Note

Isolation of a 2-Pyrone Compound as an Antioxidant from a Fungus
and Its New Reaction Product with 1,1-Diphenyl-2-picrylhydrazyl Radical

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The indophenol-reducing compound, 4-hydroxy-3,6-dimethyl-2H-pyran-2-one (I), was isolated from the culture filtrate of an unidentified fungus. I also reacted with the DPPH radical to form a reaction product IV which was determined to be 1-(4-(3,4-dihydro-3,6-dimethyl-2,4-dioxo-2H-pyran-3-yl)phenyl)-1-phenyl-2-picrylhydrazine. This is the first report describing the formation of an adduct of the DPPH radical and its scavenger.

Key words: indophenol-reducing compound; antioxidant; radical-scavenging activity

In our study to find new antioxidants produced by microorganisms, we have so far isolated a 1,3-dicarboxyl compound from Streptomyces,1 and new phenol compounds from the fungus, Mortierella sp. USF-406,2 by chemical screening with 2,6-dichloroindophenol which is used as a reagent for evaluating ascorbic acid. We have also isolated ansamycin antibiotics from Streptomyces,3 and two oxazol compounds from an unidentified fungus4 as hydroxyl radical scavengers. Furthermore, we have isolated some sorbicillin dimers from the Trichoderma sp. fungus as DPPH radical scavengers.5,6

A continuation of this study revealed that an unidentified fungus strain USF-2550, which had been isolated from a soil sample collected in Shimizu city, Shizuoka prefecture, produced the indophenol-reducing compound, USF-2550A (I).

We describe in this paper the cultivation, isolation, and bioactivity of I, and a product from the reaction between I and the DPPH radical.

USF-2550 strain was cultivated for 10 days at 30°C in the medium described in the experimental section. The culture filtrate was extracted with ethyl acetate (EtOAc) at pH 3, and the resulting extract was found to contain a 2,6-dichloroindophenol-reducing compound from decoloration after spraying indophenol by silica gel thin-layer chromatography (TLC).

The extract was concentrated in vacuo, and the obtained pinkish crude powder was subjected to silica gel column chromatography (Wako gel C-200), eluting with n-hexane (Hex)-EtOAc as the solvent. The eluate of EtOAc containing 25% Hex contained the crude indophenol-reducing compound, USF-2550A (I). Crude I was recrystallized from chloroform-methanol to yield a pure crystal of I.

The molecular formula of I was determined to be C₁₉H₁₉NO₆ from high-resolution electron ionization mass spectrometry (HREIMS). Analyses of the DEPT and HMBC spectra of I in conjunction with the 1H- and 13C-NMR spectra indicate that the chemical structure of I is 4-hydroxy-3,6-dimethyl-2H-pyran-2-one (Fig. 1), which had already been isolated from Penicillium stipitatum.7

Since a hydroxyl group at C-4 was speculated to play an important role in the reaction between I and 2,6-dichloroindophenol by TLC, I was reacted with a diazomethane solution to protect the hydroxyl group. The reaction mixture gave two compounds (II and III) after purification by silica gel column chromatography. II and III were determined to be 4-methoxy-3,6-dimethyl-2H-pyran-2-one and 2-methoxy-3,6-dimethyl-4H-pyran-4-one, respectively. As expected, these two compounds did not reduce indophenol by TLC.

We next tried to react I with 2,6-dichloroindophenol in solution, but the reaction did not seem to proceed. However, I also reacted with the DPPH radical on a TLC plate and decolorized the purple of DPPH. In the assay,8 I scavenged the DPPH radical and its ED₅₀ value was 500 μM, while II and III did not show DPPH radical-scavenging activity. I and DPPH were reacted in an ethanol solution, and a reaction product (IV) as a redish brown powder, was obtained after purification by Sephadex LH-20 column chromatography.

The molecular formula of IV was determined to be C₃₀H₂₅N₂O₅ by HREIMS. The 1H- and 13C-NMR spectra of IV indicated it to be the adduct of I and DPPH, in that the hydradyl moiety was converted to the hydradyl

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Abbreviations: HMBC, heteronuclear multiple-bond connectivity; DEPT, distortionless enhancement by polarization transfer; DPPH, 1,1-diphenyl-2-picrylhydrazyl

Fig. 1. Structures of I, II and III.
New Reaction Product from a 2-Pyrone Compound and DPPH Radical

![Diagram](image)

Fig. 2. Summary of HMBC Spectral Data of IV.

one, and that C-4 of I was ketonic. Furthermore, the HMBC spectrum showed C-3 of I bonded with C-4' of one of two phenyl groups of DPPH (Fig. 2).

