Indole Derivatives from a Marine Sponge-Derived Yeast as DPPH Radical Scavengers

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Two new indole derivatives (**3**, **4**) and three known compounds (**1**, **2**, **5**) were isolated as radical scavengers from the culture filtrate of a marine sponge-derived yeast. Their structures were determined to be tyrosol (**1**), tryptophol (**2**), 2-(1*H*-indol-3-yl)ethyl 2-hydroxypropanoate (**3**), 2-(1*H*-indol-3-yl)ethyl 5-hydroxypentanoate (**4**), and cyclo(L-Pro-L-Tyr) (**5**) on the basis of their spectroscopic data. The absolute configurations of compounds **3** and **5** were determined by chiral HPLC analysis combined with synthesis and Marfey's method, respectively. Each obtained compound was evaluated for DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, and all compounds exhibited weak activities.

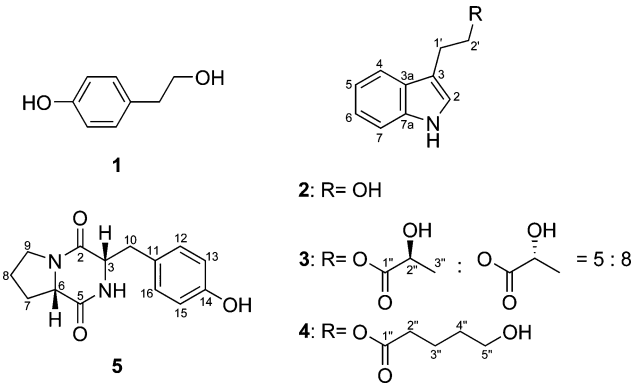
Excessive radicals are one of the risk factors for organisms, because they damage DNA and react with other biomolecules such as lipids and proteins. This unfavorable oxidative stress consequently induces various diseases including arteriosclerosis, cardiovascular disease, and cancer. Therefore, antioxidants are expected to be useful tools to combat and prevent those diseases.[1,2]

In our screening program for antioxidants, we have reported several new compounds from soil-derived microorganisms[3−7] and fermented foods[8,9] in the past. We continued to investigate new antioxidants from microorganisms, and recently a yeast strain (USF-HO25) was found as a producer of the DPPH (2,2-diphenyl-1-picrylhydrazyl)[10] radical scavenging compounds. The yeast was separated from the marine sponge *Halichondria okadai* collected at the west coast of Izu Peninsula in Shizuoka Prefecture, Japan. The USF-HO25 strain produced several indole derivatives and phenol derivatives in the culture filtrate. In this paper, we report the fermentation of the USF-HO25 strain and the isolation, structural elucidation, and radical scavenging activities of five compounds, including two novel indole derivatives.

The marine sponge-derived yeast, HO-25 strain, was identified as *Pichia membranifaciens* from the observation of cell and ascospore morphology under the light microscope and the molecular phylogenetic analysis based on the sequence of the D1/D2 region of the 26S rRNA gene. The cultivation of the USF-HO25 strain was conducted on a reciprocal shaker at 30 °C for 4 days. An EtOAc extract from the culture filtrate was successively purified by silica gel column chromatography, repeated Sephadex LH20 column chromatography, and reversed-phase HPLC to yield compounds **1**−**5**.

Compounds **1** and **2**, respectively, were identified as tyrosol[11] and tryptophol[12] on the basis of their spectroscopic data. It had been reported that both compounds were produced by a yeast, and tyrosol showed radical scavenging activity for DPPH.[13]

The molecular formula of **3** was established as $C_{13}H_{15}NO_3$ from the HR-FABMS data. The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of **2**, except that additional signals of a methyl group (δ_H 1.31, δ_C 20.6), an oxymethine group (δ_H 4.22, δ_C 67.9), and a carbonyl carbon (δ_C 176.5) were observed in the NMR spectra of **3** (Table 1). Hence, it was supported that compound **3** was a derivative of indole-3-ethanol. In addition, it was clear that a methyl group is connected to an oxymethine group, as exhibited by their coupling constants in the ^{1}H NMR spectrum of **3**. The connection of three partial structures was established by HMBC correlations from both a methyl proton (δ_H 1.31 (H-3″)) and a methylene proton (δ_H 4.38 (H-2′)) to a carbonyl carbon (δ_C 176.5 (C-1″)) (Figure 1). Thus, the planar structure of **3** was determined to be 2-(1*H*-indol-3-yl)ethyl 2-hydroxypropanoate, a novel compound.



