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Naoki ABE[a], Osamu SUGIMOTO[a], Tadaharu ARAKAWA[a], Ken-ichi TANJI[a] & Akira HIROTA[a]
[a] School of Food and Nutritional Sciences, University of Shizuoka
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Sorbicillinol, a Key Intermediate of Bisorbicillinoid Biosynthesis in *Trichoderma* sp. USF-2690

Naoki ABE,† Osamu SUGIMOTO, Tadaharu ARAKAWA, Ken-ichi TANJI, and Akira HIROTA

School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

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In the course of our screening program for free radical scavengers from *Trichoderma* sp. USF-2690, we found an unidentified metabolite (1) that appeared by the method used for HPLC analysis. Metabolite 1 gradually decreased with the production of bisorbicillinoids and was easily missed during the general isolation procedure. The LC-ESI-MS (negative) analysis for 1 gave *m/z* 247 as the $(M-1)^-$ ion peak. The hydrolysis of synthetic 6-*O*-acetylsorbicillinol (±-2) by 0.05 M KOH and acetylation of product 1 in an aqueous solution indicated that the structure of 1 was (6*S*)-4-(2,4-hexadienoyl)-3,6-dihydroxy-2,6-dimethyl-2,4-cyclohexadien-1-one, designated sorbicillinol, a quinol that has been postulated to be important in bisorbicillinoid biosynthesis.

Key words: sorbicillinol; DPPH-radical scavenger; *Trichoderma* sp. USF-2690; bisorbicillinoids biosynthesis

Nicolaou *et al.* have recently defined all dimeric natural products derived from sorbicillin as fresh "bisorbicillinoids," which included demethyl oxidized sorbicillin dimers.[1] Some of the bisorbicillinoids have been reported to exhibit unique activities: inhibition of the induction of the mitogen-induced cyclooxygenase (PGHS-2, COX-2) by a lipopolysaccharide (LPS) -stimulated human monocyte cell (THP-1)[2] and inhibition of β-1,6-glucan biosynthesis.[3]

In the course of our screening program for free radical scavengers with DPPH radical-scavenging activity,[4] we have isolated 10 active sorbicillin-related metabolites: sorbicillin (**3**), oxosorbicillinol (**6**), demethylsorbicillin (**7**), bisorbicillinol (**8**), trichodimerol (**9**), demethyltrichodimerol (**10**), bisvertinolone (**11**), bisorbibutenolide (bislongiquinolide **12**), bisorbicillinolide (**13**), and bisorbibetanone (**14**) from the fermentation broth of *Trichoderma* sp. USF-2690 strain (Fig. 1 and 2).[5–8] The variety of these structures prompted our strong interest in their biosynthesis. Oxidized sorbicillin dimers are known to be produced by a few fungal genera (*Trichoderma, Verticillium, Acremonium* and *Penicillium*). Dreiding *et al.* proposed biogeneses for metabolites derived from 2,4-dimethyl-hexaketides (defined as vertinoids), which were sorbicillin, dihydrosorbicillin, bisvertinoquinol, vertinolide and bisvertinols, in 1981,[9] 1983[10] and 1986.[11] In addition, Satake *et al.*[12] have presented a possible biosynthetic route from 2,4-dimethylhexaketides to trichotetronins and trichodimerol, and Crews *et al.*[13] have postulated a biogenesis for epoxysorbicillinol and bislongiquinolide which was first reported by Ayer *et al.*[14] We also proposed a different biosynthetic route to bislongiquinolide (bisorbibutenolide) and bisorbicillinolide *via* bisorbicillinol in 1998[6] and then reported the result of a preliminary labeling experiment that provided evidence for the biosynthetic route.[15] All of these proposed biosynthetic routes reported a quinol (**1**) to be the common key intermediate which served in tautomeric forms. On the other hand, Corey *et al.* have achieved a total synthesis of trichodimerol.[16] Nicolaou and co-workers have biomimetically synthesized trichodimerol, bisorbicillinol, and bislongiquinolide (bisorbibutenolide) on the basis of our proposed biosynthetic route.[17] Both groups introduced 6-*O*-acetylsorbicillinol (±-**2**) as a precursor of the quinol (**1**) in their key steps for bisorbicillinoid syntheses. In these studies, the hydrolysis of 6-*O*-acetylsorbicillinol (**2**) and subsequent dimerization of the generated quinol (**1**) proceeded simultaneously. Therefore, no evidence for quinol **1** as an intermediate was obtained.

