Novel Oxidized Sorbicillin Dimers with 1,1-Diphenyl-2-picrylhydrazyl-Radical Scavenging Activity from a Fungus

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Three yellow compounds with 1,1-diphenyl-2-picrylhydrazyl-radical scavenging activity were newly isolated from the fermentation broth of Trichoderma sp. USF-2690 strain that had been isolated from a soil sample: two were novel oxidized sorbicillin dimers designated as bisorubicillinolide (1) and bisorbicillinolide (2), and one was sorbicillin (3) itself. The structures of 1 and 2 were determined from spectroscopic evidence. In the DPPH-radical scavenging assay, α-tocopherol gave an ED₅₀ value of 17.0 μM after standing for 30 min, while continuing observation showed that the ED₅₀ values for bisorubicillinolide, bisorbicillinolide, and sorbicillin slowly reached 80.8, 88.8 and 152.3 μM over 24 hr.

Key words: DPPH-radical scavenger; Trichoderma sp. USF-2690; bisorubicillinolide; bisorbicillinolide; oxidized sorbicillin dimer

Free radicals have recently been found to mediate a wide range of diseases such as atherosclerosis, ischemia reperfusion injury, inflammation, carcinogenesis, and rheumatoid arthritis. A decrease in the free radical level is an important and useful treatment for these diseases, and an effective preventive and/or therapeutic agent would be expected to possess radical scavenging activity. On the other hand, antioxidants, including free radical scavengers, are required to preserve and process foods. We have screened for radical scavengers from the fermentation broth of microorganisms, and previously reported four ansamycin-type, two oxazolyl-type and an α-pyrene-type compounds as free radical scavengers. We applied our screening method that uses the bactericidal action of the hydroxyl radical. Subsequently, in the course of another screening program for free radical scavengers by detecting the radical scavenging activity of DPPH, we isolated four active yellow compounds, which were bisorbicillinol, demethyltrichodimerol, bisvertinolone and trichodimerol, from the fermentation broth of Trichoderma sp. USF-2690 strain that had been isolated from a soil sample collected in Shizuoka City, Shizuoka, Japan. Further investigation into products of the strain in several producing media suggested that the production of sorbicillin-related compounds might depend on the composition of the producing medium; chromatograms from the HPLC analysis indicated sufficiently different varieties and quantities of the minor products according to the producing medium. Sorbicillin-related compounds might be expected to be radical scavengers, so we selected two conditions, described in the materials and methods section, for isolating these minor compounds which increased in production (Fig. 2). Our continuing investigation to find sorbicillin-related radical scavengers from the strain resulted in the isolation of three additional substances: two of them were novel compounds named bisorubicillinolide (1) and bisorbicillinolide (2), and the other was known sorbicillin (3). In this paper, we report the fermentation of the strain, and the isolation, structural elucidation and DPPH-radical scavenging activity of 1, 2 and 3.

Materials and Methods

Chemicals. DPPH, α-tocopherol and the other reagents were analytical-grade products from Wako Pure Chemical Industries, Japan.

Instruments. Spectroscopic measurements were taken with the following instruments: a JEOL Alpha-400 spectrometer (NMR), a Hitachi 270-50 infrared spectrometer (IR), a Shimadzu UV-160A spectrometer (the UV and visible spectra), a JEOL DX-303 spectrometer (FAB-MS), and a Horiba SEPA-200 high-sensitivity polarimeter (optical rotation). MPLC was performed with a Yamazen BLPC-600-FC chromatograph connected to a YMC ODS-AQ 120-S50 column (250 x 340 mm), and HPLC was carried out with Jasco PU-980 pump connected to Jasco UV-970 spectrometer (370 nm) and to a Shiseido Capcell pak C18 SG120 column (4.6φ x 150 mm or 15φ x 250 mm).

