Three New Metabolites from the Marine Yeast *Aureobasidium pullulans*

Hideyuki Shigemori, Masami Tenma, Kengo Shimazaki, and Jun'ichi Kobayashi*

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

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Two new diketopiperazines (**1** and **2**) with a D-*cis*-4-hydroxyproline residue, and orcinotriol (**3**), a new 1,3-dihydroxyphenol derivative, were isolated from cultured broth of the yeast *Aureobasidium pullulans,* which was isolated from an Okinawan marine sponge. The structures of the compounds, including their absolute stereochemistries, were determined by spectroscopic data and chemical means.

Marine microorganisms have proven to be a rich source of compounds that might be useful for the development of new pharmaceutical agents.[1] In our search for bioactive compounds from marine microorganisms,[2] two new diketopiperazines (**1** and **2**) and a new 1,3-dihydroxyphenol derivative, orcinotriol (**3**), have been isolated from the yeast *Aureobasidium pullulans* (de Bary) Arnaud (Hyphomycetes), which was separated from an Okinawan marine sponge. In this paper, we describe the isolation and structure elucidation of **1**−**3**.

The yeast *A. pullulans* was separated from a marine sponge collected at Okinawa, and grown in PYG broth [peptone (1%), yeast extract (0.5%), and glucose (2%) in 50% seawater, pH 7.0] at 25 °C for 6 days. The supernatant of the cultured broth (12 L) was chromatographed on a Diaion HP-20 column (MeOH), and the eluent was partitioned between EtOAc and H_2O. The EtOAc-soluble portions were subjected to Si gel and Sephadex LH-20 column chromatography to afford compounds **1**−**3**.

Compound **1** was obtained as a colorless amorphous solid, and HREIMS analysis revealed the molecular formula to be $C_{14}H_{16}N_2O_3$. IR absorptions of **1** implied the presence of OH, NH, or both, (3260 cm^{-1}) and amide carbonyl (1650 cm^{-1}) groups. From IR and ^1H NMR data, **1** was indicated to be a peptide. Compound **1** was negative to the ninhydrin test, suggesting that it is a cyclic or an *N*-terminus-blocked peptide. Standard amino acid analysis of the hydrolysate of **1** (6N HCl) gave one mole each of phenylalanine (Phe) and *cis*-4-hydroxyproline (*cis*-Hyp). HMBC correlations of H_2-9/C-2 (δ_C 167.0) and NH-4/C-5 (δ_C 169.1) revealed that **1** was cyclo(*cis*-Hyp−Phe). The absolute configurations of the *cis*-Hyp and Phe residues were determined to be D- and L-, respectively, by chiral HPLC analysis (SUMICHIRAL OA-5000) of the acid hydrolysate of **1**. Compound **1** was thus concluded to be cyclo(D-*cis*-Hyp-L-Phe).

The molecular formula of compound **2** was established to be $C_{11}H_{18}N_2O_3$ by HREIMS. Standard amino acid analysis of the hydrolysate of **2** gave one mole each of leucine (Leu) and *cis*-Hyp. HMBC correlations of H_2-9/C-2 (δ_C 167.7) and NH-4/C-5 (δ_C 169.8) revealed that **2** was cyclo(*cis*-Hyp−Leu). Chiral HPLC analysis of the acid hydrolysate of **2** showed that **2** consisted of D-*cis*-4-hydroxyproline and L-Leu. Thus, compound **2** was determined to be cyclo(D-*cis*-Hyp-L-Leu).

Orcinotriol (**3**) was obtained as a colorless amorphous solid and showed a molecular ion peak at *m*/*z* 168 (M^+) in the EIMS spectrum, which HREIMS analysis indicated that **3** had the composition $C_9H_{12}O_3$. IR absorptions implied that **3** possesses a hydroxy group (3300 cm^{-1}) and an aromatic ring (1600 cm^{-1}). ^1H and ^{13}C NMR data (δ_H 6.16 and 6.20; δ_C 102.3, 109.7, 143.3, and 160.1) indicated that **3** possesses a 1,3-dihydroxyphenol ring. These data were similar to those of the 1,3-dihydroxyphenol ring in α-acetylorcinol (**5**).[4] $^1H-^1H$ COSY connectivities of H-7 to H-8 (δ_H 3.95) and H-8 to H-9 suggested the presence of a 2-hydroxypropyl group. HMBC correlations of H_2-7 to C-1 indicated that the 2-hydroxypropyl group was attached at C-1. Thus, the structure of orcinotriol was assigned to be **3**. The absolute configuration at C-8 of **3** was determined by the modified Mosher method. Treatment of **3** with diazomethane afforded the dimethyl ether **6**, which was then converted into the (*S*)- and (*R*)-2-methoxy-2-trifluoromethylphenylacetic acid (MTPA) esters (**7** and **8**, respectively). The values of $\Delta\delta$ [δ(*S*-MTPA ester) − δ(*R*-MTPA ester)] in the ^1H NMR (Figure 1) spectra suggested that the absolute configuration at C-8 of **3** was (*S*).[5]