In our previous communication,[18] we have indicated that our careful HPLC analysis of the bisorbicillinoids produced by *Trichoderma* sp. USF-2690 showed that an unidentified major metabolite accumulated in the early stage of the fermentation and decreased gradually with bisorbicillinoid production. We thought that this metabolite, designated as sorbicillinol, was likely to have been quinol **1**. In this

† To whom correspondence should be addressed. Fax: +81-54-264-5099; E-mail: abe@fns1.u-shizuoka-ken.ac.jp

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; MPLC, medium-pressure liquid chromatography; HPLC, high-performance liquid chromatography

present paper, we describe a detailed determination of the structure of sorbicillinol (**1**), together with the absolute stereochemistry and DPPH radical-scavenging activity of **1**, the changeable behavior of bisorbicillinol (**8**) derived from **1** in the fermentation broth, and the postulated position of **1** in bisorbicillinoid biosynthesis.

Materials and Methods

Chemicals. DPPH, 2,6-di-*t*-butyl-4-methylphenol (BHT) 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), Jeol JMS-700 spectrometer (FAB-MS), Shimadzu UV-160A spectrometer (UV and visible spectra), and Horiba SEPA-200 high-sensitivity polarimeter (optical rotation). A Shiseido Nanospace SI-1 HPLC system with a Shiseido Capcell pak C_{18} SG120 column (4.6ϕ × 150 mm) was linked to a ThermoQuest LCQ LC mass spectrometer operating in the ESI negative ion mode. HPLC was carried out with Jasco PU-980 pump connected to a Jasco UV-970 spectrometer (370 nm) and to a Shiseido Capcell pak C_{18} SG120 column (4.6ϕ × 150 mm) or to a Chiralpak AD column (4.6ϕ × 250 mm, Daisel Chemical Ind., Japan).

Fermentation. The fungal strain classified as *Trichoderma* sp. USF-2690 was cultivated on a reciprocal shaker for 3 days at 30°C in 0.5-liter flasks each containing 100 ml of a medium of 2% glucose, 0.05% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.1% trace salt mixture at pH 7.0.

HPLC analysis. A 10-μl aliquot of the filtered fermentation broth was directly injected into an analytical HPLC system under the following conditions: column, Capcell pak C_{18} SG120; solvent system, 0.15% KH_2PO_4 (pH 3.5) solution (solvent A) and CH_3CN (solvent B), a gradient program made up of linear segments with 40% of solvent B (from 0 to 10 min), from 40% to 60% of solvent B (from 10 to 20 min) and with 60% of solvent B (from 20 to

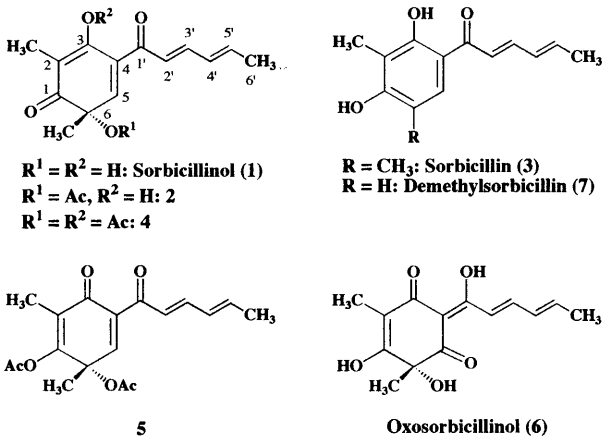

Fig. 1. Structures of Sorbicillin-related Compounds Isolated from *Trichoderma* sp. USF-2690.