Fermentation. Condition A: The fungal strain classified as Trichoderma sp. USF-2690 was cultivated on a reciprocal shaker for 12 days at 30°C in 18 0.5-liter flasks each containing 150 ml of a medium of 2.0% glucose and 0.5% polypeptide at pH 5.6. Condition B: The strain was cultivated on a reciprocal shaker for 7 days at 30°C in 30 0.5-liter flasks each containing 150 ml of a medium of 2% glycerol, 1.5% soybean meal, 0.3%
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Fig. 1. Structures of DPPH-Radical Scavengers from Trichoderma sp. USF-2690.

Fig. 2. HPLC Profile of the Products.

(A) condition A; (B) condition B a, bisorbutenolide (1); b, bisorbutenolide (4); c, demethyltrichodimerol; d, sorbicillin (3); e, trichodimerol; f, bisvertinolone; g, bisorbutenolide (2) Column, Capcell pak C18 SG120 (4.6 × 150 mm); solvent system, acetonitrile-H2O [1:1, including 0.1% trifluoroacetic acid]; flow rate, 1.0 ml/min; detection, UV at 370 nm.

CaCO₃, 0.01% KH₂PO₄, 0.04% Na₂HPO₄·12H₂O, and 0.05% MgSO₄·7H₂O at pH 7.0.

Isolation of bisorbutenolide (1), bisorbutenolide (2), and sorbicillin (3). The filtered broth (2.7 liters), which had been cultured under condition A and adjusted to pH 3.0 with HCl, was extracted with the same volume of ethyl acetate. The organic extract (1.50 g) that had been concentrated in vacuo was applied to a Sephadex LH-20 column (25g × 500 mm) and eluted with CH₃OH. The desired fraction (372.9 mg) including bisorbutenolide (1) was obtained. This fraction was concentrated in vacuo and then purified by reversed-phase MPLC under the following conditions: support, YMC-ODS-AQ 120-550 (25g × 350 mm); solvent system, acetonitrile-H₂O [1:1, including 0.1% trifluoroacetic acid]; detection, UV (370 nm). A yield of 80.8 mg of 1 was obtained.

The fermentation broth (4.5 liters) from condition B was acidified to pH 3.0 with HCl after filtration and then extracted with the same volume of ethyl acetate. The ethyl acetate layer was concentrated in vacuo to give an oily residue (3.09 g). This residue was chromatographed on Sephadex LH-20, eluting with CH₃OH, to give two desired fractions. The bisorbutenolide (2) fraction and sorbicillin (3) fraction were each concentrated in vacuo. The fraction containing 2 was applied to repetitive LH-20 column chromatography, using CHCl₃-CH₃OH (1:3) as the eluent, and subsequently to MPLC under the same conditions as those already described. The fraction containing 3 was also purified by MPLC un-
under the same conditions. Two yellowish active compounds of 2 (10.2 mg) and 3 (15.8 mg) were given.

**Bisorbutenanolide (1).** Yellowish amorphous powder, [α]D 20 +124.4° (c 0.5, in CH3OH); IR νmax (KBr) cm⁻¹: 3445, 2980, 2940, 1740, 1665, 1630, 1565, 1380, 1200, 1000; HRFA-NBS m/z: 497.2222 [M+H]⁺, 497.2176 for C24H32O8; FAB-MS m/z: 497 (M⁺), 519 (M+Na)⁺; UV λmax nm (e, CH3OH): 234 (9,100), 295 (15,900), 365 (15,700); UV λmax nm (e, basic CH3OH): 261 (18,800), 287 (19,700), 389 (9,700). The ¹H- and ¹³C-NMR spectral data are shown in Tables 1 and 2.