Compounds **1** and **2** are new diketopiperazines with a D-*cis*-4-hydroxyproline residue. Diketopiperazines with a L-*trans*-hydroxyproline residue have been isolated from rabbit skin tissue.[6] Orcinotriol (**3**) is a new 1,3-dihydroxyphenol derivative related to α-acetylorcinol, which has been obtained from the fungus *Cochliobolus lunata*.[4]

Experimental Section

General Methods. Optical rotations were determined on a JASCO DIP-370 polarimeter. UV and IR spectra were obtained on JASCO Ubest-35 and JASCO IR report-100 spectrometers, respectively. ^1H and ^{13}C NMR spectra were recorded on Bruker ARX-500 and AMX-600 spectrometers. The 7.26-ppm resonance of residual $CHCl_3$ and 77.0 ppm of $CDCl_3$ were used as internal references. EIMS was obtained on a JEOL DX-303 spectrometer operating at 70 eV. FABMS was measured on a JEOL HX-110 spectrometer by using glycerol matrix.

* To whom correspondence should be addressed. Tel.: +81-11-706-4985. Fax: +81-11-706-4989. E-mail: jkobay@pharm.hokudai.ac.jp.

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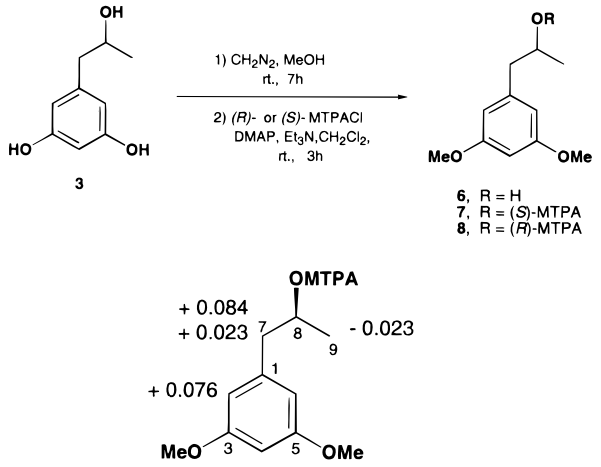
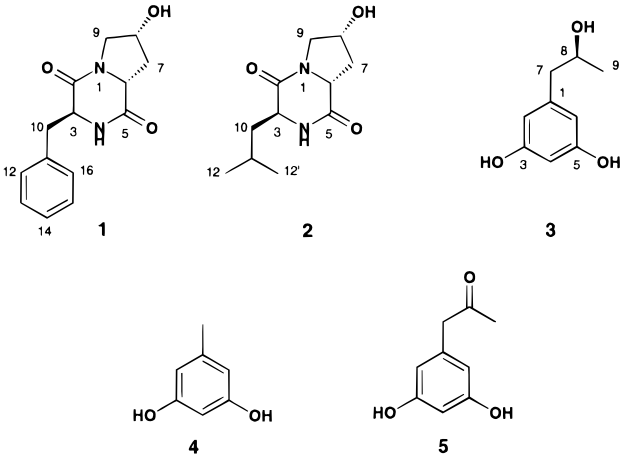


Figure 1. ^{1}H NMR chemical shift differences ($\Delta\delta$) for MTPA esters of compound **6**; $\Delta\delta$ (ppm) = δ[(*S*)-MTPA ester (**7**)] − δ[(*R*)-MTPA ester (**8**)].



Collection and Cultivation. The yeast *A. pullulans* was separated from an unidentified marine sponge collected in Okinawa. Subcultures of the organism are deposited at Faculty of Pharmaceutical Sciences, Hokkaido University. The yeast was grown in the PYG broth [peptone (1%), yeast extract (0.5%), and glucose (2%) in 50% seawater] at 25 °C for 6 days. The cultured broth (12 L) was centrifuged at 5000 rpm for 20 min.