The absolute structure of **3** was decided by comparison of the chromatogram of a natural product with those of synthetic compounds. At first, we synthesized (*S*)-2-(1*H*-indol-3-yl)ethyl 2-hydroxypropanoate (**3a**) and (*R*)-2-(1*H*-indol-3-yl)ethyl 2-hydroxypropanoate (**3b**), which were reaction products from tryptophol with L-lactic acid and D-lactic acid, respectively. Subsequently, the synthesized compounds (**3a** and **3b**) and the natural product (**3**) were analyzed by HPLC using a chiral column. Two peaks were detected in the chromatogram of **3**, and they were assigned to **3a** and **3b** on the basis of their retention times. Moreover, **3b** was 1.6 times larger than that of **3a**. These results indicate that **3** is a mixture of the *S*-form and the *R*-form, and the composition ratio is 5:8 (*S*/*R*). Each of synthetic compounds **3a** and **3b** did not convert spontaneously to the opposite configuration when they were purified in a similar manner to the natural product. This phenomenon might indicate that the mixed configuration of **3** was derived from the biosynthetic process.

The molecular formula of **4** was determined to be $C_{15}H_{19}NO_3$ by the HR-FABMS spectrum. The ^{1}H and ^{13}C NMR spectra of **4** were comparable to those of **2**, and it was suggested that **4** was an analogue of **3**. The signals of an oxymethylene moiety (δ_H 3.51, δ_C 62.4), three methylene groups (δ_H 2.33, 1.63, and 1.49, δ_C 34.9, 22.4, and 32.9), and a carbonyl carbon (δ_C 175.5) were observed besides the signals corresponding to an indole-3-ethanol moiety in the NMR spectra of **4** (Table 1). Four methylene groups represent an alkyl side chain, $-CH_2(2'')-CH_2(3'')-CH_2(4'')-CH_2(5'')-O-$,

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Table 1. NMR Spectroscopic Data of Compounds **3** and **4** in CD_3OD

	3			**4**		
position	δ_C[a], mult.	δ_H[b] (*J* in Hz)	HMBC[c]	δ_C, mult.	δ_H (*J* in Hz)	HMBC
2	123.8, CH	7.09, s	3a, 7a	123.7, CH	7.08, s	3a, 7a
3	111.6, qC			111.9, qC		
3a	128.7, qC			128.8, qC		
4	119.2, CH	7.56, d, (8.0)	6, 7a	119.3, CH	7.55, d (8.0)	6, 7a
5	119.7, CH	7.00, t (8.0, 7.5)	3a, 7	119.6, CH	7.00, t (8.0, 7.5)	3a, 7
6	122.3, CH	7.08, t[d]	4, 7a	122.3, CH	7.08, t[e]	4, 7a
7	112.2, CH	7.33, d (8.0)	3a, 5	112.2, CH	7.32, d (8.0)	3a, 5
7a	138.1, qC			138.1, qC		
1′	25.8, CH_2	3.10, t (6.9)	2, 3, 3a, 2′	25.8, CH_2	3.07, t (6.9)	2, 3, 3a, 2′
2′	66.4, CH_2	4.38, m	3, 1′, 1″	66.0, CH_2	4.32, t (6.9)	3, 1′, 1″
1″	176.5, qC			175.5, qC		
2″	67.9, CH	4.22, q (6.9)	1″, 3″	34.9, CH_2	2.33, t (6.9)	1″, 3″, 4″
3″	20.6, CH_3	1.31, d (6.9)	1″, 2″	22.4, CH_2	1.63, quint	1″, 2″, 4″, 5″
4″				32.9, CH_2	1.49, quint	2″, 3″, 5″
5″				62.4, CH_2	3.51, t (6.9)	3″, 4″

[a] ^{13}C NMR spectrum was measured at 125 MHz. [b] ^1H NMR spectrum was measured at 500 MHz. [c] HMBC correlations optimized for 8 Hz are from proton started to the indicated carbons. [d] Coupling constants are unclear due to overlaps. [e] Coupling constants are unclear due to overlaps.