**Bisorbicillinolide (2).** Yellowish amorphous powder, [α]D 20 +318.0° (c 0.1, in CH3OH); IR νmax (KBr) cm⁻¹: 3450, 3000, 2995, 1760, 1620, 1580, 1450, 1380, 1250, 1130, 1090, 1000; HRFA-NBS m/z: 497.2202 [M+H]⁺, 497.2176 for C24H32O8; UV λmax nm (e, CH3OH): 293 (20,900), 374 (17,900); UV λmax nm (e, acidic CH3OH): 293 (20,500), 373 (19,300); UV λmax nm (e, basic CH3OH): 256 (sh 17,000), 291 (24,100), 380 (12,000). The ¹H- and ¹³C-NMR spectral data are shown in Tables 1 and 2.

**Measurement of the DPPH-radical scavenging activity**

An ethanol solution of a sample (2 ml) was mixed with a 0.5 mM DPPH ethanol solution (1 ml) and 0.1 M acetate buffer (pH 5.5; 2 ml). After standing for 30 min and monitoring for 1, 8 and 24 hr, the absorbance of the mixture at 517 nm was measured. The ED₅₀ value was determined as the concentration of each sample required to give 50% of the absorbance shown by a blank test.

**Results and Discussion**

**Fermentation**

In the preceding paper,⁹ we reported that *Trichoderma* sp. USF-2690 producedisorbicillinol (4, Fig. 1), demethyltrichodimero, trichoderimol, and bisvertinolone in a medium of 0.5% glucose, 1% glycerol, 0.5% peptone, 0.3% yeast extract, 0.5% meat extract, and 0.5% sodium chloride (NaCl) at pH 7.0. Several media were tested to improve the production of sorbicillin-related compound. Under condition A, only 2% glucose as the carbon source and 0.5% peptone as the nitrogen source were adopted. The medium gave a new major compound [peak "a", as shown in Fig. 2 (A)]. On the other hand, condition B employed 1.5% soybean meal as the nitrogen source to yield unknown peak "g" in Fig. 2 (B).

**Structural determination of bisorbutenanolide (1)**

The molecular formula of bisorbutenanolide (1) obtained as a yellowish amorphous powder was established as C₂₄H₃₂O₈ from HRFA-NBS data. The UV and visible spectra indicated 1 to be an oxidized sorbicillin dimer. The characteristic absorption bands at 1740, 1665 and 1630 cm⁻¹ in the IR spectrum suggested that 1 possessed several carbonyl moieties.

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| a | Multiplicity | b-o | Assignments are interchangeable. |
(δH 1.88 and 1.92) and eight olefinic methine protons (δH 6.12-7.33). The (C-1)-(C-7)-(C-8) sequence was deduced from the 1H-1H COSY spectrum and the coupling constant values (J1,2=1.2 Hz and J7,8=4.8 Hz).

Twenty-eight signals were observed in the 13C-NMR (Table 2) and DEPT spectra of 1 in CDCl3: four singlet methyls (δC 6.3, 11.0, 23.1 and 23.5), two doublet methyls (δC 18.9 and 19.1), three sp3 methines (δC 42.4, 43.6 and 51.3), eight olefinic methines (δC 117.6, 127.0, 130.3, 131.0, 140.9, 143.9, 145.5 and 148.0), three ester or oxygen-bearing olefinic carbons (δC 169.8, 174.9 and 176.5), three carbonyls (δC 194.9, 202.7 and 208.3), and five other quaternary carbons (δC 62.6, 75.0, 83.2, 98.2 and 108.5).

The connectivity between the hydrogen and carbon atoms was confirmed from the HSQC spectrum of 1, and two partial structures (7 and 8, Fig. 3) were assembled by analyzing the long-range correlation between four singlet methyls and carbons in the HMBC spectrum. Partial structure 7 was given by interpretation of the cross peaks between 4-CH3 (δH 1.12) and C-3 (δC 194.9), C-4 (δC 62.6), C-8 (δC 51.3) and C-5 (δC 208.3), and between 6-CH3 (δH 1.29) and C-1 (δC 42.4), C-5 and C-6 (δC 75.0), while 8 was inferred through the cross peaks between 9-CH3 (δH 1.51) and C-7 (δC 43.6), C-9 (δC 83.2) and C-10 (δC 176.5), and between 11-CH3 (δH 1.29) and C-10, C-11 (δC 98.2) and C-12 (δC 174.9).

