2,3,4-Trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran, a Novel Antioxidant, from *Penicillium citrinum* F5

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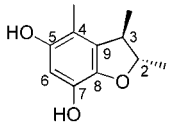
Bioassy-directed fractionation of a culture broth of *Penicillium citrinum* F5 led to the isolation of a novel antioxidant 2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran (**1**), together with gentisic acid (**2**), and their structures were determined on the basis of spectroscopic data. In the 1,1-diphenyl-picryl-hydrazyl (DPPH) assay system, compounds **1** and **2** showed free radical scavenging activity with IC_{20} values of 10.39 and 4.26 μM, respectively.

Free radicals have been known to mediate disease such as atherosclerosis,[1] ischemia-reperfusion,[2] cancer, and even aging.[3,4] Consequently, free radical scavengers are now considered to be prospects as protective or therapeutic agents against these diseases. In the course of our preliminary microbial screening program, we found that the culture broth of *Penicillium citrinum* F5 exhibited strong activity as a free radical scavenger in the 1,1-diphenyl-picryl-hydrazyl (DPPH) assay.[5,6]

Isolation of bioactive components was guided by their free radical scavenging activity in a DPPH assay.[5,6] *P. citrinum* F5 was incubated for 20 days at room temperature, and the culture broth was fractioned by sequential Diaion HP-20, Cosmosil 75C_{18}-OPN, and TSK gel HW-40 column chromatography and finally purified by preparative HPLC. Two active compounds, **1** and **2**, were isolated. Compound **1** was a new dihydrobenzofuran derivative, 2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran, whose structure was elucidated by spectral analysis. Compound **2** was identified as gentisic acid by comparison of its spectral data with those reported for gentisic acid.[7]



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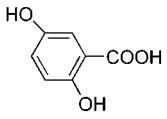

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Compound **1** was isolated as a brown solid, and the molecular formula was assigned as $C_{11}H_{14}O_3$ on the basis of its HREIMS and ^{1}H and ^{13}C NMR spectra. The IR spectrum exhibited bands at 3356 cm^{-1} (hydroxyl group) and at 1637, 1530, 1510, 1460 cm^{-1} (aromatic ring). Both ^{1}H and ^{13}C NMR spectra of compound **1** contained well-separated and easily assigned resonances and led us to believe that compound **1** possessed a dihydrobenzofuran-like skeleton.[8] Analysis of the ^{13}C NMR and DEPT spectra revealed the presence of three methyl groups, one oxygenated sp^3 carbon, one sp^3 methine, one sp^2-hybridized methine, two sp^2-hybridized quaternary carbons, and three sp^2 carbons that were both oxygenated and quaternary. The ^{1}H NMR spectrum of **1** showed signals at δ_H 1.25 (3H, d, J = 6.5 Hz), 1.30 (3H, d, J = 6.5 Hz), 3.00 (1H, m), and 4.37 (1H, m), assigned to two methyl groups, one methine signal, and one oxymethine signal, respectively, corresponding to the furan ring of the dihydrobenzofuran. These protons were correlated with the carbon signals at δ_C 18.7 (q, 3-CH_3), 20.1 (q, 2-CH_3), 44.3 (d, C-3), and 86.7 (d, C-2) in the HMQC experiment and are in agreement with a 2,3-dimethyl-2,3-dihydrobenzofuran unit. The ^{1}H NMR spectrum also showed the presence of one aromatic proton at δ_H 6.20 (1H, s), which indicated a pentasubstituted benzene ring. In addition, one methyl group at δ_H 2.05 (3H, s) appeared on the benzene ring of **1**. HMBC correlation peaks (3-CH_3/C-2, C-3, C-9; 2-CH_3/C-2, C-3; H-2/3-CH_3, C-8, C-9; H-3/2-CH_3, 3-CH_3, C-8, C-9; 4-CH_3/C-5, C-9, C-4; H-6/C-4, C-5, C-7, C-8) also supported the substitution of the benzene ring. The relative stereochemistry of the furan ring of **1** was deduced from NOESY 1D experiments. NOEs from 2-CH_3 to H-2 and H-3, and 3-CH_3 to H-2, H-3, and 4-CH_3 indicated a trans relationship for 2-CH_3 and 3-CH_3. All of the above data are compatible with the structure of **1** as 2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran.

Table 1. Free Redical Scavenging Activity of **1**, **2**, Probucol, Ascorbate, and Trolox in the DPPH Assay[a,b]

sample	free redical scavenging activity IC_{20} (μM)
1	10.39 ± 0.37
2	4.26 ± 0.04
probucol	36.11 ± 4.98
ascorbate	8.92 ± 0.40
Trolox	8.77 ± 0.34

[a] Results were given as the mean ±SD (n = 3). [b] Statistical analyses were performed using Student's t test.

