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Two *p*-Terphenyls from Mushroom *Paxillus panuoides* with Free Radical Scavenging Activity

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Abstract As part of a continuing investigation to identify free radical scavengers from the fruit bodies of basidiomycetes, we isolated two *p*-terphenyl compounds, designated as PA1 and PA21, from methanolic extract of the fruit body of *Paxillus panuoides*. The methanolic extract was processed by ethyl acetate extraction and silica gel column chromatography to yield two active fractions. PA1 was obtained from one of the fractions through Sephadex LH-20 and silica gel column chromatographies and reverse-phase HPLC. The other fraction was purified by Sephadex LH-20 and reverse-phase column chromatographies to produce PA2. The compounds PA1 and PA2 were identified as leucomentin-4 and leucomentin-2, respectively, on the basis of various spectroscopic analyses. These compounds exhibited strong inhibitory activities against lipid peroxidation in rat liver microsomes with IC_{50} values of 0.10 and 0.06 μg/ml, respectively.

Key words: *Paxillus panuoides*, leucomentins, lipid peroxidation inhibitor, chemical structure

The peroxidative disintegration of cells and organellar membranes by free radicals has been implicated in various pathological processes and particularly in the pathogenesis of diseases such as myocardial and cerebral ischemia, atherosclerosis, diabetes, rheumatoid arthritis, cancer-initiation, and the aging process [7-9]. Accordingly, free radical scavengers are potential protective agents against these diseases. Recently, many antioxidants for ameliorating free radical-mediated injuries have been identified from microbial metabolites [20-22].

In this continuing investigation for biologically active constituents from basidiomycetes [13-18], two *p*-terphenyl compounds, named PA1 and PA2, were isolated from the methanolic extract of the fruit body of *Paxillus panuoides*, which was previously reported to produce diphenyl benzoquinones, flavomentins, and spiromentins [4, 6]. This paper describes the isolation, structural elucidation, and free radical scavenging activity of these compounds.

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Materials and Methods

General Experiment

Specific rotations were determined using a Polartronic polarimeter. The high resolution mass spectra were measured using a JEOL JMS-HX 110/100A spectrometer in the FAB mode using a glycerol matrix with polyethylene glycol as the internal standard. The UV and IR spectra were recorded on a Shimadzu UV-260 and FT-IR Equinox 55 spectrophotometer, respectively. The NMR spectra were obtained on a Varian UNITY 500 NMR spectrometer with ^{1}H-NMR at 500 MHz and ^{13}C-NMR at 125 MHz. The chemical shifts were taken in ppm using TMS as the internal standard. The analytical silica gel TLC (Merck, Kiesel gel 60 F_{254}, 0.25 mm) and preparative silica gel TLC (Merck, Kiesel gel 60 F_{254}, 0.5 mm) plates were used without activation. The HPLC was performed on a Senshu pak ODS column (20×250 mm) with a flow rate of 6 ml/min using 70% and 50% aqueous methanol for PA1 and PA2, respectively, as the mobile phase, and by monitoring with a photodiode-array detector (190–650 nm).

Microorganism

Paxillus panuoides was collected at Sokri mountain in Chungbuk Province, Korea and identified by the staff of the Korea Research Institute of Bioscience and Biotechnology, Korea, according to the Hongo method [12]. After being dried in a dark and well-ventilated place, the fruit body of *P. panuoides* was extracted with methanol for the isolation of the active compounds.

Inhibitory Activity against Lipid Peroxidation in Rat Liver Microsomes

The lipid peroxidation inhibitory activity in rat liver microsomes was evaluated by the thiobarbituric acid

method [19] with minor modifications. The rat liver microsomes were prepared according to the method of Hogeboom [10] with some modifications and finally suspended in a 100 mM Tris-HCl buffer (pH 7.4). The reaction was initiated by the addition of 100 μM $FeSO_4 \cdot H_2O$ (0.1 ml) into a mixture of a Tris-HCl buffer (0.7 ml), 0.2 mM ascorbic acid (50 μl), 0.5 μg protein/ml microsomal suspension (40 μl), and 10 μl of the sample solution. The reaction mixture was incubated at 37°C for 30 min. After incubation, the reaction was stopped by the addition of 0.25 ml of TCA (3M)-HCl (2N) 1:1 mixture and then centrifuged at 3,500 ×g for 10 min. The supernatant (1 ml) was mixed with 0.67% (w/v) thiobarbituric acid (0.25 ml) and then heated in boiling water for 10 min. The lipid peroxidation was assessed by measuring the thiobarbituric acid reactive products at 532 nm. The lipid peroxidation inhibitory activity was calculated as follows: [1-(T-B)/(C-B)]×100(%), in which T, C, and B are the absorbance values at 532 nm of the sample treated, the control (without the sample), and the zero-time control, respectively. The value of IC_{50} represents the concentration (μg/ml) of the compounds required for 50% inhibition of the microsomal lipid peroxidation.