Consequently, the structure of IV was 1-[4-(3,4-dihydro-3,6-dimethyl-2,4-dioxo-2'H-pyran-3-yl)phenyl]-1-phenyl-2-picrylhydrazine.

The yield of IV from the reaction between I and DPPH in a solution was low, but the reaction mixture seemed to contain only four compounds, I, DPPH, DPPH hydrazine and IV. Studies on the reaction mechanism by HPLC analysis are now in progress.

Yoshida et al. have reported that the reaction between the DPPH radical and gallic acid produced a dimer of gallic acid. On the other hand, Sakata et al. have described the reaction between DPPH and catechins and demonstrated by NMR spectral measurements that the catechins were converted to quinones by the reaction with the DPPH radical. These two research groups, however, did not isolate the adduct of the DPPH radical and its scavenger.

Accordingly, this is the first evidence that a radical scavenger reacts with the DPPH radical to form an adduct. We have already reported that 6-pentyl-2-pyrene from a fungus scavenged a hydroxyl radical and was converted to a hydroxy acid bonded with a solvent alcohol radical.4 Judging from the evidence that 2-pyrene compounds, which are common as fungal metabolites, scavenges radicals, 2-pyrene compounds might partly protect fungi against active oxygen species and radicals.

Experimental

**Apparatus.** Melting point (mp) data were determined on the microscope hot plate of a Yanagimoto MP-13 instrument. IR spectra were recorded with a Hitachi 270-50 infrared spectrometer, and $^1$H- and $^{13}$C-NMR spectra were obtained with a Jeol α-400 spectrometer, using tetramethylsilane as an internal standard. High- and low-resolution mass spectra were measured by a Jeol JMS-SX102 spectrometer, and UV spectra were recorded with a Shimadzu UV-160A spectrometer.

**Isolation of USF-2550A (I).** Fungal strain USF-2550 was cultivated on a rotary shaker for 10 days at 30°C in five 2-liter Erlenmeyer flasks containing 0.8 liters of the following medium: glycerol, 20 g; soybean meal, 0.5 g; yeast extract, 2 g; KH$_2$PO$_4$, 1 g; MgSO$_4$·7H$_2$O, 1 g; trace salt mixtures, 1 ml; in 1000 ml of distilled water. The culture filtrate (4 l) was extracted with EtOAc at pH 3. The resulting extract was concentrated in vacuo and the obtained pinkish crude powder (2.12 g) was subjected to silica gel column chromatography (Wako gel C-200; 64.5 × 25 cm), eluting with a Hex EtOAc solvent system. The eluate of EtOAc containing 25%Hex contained crude I, and was recrystallized from CHCl$_3$ and MeOH to yield pure colorless needles of I (422 mg).

**USF-2550A (I).** Mp 218°C, colorless needles. HREIMS m/z: 140.0488 (M$^+$; calcd. for C$_7$H$_6$O$_5$: 140.0474). EIMS m/z: 140 (M$^+$, 100), 112 (75), 85 (83), 69 (53), 43 (58). UV $\lambda_{max}$ (MeOH) nm (ε): 293.9, (9,500).

**Reaction between I and diazomethane.** An ethereal diazomethane solution was added to a solution of I (100 mg) until the yellow color was retained. The reaction mixture (123.1 mg) was chromatographed by Wakogel C-200 (30 g), eluting with a Hex-EtOAc solvent system, and II (61.6 mg) and III (36.1 mg) were obtained.

**Methyl ether of I (II).** Colorless needles, mp 89°C. EIMS m/z: 154 (M$^+$). $^1$H-NMR (CD$_2$OD, 400 MHz) δ: 1.90 (3H, s, 3-CH$_3$), 2.25 (3H, s, 6-CH$_3$), 3.87 (3H, s, 4-OCH$_3$), 6.02 (1H, s, 5-H). $^{13}$C-NMR (CD$_2$OD, 100 MHz) δ: 8.3 (q, 3-CH$_3$), 20.1 (q, 6-CH$_3$), 56.1 (q, 4-OCH$_3$), 94.8 (d, C-5), 100.7 (s, C-3), 160.6 (s, C-6), 165.7 (s, C-4), 165.8 (s, C-2).