For the screening and evaluation of antioxidant activity of crude extracts and pure compounds, a DPPH assay was adopted.[5,6] In the DPPH assay system, the free radical scavenging activity of tested samples was expressed as IC_{20}. The scavenger activities of **1**, **2**, probucol, ascorbate, and Trolox were compared in Table 1. Compound **2** showed the most free redical scavenging activity (IC_{20} = 4.26 ± 0.04 μM) in these five tested compounds. Compound **1** (IC_{20} = 10.39 ± 0.37 μM) was not only more potent than probucol (IC_{20} = 36.11 ± 4.98 μM) but also as effective as ascorbate (IC_{20} = 8.92 ± 0.40 μM) and Trolox (IC_{20} = 8.77 ± 0.34 μM).

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Experimental Section

General Experimental Procedures. Optical rotation was recorded on a JASCO DIP-370 polarimeter. IR spectra were recorded on a Nicolet 50X FTIR spectrophotometer, and UV spectra on a Hitachi U-3200 spectrophotometer. NMR spectra were run on a Varian INOVA 500 MHz NMR spectrometer. EIMS and HREIMS spectra were obtained using Finnigan MAT GCQ and Finnigan MAT 95S spectrometers, respectively. Trolox was purchased from Aldrich Co. 1,1-Diphenyl-picrylhydrazyl (DPPH), probucol, and ascorbate were purchased from Sigma Co. Diaion HP-20 (Mitsubishi Chemical, Japan), Cosmosil 75C_{18}-OPN (Nacalai Tesque Co., Japan), and TSK gel HW-40S (Tosho Co., Japan) were used for column chromatography. Preparative HPLC was performed using a Cosmosil 5C-18-AR-II column (Nacalai Tesque Co., 10 × 250 mm, detector: UV 254 nm).

Organism and Culture Conditions. The producing *P. citrinum* F5 is the soil-isolated strain which was identified by the Culture Collection and Research Center of Food Industry Research and Development Institute, Hisnchu, Taiwan, Republic of China. Samples of the *P. citrinum* F5 were deposited in the Institute of Biochemistry, National Yang-Ming University, reference number TSCH-1. An inoculum culture was grown for 20 days (room temperature, standing state; glass bottles 1000 mL each containing 200 mL medium) on a liquid medium composed of (g/L) starch (30), glucose (10), yeast extract (5), β-cyclodextrin (10), KH_2PO_4 (1), $MgSO_4 \cdot 7H_2O$ (0.2), KCl (0.2), agar (20), and distilled water, pH 6.5.

Extraction and Isolation. The mold *P. citrinum* F5 was cultured on the above liquid medium for 20 days at room temperature. The filtered broth (5.6 L) was adsorbed onto the equi-volume Diaion HP-20 gel; and bioactive principles were eluted with acetone. The crude acetone extract was chromatographed on a Cosmosil 75C-18 column (170 mL, 3.6 × 16 cm) using increasing concentrations of MeOH in H_2O as eluent. The eluate of MeOH/H_2O (1:1) showed strong antioxidative activity. This active fraction was further separated by a TSK gel HW-40S column (177 mL, 1.5 × 100 cm), eluting with MeOH. HPLC purification of the active fractions afforded 2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran (**1**, 16 mg) and gentisic acid (**2**, 12 mg).

2,3,4-Trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran (1): brown solid; $[\alpha]_D^{25}$ +21° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 290 nm; IR ν_{max} (KBr) 3356, 1637, 1510, 1460, 1376, 1352, 1265, 1109, 1016 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 1.25 (3H, d, J = 6.5 Hz, 3-CH_3), 1.30 (3H, d, J = 6.5 Hz, 2-CH_3), 2.05 (3H, s, 4-CH_3), 3.00 (1H, m, H-3), 4.37 (1H, m, H-2), 6.20 (1H, s, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 10.5 (4-CH_3), 18.7 (3-CH_3), 20.1 (2-CH_3), 44.3 (C-3), 86.7 (C-2), 102.8 (C-6), 111.8 (C-4), 132.0 (C-9), 138.7 (C-8), 138.9 (C-7), 149.0 (C-5); HMBC correlations 3-CH_3/C-2, C-3, C-9; 2-CH_3/C-2, C-3; H-2/3-CH_3, C-8, C-9; H-3/2-CH_3, 3-CH_3, C-8, C-9; 4-CH_3/C-5, C-9, C-4; H-6/C-4, C-5, C-7, C-8; EIMS (positive-ion model) *m*/*z* 195 $[M + H]^+$(100), 183 (12); HREIMS *m*/*z* 194.0949 $[M]^+$ (calcd for $C_{11}H_{14}O_3$, 194.0943).

Evaluation of DPPH Radical Scavenging Activity.[5,6] The antioxidant activities of the crude extracts and pure compounds were assessed on the basis of the radical scavenging effect of the stable DPPH free radical. One milliliter of a 100 μM DPPH ethanol solution was added to 10 μL of sample solutions of different concentrations and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm using a spectrophotometer and converted into the percentage antioxidant activity (AA) using the following formula:

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}\}$$

Ethanol (1.0 mL) plus sample solution (10 μL) was used as a blank. DPPH solution plus ethanol was used as a negative control. The IC_{20} value is the concentration of test sample required to scavenge 20% DPPH free radicals. Probucol, ascorbate, and Trolox were used as positive controls.

References and Notes

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