Extraction and Separation. The aqueous supernatant of the cultured broth (12 L) was passed through a Diaion HP-20 column (Mitsubishi Chemicals, Co., Ltd.), which was subsequently eluted with MeOH. The eluent was partitioned between EtOAc and H_2O. The EtOAc-soluble portions were subjected to a Si gel column ($CHCl_3$−MeOH, 90:10) to afford two fractions, **a** (49 mg) and **b** (43 mg). Fraction **b** was separated by a Si gel column ($CHCl_3$−Me_2CO) to give compounds **1** (10.2 mg) and **2** (9.7 mg), and orcinotriol (**3**, 8.2 mg). Fraction **a** was subjected to a Si gel column ($CHCl_3$−EtOAc) to afford orcinol (**4**, 1.2 mg) and α-acetylorcinol (**5**, 2.2 mg).

Compound 1: colorless amorphous solid; $[\alpha]^{24}_D$ +34.7° (*c* 1.02, MeOH); UV (MeOH) λ_{max} 281 (ϵ 2900) and 208 (28 000) nm; IR (film) ν_{max} 3260 and 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.1−7.3 (5H, m, H-12−H-16), 6.82 (1H, d, *J* = 3.3 Hz, 4-NH), 4.31 (1H, m, H-8), 4.22 (1H, m, H-3), 3.87 (1H, d, *J* = 12.6 Hz, H-9a), 3.21 (1H, dd, *J* = 4.9, 12.6 Hz, H-9b), 3.14 (1H, dd, *J* = 6.3, 13.7 Hz, H-10a), 3.04 (1H, dd, *J* = 4.3, 13.7 Hz, H-10b), 2.95 (1H, dd, *J* = 7.2, 8.8 Hz, H-6), 2.26 (1H, m, H-7a), and 2.18 (1H, m, H-7b); ^{13}C NMR ($CDCl_3$) δ 169.4 (C-5), 165.8 (C-2), 135.4 (C-11), 130.0 (C-12 and C-16), 128.8 (C-13 and C-15), 127.6 (C-14), 67.9 (C-8), 58.8 (C-3), 55.8 (C-6), 53.7 (C-9), 40.2 (C-10), and 37.2 (C-7); EIMS *m*/*z* 260 (M^+); HREIMS *m*/*z* 260.1172 (M^+) (calcd for $C_{14}H_{16}N_2O_3$, 260.1161).

Compound 2: colorless amorphous solid; $[\alpha]^{24}_D$ +40.5° (*c* 1.04, MeOH); IR (film) ν_{max} 3280 and 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.56 (1H, d, *J* = 4.2 Hz, 4-NH), 4.45 (1H, m, H-8), 4.16 (1H, dd, *J* = 5.8, 9.1 Hz, H-6), 3.95 (2H, m, H-3 and H-9a), 3.34 (1H, dd, *J* = 4.6, 12.5 Hz, H-9b), 2.45 (2H, m, H-7), 1.75 (1H, m, H-11), 1.63 (2H, t, *J* = 6.9 Hz, H-10), 0.98 (3H, d, *J* = 6.5 Hz, H-12), and 0.95 (3H, d, *J* = 6.5 Hz, H-12′); ^{13}C NMR ($CDCl_3$) δ 169.8 (C-5), 167.7 (C-2), 68.1 (C-8), 56.2 (C-3 or C-6), 56.1 (C-6 or C-3), 54.0 (C-9), 42.2 (C-10), 36.8 (C-7), 24.6 (C-11), 23.0 (C-12), and 21.5 (C-12′); EIMS *m*/*z* 226 (M^+); HREIMS *m*/*z* 226.1290 (M^+) (calcd for $C_{11}H_{18}N_2O_3$, 226.1318).

Orcinotriol (3): colorless amorphous solid; $[\alpha]^{25}_D$ +6.0° (*c* 1.1, MeOH); UV (MeOH) λ_{max} 276 (ϵ 5900) and 208 (17 000) nm; IR (film) ν_{max} 3300 and 1600 cm^{-1}; ^{1}H NMR (CD_3OD) δ 6.20 (2H, d, *J* = 2.1 Hz, H-2 and H-6), 6.16 (1H, t, *J* = 2.1 Hz, H-4), 3.95 (1H, m, H-8), 2.70 (1H, dd, *J* = 6.4, 13.2 Hz, H-7a), 2.50 (1H, dd, *J* = 6.8, 13.2 Hz, H-7b), and 1.17 (3H, d, *J* = 6.2 Hz, H-9); ^{13}C NMR (CD_3OD) δ 160.1 (C-3 and C-5), 143.3 (C-1), 109.7 (C-2 and C-6), 102.3 (C-4), 70.6 (C-8), 47.7 (C-7), and 23.7 (C-9); EIMS *m*/*z* 168 (M^+), 153, 123, and 110; HREIMS *m*/*z* 168.0775 (M^+) (calcd for $C_9H_{12}O_3$, 168.0787).