Furthermore, four partial structures 5, 6, 7 and 8 were linked through the cross peaks between 1-H (δH 3.36) and C-2 (δC 108.5), C-3, C-5, C-6, C-7, C-8, C-9, C-1' (δC 169.8) and 6-CH3 (δC 23.5), between 7-H (δH 3.21) and C-1, C-2, C-9, C-10 and C-1" (δC 202.7), between 8-H (δH 3.43) and C-3, C-4, C-5, C-9 and C-1", between 2'-H (δH 6.12) and C-1", and between 2''-H (δH 6.13) and C-1". The (C-9)-O-(C-12) connection, the presence of γ-lactone and the hydroxyl group at C-6 were determined from the absorption band at 1740 cm⁻¹ in the IR spectrum and from the molecular formula of 1.

The NOESY spectrum of 1 (9, Fig. 3) suggests that the relative configuration of 1 was 1S*, 4R*, 6S*, 7S*, 8R* and 9S*. In particular, NOE between 4-CH3 and 11-CH3 could directly infer the stereochemistry of the asymmetric carbons at C-7 and C-9. The relative configuration between C-7 and C-8 is supported by the J1,2 value of 4.8 Hz. Consequently, we determined the structure of 1 as shown in Fig. 1. Compound 1 was a novel compound and is named bisorbibutenolide.

After we presented the structure of 1 at the 120th Meeting of Central Branch of Japan Society for Bioscience, Biotechnology and Agrochemistry at Nagoya City in Oct. 1997, we found that O. Shirota et al. had reported a novel fungal metabolite as trichotetronine, which was a very similar compound having an opposite relative configuration at C-9 to that of bisorbibutenolide (1), in July 1997. The NOESY spectra of trichotetronine showed the cross peak between 3'-H and 11-CH3 substituting for that between 4-CH3 and 11-CH3 of bisorbibutenolide (1). Interestingly, the structural differences between bisorbibutenolide (1) and trichotetronine appeared in two partial structures as (C-1')-(C-2)-(C-3) and (C-9)-(C-10)-(C-11)-(C-12), which were observed as two enolized β-diketone moieties for trichotetronine. The physico-chemical and spectroscopic data for 1 are not in agreement with those for trichotetronine; for example, the chemical shifts at C-1' (δ 181.0), C-3 (δ 184.6), C-10 (δ 194.9), C-11 (δ 90.3) and C-12 (δ 182.2) in the 13C-NMR spectrum and at 8-H (δ 4.01) in the 1H-NMR spectrum of trichotetronine. In
In particular, the optical rotation of the compound (+456°, in CH₂OH) was completely different from that of 1 (see the materials and methods section). We postulate that the difference between the biosynthetic pathway to compound 1 via sorbicillinol (4), which is described later, and that of trichotetronine reported to gave two similar molecules.

**Structural determination of sorbicillinolide (2)**

Compound 2 was obtained as a yellowish amorphous powder and formulated as C₆₈H₆₂O₁₂ from the HRFAB-MS data. The UV and visible spectra of 2 suggested that 2 was an oxidized sorbicillin dimer. The IR spectrum of 2 demonstrated characteristic absorption bands at 3450 cm⁻¹ (hydroxyl), 1770 cm⁻¹ (γ-lactone), 1680 and 1620 cm⁻¹ (carbonyls), and 1590 cm⁻¹ (β-diketone).