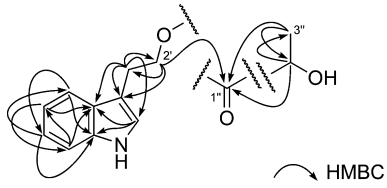

Figure 1. Partial structures and HMBC correlations of compound **3**.

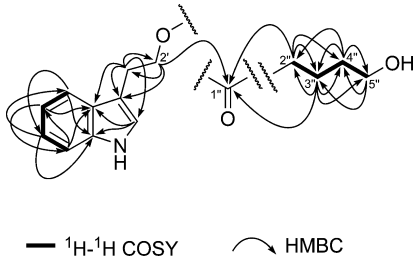

Figure 2. Partial structures and $^1H-^1H$ COSY and HMBC correlations of compound **4**.

as determined by analysis of the $^1H-^1H$ COSY spectrum of **4**. The key HMBC correlations were observed from the oxymethylene proton at δ_H 4.32 (H-2′) and two methylene protons (δ_H 2.33 (H-2″) and 1.63 (H-3″)) of the alkyl chain to a carbonyl carbon (δ_C 175.5 (C-1″)) (Figure 2). For this reason, it was demonstrated that the alkyl chain was attached to an indole-3-ethanol moiety via a carbonyl carbon (δ_C 175.5). Therefore, the structure of **4** was determined to be 2-(1*H*-indol-3-yl)ethyl 5-hydroxypentanoate as a novel compound.

HR-FABMS, ^1H and ^{13}C NMR, $^1H-^1H$ COSY, HMQC, and HMBC spectroscopic data showed that compound **5** was a cyclic dipeptide consisting of a proline and a tyrosine. Both chiral centers of **5** were determined to have *S* configurations by HPLC analysis of Marfey's derivatives[14] from the hydrolysate. Therefore, the structure of **5** was identified as cyclo(L-Pro-L-Tyr).[15]

The five compounds (**1**−**5**) obtained in this study and BHT as a positive control were tested for DPPH radical scavenging activities at 300 μM (final concentration), and their percentages of scavenged DPPH radical were calculated (Table 2). As a result, the activities of the indolic metabolites (**2**, **3**, and **4**) were slightly higher than the isolated phenolic compounds (**1** and **5**). However, between the three indolic derivatives (**2**, **3**, and **4**) there was no significant difference in their DPPH radical scavenging activities. Therefore, the presence of a side chain does not affect the antioxidative activity of an indolic derivative. In contrast, a high antioxidative activity

Table 2. DPPH Radical Scavenging Activities of Compounds **1**−**5** and BHT

compound[a]	scavenged DPPH radical (%)[c]
1	26
2	52
3	33
4	47
5	16
BHT[b]	86

[a] Compounds were used at a final concentration of 300 μM. [b] Butylhydroxytoluene; 2,6-di-*tert*-butyl-4-methylphenol, used as a positive control. [c] Percentage of scavenged DPPH radical after 48 h.

of tyrosol (**1**) was shown in the ORAC (oxygen radical absorbance capacity) assay, in spite of no activity for scavenging the DPPH radical.[16] High antioxidative activity of tryptophol (**2**) was shown in the ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) assay under hydrophilic conditions.[17] Many indole-containing compounds are known to present various activities such as anticancer, antibiotic, and anti-inflammatory activities. Accordingly, the two novel indole derivatives (**3** and **4**) are expected to exhibit other biological activities along with their antioxidative properties.