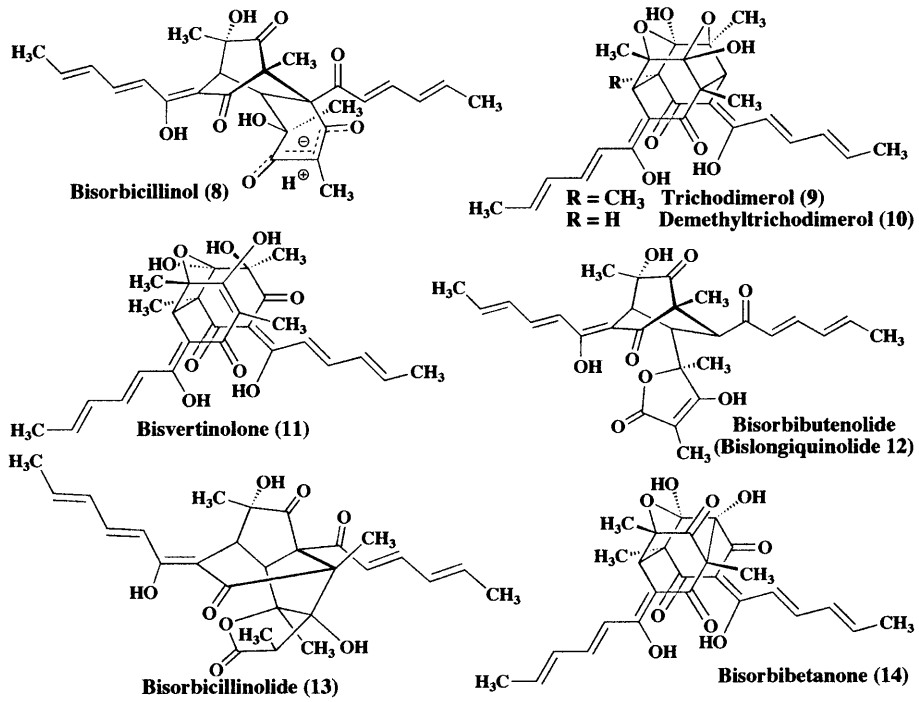

Fig. 2. Structures of Bisorbicilinoids Isolated from *Trichoderma* sp. USF-2690.

25 min); flow rate, 1.0 ml/min; detection, 370 nm.

Purification of sorbicillinol (1). The filtered broth (100 ml), which had been cultured under the conditions just described and had subsequently been concentrated *in vacuo* to 20 ml, was applied to a Sephadex LH-20 column (25ϕ × 500 mm) and eluted with H_2O. The desired fraction (20 ml) including sorbicillinol (**1**) of 80% purity was obtained by the HPLC analysis.

LC-ESI-MS analysis. A Shiseido Capcell pak C_{18} SG120 column (4.6ϕ × 150 mm) was used at a flow rate of 0.5 ml/min with a solvent system of CH_3CN-0.5% CH_3COOH (4:6). A 10-μl aliquot of the LH-20 fraction including sorbicillinol (**1**) was injected into the column, and the structure of sorbicillinol (**1**) was analyzed by liquid chromatography combined with mass spectrometry (LC/MS) with UV and visible detection (370 nm).

Chiral HPLC analysis. A 10-μl aliquot of 1.0 mg/ml of each sample was injected into an analytical HPLC system under the following conditions: column, Chiralpak AD; solvent system, *n*-hexane/*i*-PrOH/CH_3OH (80:15:5) containing 0.1% TFA; flow rate, 0.5 ml/min; detection, 370 nm.