The ¹H- and ¹³C-NMR spectra in CDCl₃ exhibited 32 proton and 28 carbon signals. The assignments of the ¹H- and ¹³C-NMR spectral data of 2, which were obtained from the 2D-NMR experiments (HSQC, ¹H-¹H COSY and HMBC), are shown in Tables 1 and 2. The ¹H-NMR spectrum of 2 displays the proton forms as three singlet methyls, three doublet methyls, three methines [two of which (δH 3.38 and 3.73) were coupled to each other with a coupling constant of 5.4 Hz], three exchangeable hydroxyls, and eight olefinic methines arising from two (E, E)-1,3-pentadienyl moieties proven through the ¹H-¹H COSY spectrum. The analysis of the ¹³C-NMR spectrum of 2 clarified the presence of six methyl (δC 10.3, 13.7, 18.1, 18.9, 19.0 and 24.3), three sp² methine (δC 41.1, 44.4 and 56.9), two sp² quaternary (δC 68.0 and 73.5), three oxygenated sp² quaternary (δC 84.1, 88.3 and 93.8), an sp² quaternary (δC 107.3), eight sp² quaternary (δC 119.0, 123.8, 130.3, 131.0, 141.5, 145.0, 144.6 and 147.9), two carboxyl or enol (δC 177.3 and 177.9), and three carbonyl (δC 194.5, 199.4 and 210.3) carbons.

The HMBC experiment for 2 allowed partial structures 10 and advanced 11 to be built up as shown in Fig. 4. The cross peaks between four methyl groups and a hydroxyl group and the carbons, given from the HMBC spectrum, enabled planar 10 to be constructed; the cross peaks between 4-CH₃ (δH 1.36) and C-3 (δC 194.5), C-4 (δC 68.0), C-5 (δC 73.5) and C-10 (δC 88.3), between 7-CH₃ (δH 1.23) and C-1 (δC 44.4), C-6 (δC 210.3) and C-7 (δC 84.1), between 9-CH₃ (δH 1.39) and C-8 (δC 56.9), C-9 (δC 93.8) and C-10, between 11-CH₃ (δH 1.21) and C-10, C-11 (δC 41.1) and C-12 (δC 177.9), and between 10-OH (δH 6.25) and C-10 and C-11. Partial structure 11 was confirmed by elucidating the 3JCH or 1JCH correlation between 1-H (δH 3.38), 8-H (δH 3.73), 11-H (δH 2.66) and 1'-OH (δH 16.01) and the carbons; between 1-H and C-2 (δC 107.3), C-3, C-5, C-6, C-7, C-8 and C-1' (δC 177.3), between 8-H and C-1, C-2, C-4, C-10, 9-CH₃ (δC 24.3) and C-1" (δC 199.4), between 11-H and C-4, C-9, C-10, C-12 and 11-CH₃ (δC 13.7), and between 1'-OH and C-2, C-1' and C-2" (δC 119.0). We think that the expected γ-lactone moiety would have to include the carbonyl group at C-12, this situation being based on the chemical shift at C-12 (δC 177.9), and that the five-membered lactone would require the linkage between C-9 and C-12 via an oxygen atom. In addition, the ketone carbon at C-6 (δC 210.3) was obviously bonded to the quaternary carbon at C-7, and the molecular formula of 2 clarified the presence of 7-OH (δH 6.25).

The NOESY spectrum of 2 could give the relative configuration of 2 as 1S*, 4R*, 5R*, 7S*, 8S*, 9S*, 10S* and 11R* (11, Fig. 4).

![Fig. 4. Summary of the HMBC and ¹H-¹H COSY Results for Sorbicillinolide (2).](image-url)
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Fig. 5. Summary of the HMBC Results for Sorbicillin (3).

As a result, we determined the structure of unknown 2, named bisorbicillinolide, as that shown in Fig. 1.

Structural identification of sorbicillin (3)
Compound 3 was also yielded as a yellowish amorphous powder. All the physico-chemical and spectral data indicated that 3 was identical to sorbicillin which had been isolated as a fungal metabolite.\textsuperscript{10,11} Our HMBC experiment on 3 (Fig. 5) revised the \textsuperscript{13}C-NMR spectral assignments at C-2 and C-4 that had been reported by L. S. Trifonov et al.\textsuperscript{11} (Table 2).