Superoxide Dismutase (SOD) Radical Scavenging Activity

The method chosen for the assay of the SOD activity was a modification of the indirect inhibition assay developed by Beauchamp and Fridovich [3]. A xanthine/xanthine oxidase system was utilized to generate a superoxide flux. Each well of a 96-well plate contained a final concentration of the following reagents: 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 5.6×10^{-5} M NBT (nitroblue tetrazolium), 0.1 mM xanthine, enough xanthine oxidase to achieve the required reference rate (0.020 absorbance/min), and each concentration of the sample. NBT reduction to blue formazan by a superoxide was followed at 560 nm in a microplate reader at room temperature. The inhibitory ratio of each compound to the formation of diformazan from NBT was calculated. The amount of inhibition was defined as a percentage of the reference rate of the NBT reduction when the sample was absent. The data were plotted as the percentage inhibition vs the sample concentration.

Measurement of DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity [5]

Each concentration of the test sample solution in DMSO (20 μl) was added to 980 μl of a 150 μM DPPH ethanol solution. After vortex mixing, the mixture was incubated for 20 min at room temperature and the absorbance at 517 nm was measured. The differences in the absorbance between a test sample and a control (DMSO) were recorded and the ED_{50} values were determined as the concentration of the compound that produced a 50% decrease in the absorbance from a blank test.

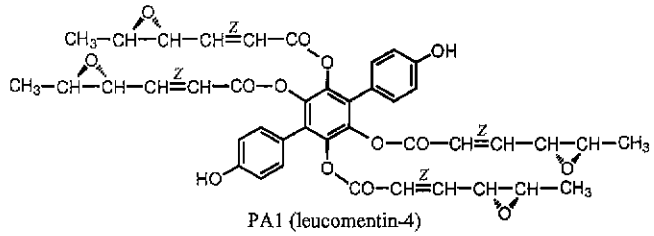
Results and Discussion

Isolation and Purification

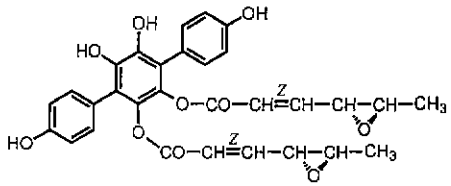
The fruit body of *P. panuoides* (690 g, fresh weight) was extracted twice using methanol (Fig. 2). The methanolic extract was concentrated *in vacuo* and the residue was partitioned between ethyl acetate and water. After concentrating the solvent layer, the concentrate was subjected to a column of silica gel and the column was eluted with $CHCl_3$-MeOH (30:1-5:1) to obtain two active fractions. One of the fractions was further purified by column chromatographies of Sephadex LH-20 eluted with $CHCl_3$-MeOH (1:1) and silica gel eluted with $CHCl_3$-MeOH (20:1). Finally, reverse-phase HPLC using 70% MeOH as the eluent produced pure PA1 (30 mg) as a gray powder. The other fraction was purified by Sephadex LH-20 column chromatography with MeOH, followed by reverse-phase (ODS) open column chromatography with 50% aq. MeOH which obtained PA2 (110 mg) as a brown powder. PA1: Gray powder; UV λ_{max} nm (ε) in MeOH:209 (42,100), 222 (45,500), 259 (18,800); IR (KBr): 3397, 1754, 1639, 1612, 1525, 1446, 1414, 1394, 1242, 1128, 1002, 951, 851 cm^{-1}; $[\alpha]_D$=73° (*c*=2.95, MeOH); ^{1}H-NMR ($CDCl_3$, 500 MHz) 1.34 (12H, d, *J*=5.1 Hz), 2.91 (4H, dq, *J*=5.1, 2.0), 3.94 (4H, dd, *J*=7.9, 2.0), 5.81 (4H, dd, *J*=11.6, 7.9), 5.91 (4H, d, *J*=11.6), 6.81 (4H, d, *J*=8.5), 7.19 (4H, d, *J*=8.5); For ^{13}C-NMR data, see Fig. 3; HRFAB-MS: *m/z* 767.2336 $(M+H)^+$, $C_{42}H_{38}O_{14}$ requires 767.2339. PA2: Dark brown powder; UV λ_{max} nm (ε) in MeOH:209 (58,900), 223 (51,900), 259 (26,400); IR (KBr): 3378, 1747, 1639, 1612, 1525, 1455, 1430, 1376, 1267, 1240, 1153, 1002, 975, 850 cm^{-1}; $[\alpha]_D$=13° (*c*=0.6, MeOH); ^{1}H-NMR (DMSO-d_6, 500 MHz) 1.23 (6H, d, *J*=4.8 Hz), 2.96 (2H, dq, *J*=5.1, 2.0), 3.75 (2H, dd, *J*=7.8, 2.0), 5.86 (2H, dd, *J*=11.7, 7.8), 5.93 (2H, d, *J*=11.7), 6.78 (4H, d, *J*=8.4), 7.08 (4H, d, *J*=8.4), 8.50 (2H, s), 9.47 (2H, s); For ^{13}C-NMR data, see Fig. 4; HRFAB-MS: *m/z* 547.1566 $(M+H)^+$, $C_{30}H_{26}O_{10}$ requires 547.1604.