Sorbicillinol [(6S)-4-(2,4-hexadienoyl)-3,6-dihydroxy-2,6-dimethyl-2,4-cyclohexadien-1-one] (1). The optical rotation showed negative value, but the dilute solution of **1** could not give an accurate value for $[\alpha]_D$; LC-ESI-MS (negative) *m/z*: 247.3 $(M-H)^-$; UV λ_{max} nm (ε, H_2O)*: 218 (8,000), 293 (12,000); UV λ_{max} nm (ε, 0.1 N HCl): 295 (9,600); UV λ_{max} nm (ε, 0.1 N NaOH): 292 (11,000).

*The evaluation of the amount of sorbicillinol (**1**) in the aqueous solution was performed by assuming that the 6-*O*-acetylsorbicillinol (**2**) hydrolysis was quantitative; hence, the UV spectra were measured in a 0.05 M KCl solution.

6-O-Acetylation of sorbicillinol (1). The aqueous sorbicillinol fraction (0.43 mg/ml, 10 ml) was added to 55 ml of acetic anhydride while stirring for 1 hr at ambient temperature. After the reaction mixture had turned into a pale yellow homogeneous solution, 55 ml of pyridine was added, and the mixture was then stirred for 1 hr. The resulting solution was poured into 2 l of distilled water, extracted with 400 ml of $CHCl_3$, and dried over Na_2SO_4. The concentrate monitored by preparative TLC (Merck Art No. 13794, *n*-hexane/EtOAc = 1:1) gave 2.3 mg of **2** (45.2% yield).

(6S)-6-O-Acetylsorbicillinol [(6S)-6-acetoxy-4-(2,4-hexadienoyl)-3-hydroxy-2,6-dimethyl-2,4-cyclohexadien-1-one] (2). Yellowish amorphous powder, $[\alpha]_D^{20}$ – 80.0° (*c* 0.05, in CH_3OH); IR ν_{max} (KBr) cm^{-1}: 3440, 1735, 1650, 1555, 1380, 1275, 1240, 1210, 1070, 1020; ESI-MS (negative) *m/z*: 289.0 $(M-H)^-$ UV λ_{max} nm (ε, CH_3OH): 314.8 (28,100); NMR δ_H ($CDCl_3$): 11.77 (1H, s, 3-OH), 7.45 (1H, dd, *J* = 10.6 and 14.8, 3′-H), 7.24 (1H, s, 5-H), 6.65 (1H, d, *J* = 14.8, 2′-H), 6.39 (1H, dq, *J* = 15.2 and 6.0, 5′-H), 6.30 (1H, dd, *J* = 10.6 and 15.2, 4′-H), 2.15 (3H, s, CH_3CO), 1.93 (3H, d, *J* = 6.0, 6′-H_3), 1.86 (3H, s, 2-CH_3), 1.49 (3H, s, 6-CH_3). Chiral HPLC analysis showed a single peak at 9.6 min as the *S*-isomer.[16]

Lead tetra-acetate oxidation of sorbicillin (3) to (±)-6-O-acetylsorbicillinol (±-2). A solution of synthetic sorbicillin (**3**) (182.9 mg, 0.79 mmol), which had been prepared according to Corey's method,[16] in acetic acid (20 ml) was treated with lead acetate (412.9 mg, 0.95 mmol) for 3 hr at ambient temperature. The resulting solution was poured into distilled water (100 ml) and then extracted with $CHCl_3$ (100 ml × 2). The combined organic layers were successively washed with distilled water and a saturated NaCl solution, dried over Na_2SO_4, and concentrated to dryness. The crude mixture (225.4 mg) was chromatographed by reversed-phase MPLC [support, YMC-ODS-AQ 120-s50 (25ϕ × 350 mm); solvent system, acetonitrile-0.15% KH_2PO_4 (pH 3.5); buffer solution (4:6); detection, UV (370 nm)] to give 39.6 mg of desired (±)-6-*O*-acetylsorbicillinol (±-**2**; yield, 17.3%).