**Methyl ether of I (III).** Brownish oil. EIMS m/z: 154 (M$^+$). $^1$H-NMR (CD$_2$OD, 400 MHz) δ: 1.88 (3H, s, 3-CH$_3$), 2.36 (3H, s, 6-CH$_3$), 4.08 (3H, s, 2-OCH$_3$), 6.48 (1H, s, 5-H). $^{13}$C-NMR (CD$_2$OD, 100 MHz) δ: 6.5 (q, 3-CH$_3$), 19.0 (q, 6-CH$_3$), 56.3 (q, 2-OCH$_3$), 100.8 (s, C-3), 110.7 (d, C-5), 161.3 (s, C-6), 164.6 (s, C-2), 181.6 (s, C-4).

**Reaction between I and indophenol in a solution.** I (20 mg) and sodium 2,6-dichloroindophenol (20 mg) were dissolved in a 50% ethanol solution (20 ml), and the solution was stirred under shaded light at room temperature. After standing for 1, 2, 4 and 24 h, the solution was analyzed on a TLC plate.

**Reaction between I and DPPH in a solution.** I (140 mg) and DPPH (394 mg) were dissolved in ethanol (50 ml), and the solution was stirred under shaded light at room temperature. After standing for 24 h, the reaction mixture was concentrated in vacuo. The concentrate (527.4 mg) was subjected to Sephadex LH-20 column chromatography (ethanol). The fraction containing IV was further subjected to Sephadex LH-20 column chro-
matography (ethanol), and IV (44.3 mg) was obtained.

Reaction product (IV). Reddish-brown powder, \([\alpha]_D^0\) 0°. HR-MS: m/z: 553.1183 (M+); calcd. for 

\[2H_2N(CH_3)O: 553.1184.\] EIMS m/z: 553 (M+, 23), 449 (32), 307 (11), 228 (20), 222 (100), 195 (58), 167 (15), 77 (10). UV \(\lambda_{max}\) (CHCl3) nm (ε): 261.5 (17,800), 317 (sh, 11,100). IR \(\nu_{max}\) (KBr) cm⁻¹: 3300, 1780, 1680, 1660, 1620, 1600, 1540, 1510, 1340, 1300, 1260. 1H-NMR (CDCl3, 400 MHz) \(\delta\) 1.77 (3H, s, 3-CH3), 2.07 (3H, s, 6-CH3), 5.57 (1H, s, 5-H), 6.96 (2H, d, \(J=8.8\) Hz, 2'-H and 6'-H), 7.04 (2H, dd, \(J=1.6\) and 7.6 Hz, 2''-H and 6''-H), 7.11 (2H, d, \(J=8.8\) Hz, 3'-H and 5'-H), 7.16 (1H, br tt, \(J=1.6\) and 7.6 Hz, 4''-H), 7.28 (2H, dd, \(J=7.6\) and 7.6 Hz, 3''-H and 5''-H), 8.42 (1H, br s, 3''-H or 5''-H), 9.11 (1H, br s, 5''-H or 3''-H), 10.04 (1H, s, NH). 13C-NMR (CDCl3, 100 MHz) \(\delta\) 19.2 (q, 6-CH3), 20.3 (q, 3-CH3), 59.5 (s, C-3), 104.8 (d, C-5), 118.4 (d, C-2' and C-6'), 121.0 (d, C-2'' and C-6''), 124.0 (d, C-3'' or C-5''), 125.2 (d, C-5'' or C-3''), 125.9 (d, C-4''), 126.0 (d, C-3' and C-5'), 128.9 (d, C-3'' and C-5''), 132.7 (s, C-2'' or C-6''), 133.5 (s, C-4'), 135.8 (s, C-4''), 138.9 (s, C-6'' or C-2''), 140.8 (s, C-1''), 144.2 (s, C-1'), 145.1 (s, C-1'), 166.2 (s, C-6), 169.4 (s, C-2'), 191.6 (s, C-4).

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