Experimental Section

General Experimental Procedures. Optical rotation was recorded with a Horiba SEPA-200 spectrometer. UV spectra were recorded with a Shimadzu UV-160A spectrometer. NMR spectra were obtained with a JEOL JMN-ECA-500 spectrometer, using tetramethylsilane as an internal standard. High- and low-resolution mass spectra were measured by a JEOL JMS-700 spectrometer, using glycerol as a matrix. The HPLC analyses were carried out on a JASCO HPLC system: PU-2080 Plus and UV-2077 Plus. HPLC for preparation was done by JASCO 880-PU and JASCO UV-970 apparatus.

Fungal Material. Morphological characteristics of the yeast strain were examined by using standard methods described by Yarrow.[18] Its nuclear DNA was isolated from colonies on a YM agar slant incubated at 25 °C for 4 days by using a MasterPure yeast DNA purification kit (Epicenter Technologies, Madison, WI) according to the manufacture's instructions. The D1/D2 domain of the large-subunit (26S) rRNA gene from DNA was amplified and sequenced as described by Kurtzman and Robnett.[19] A sequence-similarity search was performed by using the BLAST 2.2.21 (Altschul et al.)[20] in GenBank. The nomenclature of the yeast strain identified follows the classification of Kurtzman.[21] The D1/D2 sequence of the yeast strain was identical to that of the type strain of *Pichia membranifaciens* (GenBank accession number U75725).

Fermentation. The USF-HO25 strain was inoculated into a medium (100 mL) that consisted of glucose 1%, polypeptone 0.2%, yeast extract 0.1%, meat extract 0.1%, and NaCl 3%, pH 7.2 in a 500 mL flask; 100 flasks were inoculated and incubated at 30 °C on a reciprocal shaker at 130 rpm for 4 days.

Isolation of DPPH Radical Scavengers from the Culture Broth of USF-HO25. The culture broth (10 L) was filtered, and the filtrate was adjusted to pH 3 and extracted with EtOAc (5 L × 3). The combined EtOAc layer was dried over anhydrous Na_2SO_4 and filtered, and the solvent was removed under reduced pressure. The EtOAc extract (1.8 g) was applied to a silica gel column and eluted with *n*-hexane/EtOAc at 3:1, 1:1, and 1:3, then with EtOAc and MeOH. Two fractions that eluted with *n*-hexane/EtOAc (1:1) (fraction II, 568.0 mg) and MeOH (fraction V, 399.0 mg) showed DPPH radical scavenging activity in the TLC assay.[22] Fractions II and V were further purified three times by Sephadex LH20 column chromatography using MeOH as an eluent. Subfractions 17 and 18 of fraction II obtained from the third Sephadex LH20 column were combined and further purified by preparative RP-HPLC (column: Capcell-Pak C_{18} SG120, 15 × 250 mm, Shiseido; mobile phase: 31% CH_3CN/H_2O), to yield compounds **1** (51.6 mg), **2** (44.3 mg), **3** (0.6 mg), and **4** (4.9 mg). Subfractions 6−11 of fraction V were combined and evaporated to dryness *in vacuo*, and the resulting residue was purified by preparative RP-HPLC (column: Capcell-Pak C_{18} SG120, 15 × 250 mm, Shiseido; mobile phase: 8% CH_3CN/H_2O) to yield **5** (60.7 mg).

2-(1*H*-Indol-3-yl)ethyl 2-hydroxypropanoate (3): colorless oil; UV (MeOH) λ_{max} (log ϵ) 222 (4.48), 282 (3.73) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; HR-FABMS $[M + H]^+$ *m/z* 234.1127 (calcd for $C_{13}H_{16}NO_3$, 234.1124).

2-(1*H*-Indol-3-yl)ethyl 5-hydroxypentanoate (4): colorless oil; UV (MeOH) λ_{max} (log ϵ) 222 (4.33), 282 (3.57) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; HR-FABMS $[M + H]^+$ *m/z* 262.1444 (calcd for $C_{15}H_{20}NO_3$, 262.1445).