(±)-6-O-Acetylsorbicillinol [(±)-6-acetoxy-4-(2,4-hexadienoyl)-3-hydroxy-2,6-dimethyl-2,4-cyclohexadien-1-one] (±-2). NMR δ_C ($CDCl_3$): 195.0 (C-1), 193.3 (C-1′), 170.0 (CH_3CO), 162.5 (C-3), 151.8 (C-5), 148.3 (C-3′), 144.6 (C-5′), 130.1 (C-4′), 125.6 (C-4), 120.4 (C-2′), 111.8 (C-2), 78.2 (C-6), 24.1 (6-CH_3) 20.5 (CH_3CO), 19.1 (C-6′), 7.2 (2-CH_3). A chiral HPLC analysis showed two separable peaks in equal parts at 9.6 min (*S*-isomer) and 11.2 min (*R*-isomer).[16]

Hydrolysis of (±)-6-O-acetylsorbicillinol (±-2). Two milligrams of (±)-6-*O*-acetylsorbicillinol (±-**2**) was dissolved in 2.0 ml of a 0.05 M KOH aqueous solution, and the mixture stirred for 6 hr at ambient temperature. The reaction mixture was diluted with 30 ml of distilled water. The resulting solution was adjusted to pH 7 with a 0.1 N HCl aqueous solution, giving an aqueous solution including (±)-sorbicillinol (±-**1**, 0.057 mg/ml, by assuming that the hydrolysis of ±-**2** was quantitative).

(±)-Sorbicillinol (±-1). LC-ESI-MS (negative) *m/z*: 247.2 $(M-H)^-$; UV λ_{max} nm (ε, H_2O): 215.5 (13,100), 292 (14,800).

Acetylation of (±)-6-O-acetylsorbicillinol (±-2).

(±)-6-*O*-Acetylsorbicillinol (±-**2** 11.2 mg) was dissolved in 0.3 ml of pyridine, and then 0.3 ml of acetic anhydride was added to the pyridine solution. The reaction mixture was stirred for 0.5 hr at ambient temperature. After the resulting solution had directly been applied to a preparative TLC plate (Merck Art No. 13794), two diacetates were separated on the plate that was developed in *n*-hexane/EtOAc (1:1) to give 7.0 mg of **4** (yield, 54.6%) and 1.2 mg of **5** (yield, 9.4%).

(±)-3,6-Diacetoxy-4-(2,4-hexadienoyl)-2,6-dimethyl-2,4-cyclohexadien-1-one (±-4). Yellowish amorphous powder. IR ν_{max} (KBr) cm^{-1}: 1770, 1740, 1670, 1630, 1590, 1370, 1240, 1200, 1160, 1070, 1010; FAB-MS *m/z*: 333 $(M+H)^+$, 355 $(M+Na)^+$; HRFAB-MS *m/z* 333.1300 [$(M+H)^+$, 333.1338 for $C_{18}H_{21}O_6$]; UV λ_{max} nm (ε, CH_3OH): 290 (20,100); NMR δ_H ($CDCl_3$): 7.25–7.18 (1H, m, 3′-H), 6.47 (1H, s, 5-H), 6.30–6.19 (3H, m, 2′-H, 4′-H, and 5′-H), 2.22 (3H, s, C*H*$_3$CO), 2.11 (3H, s, C*H*$_3$CO), 1.88 (3H, d, $J=5.2$, 6′-H_3), 1.84 (3H, s, 2-CH_3), 1.50 (3H, s, 6-CH_3); NMR δ_C ($CDCl_3$): 196.8 (C-1), 190.3 (C-1′), 169.7 (CH_3CO), 167.4 (CH_3CO), 154.0 (C-3), 148.0 (C-3′), 142.4 (C-5 or C-5′), 142.2 (C-5 or C-5′), 131.8 (C-4), 130.3 (C-4′), 126.0 (C-2′), 124.4 (C-2), 78.4 (C-6), 23.6 (6-CH_3), 20.6 (*C*H$_3$CO), 20.4 (*C*H$_3$CO), 18.9 (C-6′), 8.7 (2-CH_3). A chiral HPLC analysis showed two separable peaks in equal parts at 11.8 min (*S*-isomer) and 14.0 min (*R*-isomer).