Structure Determination

The molecular formula of PA1 was established as $C_{42}H_{38}O_{14}$ by high-resolution FAB mass spectroscopy (*m/z* 767.2336 $(M+H)^+$ -0.3 mmu). The IR absorptions at 3,400, 1,754, and 1,128 cm^{-1} suggested the presence of hydroxyl, carbonyl, and C-O groups, respectively. The ^{1}H-NMR spectrum in $CDCl_3$ revealed signals attributable to nine protons including two aromatic, two olefinic, two methine, and one methyl protons, while only twelve carbons were observed in the ^{13}C-NMR spectrum, suggesting that this compound is a symmetrical dimer with pairs of equivalent



PA1 (leucomentin-4)



PA2 (leucomentin-2)

Fig. 1. Structures of compounds PA1 and PA2.

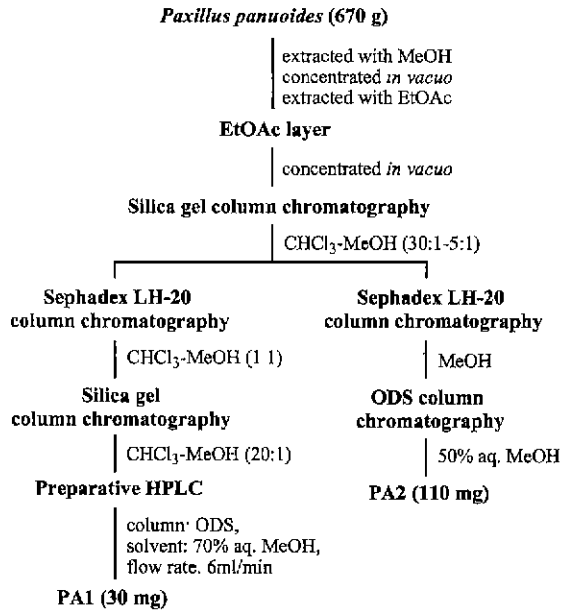


Fig. 2. Purification procedure of compounds PA1 and PA2.

carbons. Based on the DEPT spectrum, the carbon signals were assigned as a carbonyl carbon at 162.6 ppm, four sp^2 quaternary carbons at 156.0, 139.1, 130.2, and 123.1 ppm, four sp^2 methines at 149.8, 130.9, 121.1, and 115.3 ppm, two sp^3 methines at 56.2 and 55.0 ppm that were assumed to be oxirane on the basis of their chemical shift values, and one methyl at 17.3 ppm. Among these ^{13}C signals, three sp^2 quaternary carbons at 123.1, 130.2, and 156.0 ppm showed a relatively low intensity, that is, half of other peaks. The HMQC experiments [2] established all one-bonded ^1H-^{13}C connectivities, whereas the HMBC [1] and ^1H irradiation experiments revealed two partial units, 4,5-epoxy-2-hexenoic acid (**1**) and 1,4-disubstituted benzene (**2**), as shown in Fig. 3.

The ratio of the partial structures **1** and **2** was established as 2:1 based on ^1H-NMR integration and the ^{13}C peak intensity. However, this ratio only satisfied half of the molecular weights measured according to the FAB-mode, therefore, it was found that PA1 was just partially composed of units **1** and **2** with a ratio of 4:2. By a process of elimination for the ^{13}C signals, one remaining unassignable carbon at 139.1 ppm implied that PA1 was a symmetrically dimeric *p*-terphenyl. Two or more remaining quaternary carbons would be required for *o*- or *m*-terphenyl. Consequently, the structure of PA1 was unambiguously determined as a *p*-terphenyl compound, 4,5-epoxy-2-hexenoic acid 4,4"-dihydroxy-3',5',6'-tris(4,5-epoxy-2-hexenoyloxy)-1,1':4',1"-terphenyl-2'-yl ester, as shown in Fig. 1. The geometries of the double bonds were assigned as *cis* on the basis of the vicinal coupling constants of 11.6 Hz. Furthermore, the relative configurations of the oxiranes were determined to be *trans* by the small coupling constants of J=2.0 Hz.