Amino Acid Analysis of Hydrolysate of 1. Compound **1** or **2** (0.05 mg, each) was dissolved in 6N HCl (100 mL) and heated to 110 °C for 24 h in a sealed tube. Of *cis*-Hyp and Phe 1 mol each was found in the hydrolysate of **1**, while 1 mol each of *cis*-Hyp and Leu was found in the hydrolysate of **2** by standard amino acid analysis.

Determination of Absolute Configuration of 1 and 2. Compound **1** or **2** (0.1 mg, each) was hydrolyzed with 6N HCl aqueous at 110 °C for 18 h in a sealed tube. The residue was dissolved in H_2O for chiral HPLC analysis. The chiral HPLC analysis was carried out using a SUMICHIRAL OA-5000 column [Sumitomo Chemical Industry, 4 × 150 mm; flow rate, 0.5 mL/min; eluent, MeOH−H_2O (15:85) containing 2.0 mmol $CuSO_4$; detection, UV at 254 nm; column temperature, 40 °C]. Retention times of standard L- and D-*cis*-Hyp, L- and D-Phe, and L- and D-Leu were 5.2, 3.6, 22.1, 30.0, 9.9, and 13.7 min, respectively, and those of hydrolysis products of **1** were found to be 3.6 and 22.1 min, and those of hydrolysis products of **2** were 3.6 and 9.9 min.

Dimethyl Ether (6) of Orcinotriol (3). To a MeOH solution (100 μL) of compound **3** (1.1 mg), CH_2N_2−Et_2O solution (200 μL) was added at room temperature for 7 h. After evaporation of solvent, the residue was passed through a Si gel column (hexane−EtOAc, 1:1) to afford the dimethyl ether (**6**, 0.7 mg); ^{1}H NMR (CD_3OD) δ 6.42 (2H, d, *J* = 2.2 Hz, H-2 and H-6), 6.36 (1H, t, *J* = 2.2 Hz, H-4), 3.99 (1H, m, H-8), 3.79 (6H, s, MeO × 2), 2.76 (1H, dd, *J* = 6.7, 13.3 Hz, H-7a), 2.62 (1H, dd, *J* = 6.4, 13.3 Hz, H-7b), and 1.18 (3H, d, *J* = 6.2 Hz, H-9); EIMS *m*/*z* 196 (M^+).

(*S*)-MTPA Ester (7) of Compound 6. To a CH_2Cl_2 solution (100 μL) of compound **6** (0.3 mg), DMAP (1.0 mg), and Et_3N (2 μL) was added (*R*)-(−)-MTPACl (2.0 mg) at room temperature, and stirring was continued for 3 h. After evaporation of solvent, the residue was purified by Si gel TLC (hexane−EtOAc, 2:1) to afford the (*S*)-MTPA ester (**7**) as colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.3−7.5 (5H, m, Ph), 6.364 (2H, d, J = 2.2 Hz, H-2 and H-6), 6.358 (1H, t, J = 2.2 Hz, H-4), 5.393 (1H, m, H-8), 3.732 (6H, s, MeO × 2), 3.413 (3H, s, MeO of MTPA), 2.915 (1H, dd, J = 8.4, 13.9 Hz, H-7a), 2.831 (1H, dd, J = 5.4, 13.9 Hz, H-7b), and 1.332 (3H, d, J = 6.2 Hz, H-9); EIMS *m*/*z* 412 (M^+).

(*R*)-MTPA Ester (8) of Compound 6. Compound **6** (0.3 mg) was treated with (*S*)-(+)-MTPACl (2.0 mg) by the same procedure as described above to afford the (*R*)-MTPA ester (**8**) as colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.3−7.5 (5H, m, Ph), 6.312 (1H, t, J = 2.2 Hz, H-4), 6.288 (1H, 2H, d, J = 2.2 Hz, H-2 and H-6), 5.414 (1H, m, H-8), 3.716 (6H, s, MeO × 2), 3.481 (3H, s, MeO), 2.892 (1H, dd, J = 7.2, 13.8 Hz, H-7a), 2.747 (1H, dd, J = 6.0, 13.8 Hz, H-7b), and 1.355 (3H, d, J = 6.3 Hz, H-9); EIMS *m*/*z* 412 (M^+).

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