(±)-1,6-Diacetoxy-4-(2,4-hexadienoyl)-2,6-dimethyl-2,4-cyclohexadien-3-one (±-5). Pale yellowish amorphous powder. IR ν_{max} (KBr) cm^{-1}: 1775, 1750, 1680, 1675, 1380, 1240, 1180, 1020, 1060, 1010; FAB-MS *m/z*: 333 $(M+H)^+$, 355 $(M+Na)^+$; HRFAB-MS *m/z* 333.1254 [$(M+H)^+$, 333.1338 for $C_{18}H_{21}O_6$]; UV λ_{max} nm (ε, CH_3OH): 240.5 (7,600), 279.5 (7,400); NMR δ_H ($CDCl_3$): 7.32–7.27 (1H, m, 3′-H), 7.10 (1H, s, 5-H), 6.51 (1H, d, $J=15.6$, 2′-H), 6.31-6.19 (2H, m, 4′-H and 5′-H), 2.31 (3H, s, C*H*$_3$CO), 2.02 (3H, s, C*H*$_3$CO), 1.87 (3H, d, $J=4.8$, 6′-H_3), 1.76 (3H, s, 2-CH_3), 1.55 (3H, s, 6-CH_3); NMR δ_C ($CDCl_3$): 191.0* (C-1′), 184.6* (C-3), 169.0* (CH_3CO), 166.4* (CH_3CO), 157.1 (C-1), 148.6 (C-5), 146.5 (C-3′), 141.7 (C-5′), 130.7 (C-4′), 127.3 (C-2′), 126.9* (C-2), 73.8 (C-6), 24.5 (6-CH_3), 21.0 (*C*H$_3$CO), 20.4 (*C*H$_3$CO), 18.9 (C-6′), 9.5 (2-CH_3). *These chemical shifts were estimated inversely *via* HMBC cross peaks, because the quarternary carbons at C-1′, C-2, C-3 and C-4, and the two carbonyl carbons in acetyl groups could not be detected in the ^{13}C-NMR spectrum of ±-**5**.

A chiral HPLC analysis showed two separable peaks in equal parts at 9.6 min and 11.3 min.

Acetylation of sorbicillinol (1). The aqueous sorbicillinol fraction (0.41 mg/ml, 20 ml) was added to 105 ml of acetic anhydride while stirring overnight at ambient temperature. After the reaction mixture had been concentrated *in vacuo* to dryness at 50°C, 50 ml of distilled water was added to the residue. The aqueous solution was extracted with 50 ml of $CHCl_3$, and the $CHCl_3$ layer was dried over Na_2SO_4. Solvent extraction monitored by preparative TLC (Merck Art No. 13794, *n*-hexane/EtOAc = 1:1) gave 0.5 mg of **4** (yield, 4.6 %).

(6S)-3,6-Diacetoxy-4-(2,4-hexadienoyl)-2,6-dimethyl-2,4-cyclohexadien-1-one (4). Yellowish amorphous powder, $[\alpha]_D$ could not be accurately measured. A chiral HPLC analysis showed a single peak at 11.8 min as the *S*-isomer.