PA2 was closely related in its physico-chemical properties and ^1H- and ^{13}C-NMR spectra, suggesting that PA2 was also a terphenyl. The molecular formula was established as $C_{30}H_{26}O_{10}$ by high resolution FAB-mass spectrometry (*m/z* 547.1566 $(M+H)^+$ -3.8 mmu). The IR absorptions of PA2 at 3,378 and 1,747 cm^{-1} suggested the presence of hydroxyl and carbonyl groups, respectively. The ^1H-NMR spectrum in DMSO-d_6 revealed the presence of two hydroxyls at 9.47 and 8.50 ppm, which collapsed on shaking with D_2O, one 1,4-disubstituted benzene, two olefinic protons at 5.93 and 5.86 ppm that were *cis*-coupled to each other, two methines at 3.75 and 2.96 ppm, and one doublet methyl, while only thirteen carbons were observed in the ^{13}C-NMR spectrum. The ^{13}C signals were characterized by the DEPT spectrum as one methyl at 17.7 ppm, six methines at 56.1, 57.1, 116.1, 122.7, 132.5, and 149.9 ppm, and six quaternary carbons at 123.9, 124.9, 134.4, 142.7, 158.2, and 165.2 ppm. The HMQC experiments established all $^1J_{CH}$ connectivities, and the HMBC and ^1H-

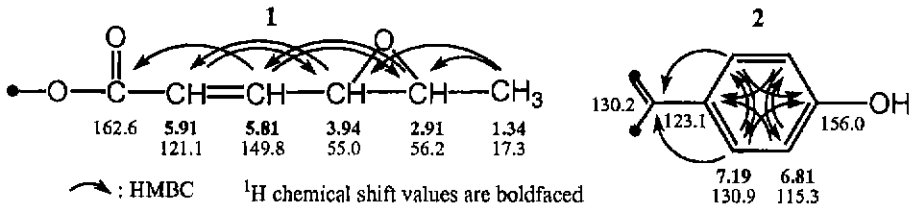


Fig. 3. Partial structures of compound PA1 elucidated by HMBC experiment.

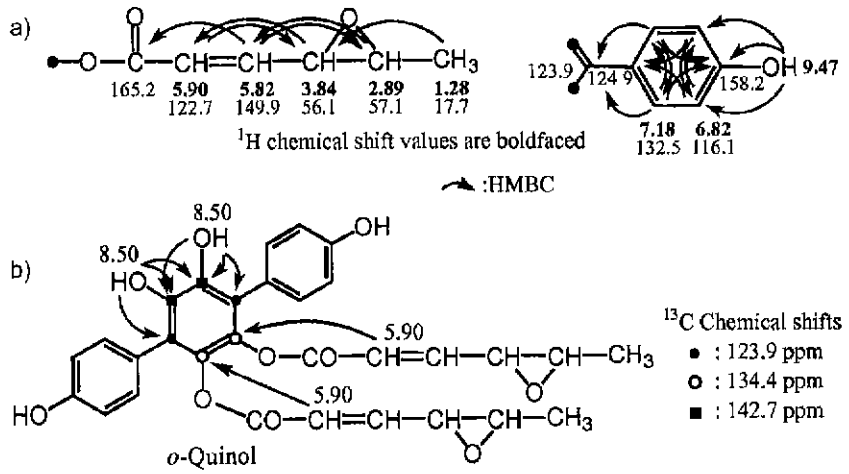


Fig. 4. Partial structures (a) and gross structure (b) of compound PA2 elucidated by HMBC experiment.

NMR data revealed the presence of two partial structures, 4,5-epoxy-2-hexenoic acid and 1,4-disubstituted benzene, the same as those of PA1 (Fig. 4a).

