoside. This compound (**1**) has been reported from several plants, e.g. *Bauhinia tarapotensis* Benth. (Leguminosae), Latin American medicinal plants (Braca *et al.*, 2001), *Stauntonia hexaphylla* Dence (Lardizabalaceae), traditional Chinese medicine (Wang *et al.*, 1993), the bark of *Olea* plants (Oleaceae) (Tsukamoto *et al.*, 1985) and sage, *Salvia officinalis* (Lamiaceae) (Wang *et al.*, 1998). However, this is the first report of its occurrence in *S. miltiorrhiza*.

Free or non-free radicals including ROS cause a variety of diseases such as inflammation, cardiovascular diseases, cancer, Alzheimers disease, rheumatoid arthritis, and atherosclerosis (Beckman *et al.*, 1996; Podrez *et al.*, 1999). These diseases have been reported to be ameliorated by radical scavengers (Aruoma, 1999; Hermann *et al.*, 1999). The compound **1** exhibited scavenging activities on peroxynitrite twice as strong, with an IC_{50} value of 3.23 ± 0.04, as that of Penicillamine, the positive control with an IC_{50} value of 6.72 ± 0.25. In addition, **1** showed the total ROS and DPPH radical scavenging activities with IC_{50} values of 2.26 ± 0.07 and 32.3 ± 0.13 μM, respectively. The positive controls on the total ROS and DPPH radical, Trolox and L-ascorbic acid, exhibited IC_{50} values of 1.43 ± 0.04 and 11.4 ± 0.07 μM, respectively (Table I). The radical scavenging abilities of (+)-1-hydroxypinoresinol-1-*O*-β-D-glucoside can be useful in the prevention and treatment of free radical related diseases.

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