Conversion of bisorbicillinol (8) from sorbicillinol (1) or 6-O-acetylsorbicillinol (2). The aqueous solution including sorbicillinol (**1**, 0.083 mg/ml, 50 ml) was adjusted to pH 3.0 with 1 N HCl and then extracted with ethyl acetate (50 ml). After the organic layer had been dried over Na_2SO_4 and concentrated to dryness *in vacuo*, (+)-bisorbicillinol (**8**) was quantitatively given (4.2 mg). (±)-6-*O*-Acetylsorbicillinol (±-**2** 6.0 mg) was dissolved in 6.0 ml of a 0.05 M KOH aqueous solution, and the solution stirred for 6 hr at ambient temperature. Distilled water (54 ml) was added to the reaction mixture, and the resulting solution was adjusted to pH 3.0. After ethyl acetate extraction and concentration to dryness *in vacuo*, 4.8 mg of (±)-bisorbicillinol (±-**8**) was obtained (93.5% yield).

(±)-Bisorbicillinol (±-8). A chiral HPLC analysis showed two separable peaks in equal parts at 13.4 min as the (−)-isomer and 15.8 min as the (+)-isomer.

(+)-Bisorbicillinol (8). A chiral HPLC analysis showed a single peak at 15.8 min as the (+)-isomer.

Conversion of trichodimerol (9) from sorbicillinol (1) or 6-O-acetylsorbicillinol (2). The aqueous sorbicillinol (**1**) solution (0.14 mg/ml, 50 ml) was concentrated *in vacuo* to dryness at 50°C, giving a crude mixture including (−)-trichodimerol (**9**). The mixture was chromatographed by preparative HPLC under the following conditions: column, Capcell pak C_{18} SG120 (15ϕ × 250 mm, Shiseido); solvent system, acetonitrile-0.15% KH_2PO_4 at pH 3.5 (4:6); detection, UV at 370 nm; flow rate, 10.0 ml/min. Finally, 1.3 mg of **9** was given (yield, 18.6%). An aqueous (±)-sorbicillinol (±-**1**) solution (0.057 mg/ml), which had been prepared from 5.0 mg of (±)-6-*O*-acetylsorbicillinol (±-**2**), was evaporated *in vacuo* at 50°C to dryness. The residue dissolved in CH_3OH (10 ml) was stirred for 20 hr at ambient temperature. The resulting methanolic solution was concentrated

in vacuo to dryness, to give (±)-trichodimerol (±-**9**, 37.8% yield by HPLC). Crude ±-**9** was purified by preparative HPLC under the same conditions as those just quoted to yield pure ±-**9** (0.7 mg, 16.3% yield).

Time-course characteristics for the bioconversion from bisorbicillinol (8) to bisorbibutenolide (12) and bisorbicillinolide (13). The fungus, which had been inoculated into 0.5-liter flasks containing 150 ml of a medium (pH 7) composed of 2.0% glucose and 0.5% polypeptone, was preincubated on a reciprocal shaker at 30°C for 9 days. The resulting mycelia were washed with sterilized water and then the washed mycelia were inoculated into a 0.5-liter flask containing 150 ml of sterilized water with 24.0 mg of bisorbicillinol (**8**). The culture was incubated on a reciprocal shaker at 30°C and monitored at 0, 2, 4, 6, 8, and 24 hr by HPLC under the conditions just described.

Measurement of the DPPH-radical scavenging activity. An ethanol or an aqueous 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, the absorbance of the mixture at 517 nm was measured. The ED_{50} 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 and isolation

In the preceding paper,[5-8] we have reported that *Trichoderma* sp. USF-2690 produced 10 sorbicillin-related compounds in the adopted distinguishable media. The products were monitored by an HPLC analysis in each bisorbicillinoid-producing medium, and a common precursor at 4.3 min was detected in the early stage of fermentation of all the media. The area of the precursor peak gradually reduced with the production of bisorbicillinoids. The strain was cultivated under the conditions described in the Materials and Methods section to isolate the common precursor. The precursor was highly sensitive to concentration, solvent extraction, and storage in freezing water and quickly changed into other compounds. Therefore, a filtered broth (100 ml) was concentrated *in vacuo* to 1/5 volume, and the concentrated aqueous solution was applied to a Sephadex LH-20 column, using H_2O as an eluent, to give the desired fraction with over 80% purity of the common precursor (0.08 mg/ml, 50 ml of aqueous solution) by an HPLC analysis.