The ratio of these two partial structures was established as 1:1 based on ^{1}H integration, and the ^{13}C numbers and molecular formula suggested that PA2 was also a symmetrical dimer. By a process of elimination, the two remaining quaternary carbons at 134.4 and 142.7 ppm implied that PA2 could be an *o*- or *p*-terphenyl moiety. *p*-Terphenyl moiety was deduced from the physico-chemical properties similar to PA1. In addition, PA2 was isolated from a PA1-producing organism and *p*-terphenyl is ubiquitous among microbial metabolites. PA2 still exhibited two possible structures of *o*- or *p*-quinols, however, the HMBC data suggested that PA2 should be a *o*-quinol as it only showed two strong long-range correlations from the hydroxyl protons at 8.50 ppm to the quaternary carbons at 142.7 and 123.9 ppm, and did not show any correlation to the quaternary carbon at 134.4 ppm, as shown in Fig. 4b. *p*-Quinol generally requires long-range correlations to three quaternary carbons at 142.7, 134.4, and 123.9 ppm.

In addition to this, four-bonded long-range correlations of weak peaks were observed in the HMBC experiment, which showed correlations from the methine protons at 5.90 ppm to the quaternary carbons at 134.4 ppm and from the aromatic methine protons at 7.18 ppm to the quaternary carbons at 142.7 ppm, thereby completely establishing all ^{1}H and ^{13}C assigments. From the above results, the structure of PA2 was assigned as a *p*-terphenyl with an *o*-quinol moiety, 4,5-epoxy-2-hexenoic acid 4,5',6',4"-tetrahydroxy-3'-(4,5-epoxy-2-hexenoyloxy)-1,1':4',1"-terphenyl-2'-yl ester. The geometries of the double bonds were assigned as *cis* based upon the vicinal coupling constants of J=11.7 Hz. The relative configurations of the oxiranes were determined as *trans* by the coupling constants with J=2.0 Hz.

Through a database and literature search, it was found that compounds PA1 and PA2 had the same structures as leucomentin-4 and leucomentin-2, respectively, which had been previously isolated from *Paxillus atrotomentosus* [11]. However, the complete assigment of the ^{1}H and ^{13}C chemical shift values of leucomentin-4 and leucomentin-2 were established in the current study. This was the first report that these compounds were isolated from *Paxillus panuoides*.

Biological Activity

For the purpose of evaluating the antioxidative activity of compounds PA1 and PA2, their lipid peroxidation inhibitory activity, superoxide scavenging activity, and DPPH radical scavenging activity were investigated. The lipid peroxidation inhibitory activity was evaluated based on the inhibitory activities of the compounds against lipid peroxidation induced by a nonenzymic Fe(II)-ascorbic acid system in rat liver microsomes according to the method of Yagi *et al.* [19]. PA1 and PA2 showed strong lipid peroxidation inhibitory activities with IC_{50} values of 0.10 and 0.06 μg/ml, respectively, in a dose-dependent fashion. PA2 was twenty-five times as active as vitamin E (IC_{50}=1.5 μg/ml) which was used as the control, as shown in Fig. 5.

The superoxide radical scavenging activities of PA1 and PA2 were also investigated and compared with those of well-known free radical scavengers such as caffeic acid, catechin, and vitamin E. Caffeic acid, catechin, and vitamin E showed strong scavenging effects of 92, 75, and 88%, respectively, at 10 μg/ml on superoxide radicals generated by a xanthine/xanthine oxidase system, whereas the compounds PA1 and PA2 exhibited very low superoxide radical scavenging activities of <15% at 10 μg/ml and 25–30% at 100 μg/ml. Furthermore, the PA1 and PA2 compounds did not exhibit any DPPH radical scavenging activity. The antioxidative test with DPPH used in this experiment is based on the proton radical scavenging action, which is one of the various mechanisms of antioxidation. This implies that PA1 and PA2 are not proton-donating antioxidative

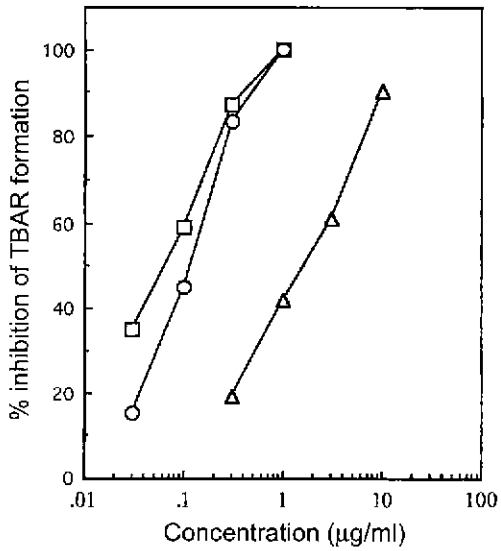


Fig. 5. Inhibitory effects of PA1 and PA2 against lipid peroxidation in rat liver microsomes. PA1 (○). PA2 (□), Vitamin E (△).

compounds. From the above results, compounds PA1 and PA2 are suggested to be specific lipid peroxidation inhibitors.

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