Preparations of (*S*)-2-(1*H*-Indol-3-yl)ethyl 2-hydroxypropanoate (3a) and (*R*)-2-(1*H*-Indol-3-yl)ethyl 2-hydroxypropanoate (3b). L-Lactic acid (270 mg) was heated in thionyl chloride (30 mL) for 6 h at 55 °C. After the thionyl chloride was evaporated at low temperature, CH_2Cl_2 (20 mL) containing tryptophol (480 mg) and 4-dimethylaminopyridine (366 mg) was added, and the mixture was stirred for 6 h at room temperature. The reaction mixture was diluted with EtOAc, washed with 0.1 M HCl, and dried over anhydrous Na_2SO_4. The filtrate was evaporated to give crude materials, and they were applied to silica gel column chromatography. The fraction was eluted with *n*-hexane/EtOAc (2:1) and was further purified by preparative RP-HPLC (column: Capcell-Pak C_{18} SG120, 15 × 250 mm, Shiseido; mobile phase: 32% CH_3CN/H_2O) to yield **3a** (47.4 mg). Similarly, (*R*)-2-(1*H*-indol-3-yl)ethyl 2-hydroxypropanoate (**3b**, 4.0 mg) was prepared from D-lactic acid (45 mg) and tryptophol (81 mg).

(*S*)-2-(1*H*-Indol-3-yl)ethyl 2-hydroxypropanoate (3a): colorless oil; $[\alpha]^{25}_D$ −8.8 (*c* 0.23, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ 7.56 (1H, d, J = 8.0 Hz, H-4), 7.32 (1H, d, J = 8.0 Hz, H-7), 7.09 (1H, s, H-2), 7.08 (1H, t, H-6), 7.00 (1H, t, J = 8.0, 7.5 Hz, H-5), 4.38 (2H, m, H-2′), 4.22 (1H, q, J = 6.9 Hz, H-2″), 3.11 (2H, t, J = 6.9 Hz, H-1′), 1.31 (3H, d, J = 6.9 Hz, H-3″); ^{13}C NMR (CD_3OD, 125 MHz) δ 176.5 (C-1″), 138.1 (C-7a), 128.7 (C-3a), 123.8 (C-2), 122.3 (C-6), 119.7 (C-5), 119.2 (C-4), 112.2 (C-7), 111.6 (C-3), 67.9 (C-2″), 66.4 (C-2′), 25.8 (C-1′), 20.6 (C-3″); HR-FABMS *m/z* 234.1137 $[M + H]^+$ (calcd for $C_{13}H_{16}NO_3$, 234.1130).

(*R*)-2-(1*H*-Indol-3-yl)ethyl 2-hydroxypropanoate (3b): colorless oil; $[\alpha]^{21}_D$ +9.3 (*c* 0.11, MeOH); ^{1}H and ^{13}C NMR data are identical to those for **3a**; HR-FABMS *m/z* 234.1142 $[M + H]^+$ (calcd for $C_{13}H_{16}NO_3$, 234.1130).

Determination of the Configuration of Compound 3. The natural product (**3**) and the synthetic compounds (**3a** and **3b**) were analyzed by chiral HPLC (column: Ceramospher Chiral RU-2, 4.6 × 250 mm, Shiseido; mobile phase: MeOH; flow rate: 0.5 mL/min; detection: 280 nm). The retention times of synthetic compounds **3a** and **3b** were 21.9 and 17.6 min, respectively. Two peaks that correspond to **3a** and **3b** were detected in the chromatogram of the natural product (**3**). The ratio of the *S*/*R* enantiomers in the natural product mixture was calculated from the area of their peaks.

Measurement of DPPH Radical Scavenging Activity. DPPH radical scavenging assay was performed by the modified method presented previously.[3−9] An ethanolic solution of the sample (750 μM, 1 mL) was mixed with a 500 μM DPPH ethanolic solution (0.5 mL) and 0.1 M acetate buffer (pH 5.5, 1 mL). The reaction mixture was left to stand for 48 h, at which time the absorbance of the mixture was measured at 517 nm. The DPPH radical scavenging activity was determined by comparison of the absorbance of sample with that of a blank control. BHT (butylhydroxytoluene; 2,6-di-*tert*-butyl-4-methylphenol) was used as a positive control at the same concentration as other samples.

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Supporting Information Available: NMR spectra of **1**−**5** and spectroscopic data and additional information on the known compounds are available free of charge via the Internet at http://pubs.acs.org.

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