DPPH-radical scavenging activity
Compounds 1, 2 and 3, bisorbicillinol (4), and \( \alpha \)-tocopherol were submitted to the assay system with DPPH.\textsuperscript{10} After standing for 30 min, bisorbicillinol (4) and \( \alpha \)-tocopherol gave ED\textsubscript{50} values of 31.4 and 17.0 \( \mu \)M, respectively. However, compounds 1, 2 and 3 did not give ED\textsubscript{50} values at a dosage below 100 \( \mu \)M. We continued to observe the DPPH-radical scavenging activity of 1, 2 and 3 for 24 hr; consequently, we found that bisorbibutenolide (1), bisorbicillinolide (2) and sorbicillin (3) slowly scavenged the DPPH radical. After standing for 24 hr, the evaluation of their ED\textsubscript{50} values in this assay at less than a 500 \( \mu \)M dosage is as follows: bisorbibutenolide (1), 80.8 \( \mu \)M; bisorbicillinolide (2), 88.8 \( \mu \)M; and sorbicillin (3), 152.3 \( \mu \)M. The results of these experiments are illustrated in Fig. 6 and suggest that the sorbicillin-related compounds used two different types of mechanism for their DPPH-radical scavenging activity, one of which afforded radical scavengers 1, 2 and 3 showing a slow effect. It has previously been reported that the rapid DPPH-radical scavenging activity of bisorbicillinol (4) was lost by methylation of the enolized \( \beta \)-diketone partial structure at 9-OH or 11-OH.\textsuperscript{9} The effect of methylation suggests that the movable hydrogen atom was necessary to achieve rapid DPPH-radical scavenging activity in the case of bisorbicillinol (4). Bisorbibutenolide (1), bisorbicillinolide (2) and sorbicillin (3) possessed an enolized sorbyl side chain in each molecule. We consider that the slow DPPH-radical scavenging activity of 1, 2 and 3 might have been caused by each enolized sorbyl side chain.

Biogenetic pathway for the oxidized sorbicillin dimers
We propose the biogenetic pathway for the oxidized sorbicillin dimers that is illustrated in Fig. 7. Two oxidized sorbicillin (13), one molecule playing the role of a diene and the other a dienophile, react with each other according to the Diels-Alder reaction to produce bisorbicillinol (4).\textsuperscript{10} The hydroxyl group at C-12 of 4 may attack the carbonyl group at C-9, and then cleavage of the bond between C-8 and C-9 would occur. The resulting anion at C-8 (14) would then catch a proton to yield bisorbibutenolide (1). On the other hand, the second anion intermediate (15) was inferred from the structure of bisorbicillinolide (2), following a Michael-type reaction of the anion at C-8 with a carbonyl at C-5 and then cleavage of the bond between C-4 and C-5 in 14 (route 1,
Fig. 7). As to the Michael-type reaction, the first anion intermediate (14) can be expected to react easily through route II in Fig. 7. The HMBC spectrum of bisorbicillinolide (2) shows cross peaks between 4-CH$_3$ and C-10 and between 11-H and C-4, while the cross peaks between 4-CH$_3$ and C-6 and between 11-H and C-5 were not evident. These results indicate to us that the structure of bisorbicillinolide was 2, not 16, and supports the existence of route I (Fig. 7).

In conclusion, we found two novel DPPH-radical scavenging compounds, bisorbibutenolide (1) and bisorbicillinolide (2), from the culture broth of Trichoderma sp. USF-2690 and determined their chemical structures, which were expected to be oxidized dimers of sorbicillin. The observation of free-radical scavenging activity for 24 hr revealed that bisorbicillinol (4) rapidly scavenged the DPPH radical, and that bisorbibutenolide (1), bisorbicillinolide (2) and sorbicillin (3) gradually did.

Acknowledgment
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References