LC-ESI-MS analysis

The behavior of the common precursor in the bisorbicillinoid-producing media suggested that it was quinol **1** (named sorbicillinol) which has been postulated as a key precursor in bisorbicillinoid biosynthesis.[6,9-13] Quinol **1** was expected to have a molecular weight of 248. As we expected, an LC-ESI-MS (negative) analysis of the LH-20 fraction including the precursor gave m/z 247 as an $(M-1)^-$ ion peak that was eluted at 8.5 min under the condition already described. To confirm the chemical structure of the precursor, we designed an experiment to derive expected quinol **1** from synthetic 6-*O*-acetylsorbicillinol (±-**2**). Our observation that the common precursor could exist in the aqueous LH-20 fraction at pH 7 for several weeks suggested how to obtain an aqueous solution of quinol **1** from 6-*O*-acetylsorbicillinol (±-**2**). The aqueous solution of quinol **1** was consequently prepared by treating 6-*O*-acetylsorbicillinol (±-**2**), which had been synthesized according to Corey's method,[16] with a 0.05 M KOH aqueous solution for 6 hr, this being followed by dilution with distilled water and then careful neutralization. An LC-ESI-MS (negative) analysis of the resulting aqueous solution of quinol **1** showed m/z 247 as an $(M-1)^-$ ion peak eluted at 8.5 min, which was completely compatible with that of the precursor.

Acetylation of quinol (1) and 6-O-acetylsorbicillinol (2)

The quinol (**1**) could not exist in a stable state under the concentrated condition and rapidly changed into other compounds. To elucidate the structure of the quinol (**1**) by ^{1}H- and ^{13}C-NMR spectra, we tried to derive stable acetates of the quinol (**1**) in an aqueous dilute solution. The LH-20 aqueous fraction including the quinol (**1**) was slowly added to an excess volume of acetic anhydride, and then the reaction mixture was stirred overnight at ambient temperature. The residue obtained from concentration was partitioned between $CHCl_3$ and water, and then the $CHCl_3$ extract was purified by preparative TLC to give a small amount of a major product (yield, 4.6%) which was presumed to be 3,6-diacetoxy-4-(2,4-hexadienoyl)-2,6-dimethyl-2,4-cyclohexadien-1-one (**4**) from the ^{1}H-NMR spectrum of the product.

On the other hand, acetylation of synthetic 6-*O*-acetylsorbicillinol (±-**2**) was performed by treating with pyridine and acetic anhydride; however, the resulting residue after general post-treatments such as dilution with water, solvent extraction with $CHCl_3$, and concentration *in vacuo* to dryness included small quantities of the expected diacetates (±-**4** and ±-**5**). Further acetylation with 4-dimethylaminopyridine also brought about insufficient yield of diacetates ±-**4** and ±-**5**. Our careful observation of each process during acetylation led to acetates ±-**4** and ±-**5** being easily hydrolyzed to 6-*O*-acetylsorbicillinol (±-**2**) again by the remaining pyridine dur-

ing the process of concentration to dryness *in vacuo*. Therefore, the reaction mixture treated with pyridine and acetic anhydride was applied to and developed on a preparative TLC plate to obtain diacetates ±-**4** and ±-**5** (a ratio of approximately 6:1). The structures of ±-**4** and ±-**5** were determined from spectroscopic evidence, mainly based on ^{1}H- and ^{13}C-NMR and HMBC spectra, as (±)-3,6-diacetoxy-4-(2,4-hexadienoyl)-2,6-dimethyl-2,4-cyclohexadien-1-one (±-**4**) and (±)-1,6-diacetoxy-4-(2,4-hexadienoyl)-2,6-dimethyl-2,4-cyclohexadien-3-one (±-**5**). The ^{1}H-NMR spectrum of the acetylation product of the quinol (**1**) was completely consistent with that of (±)-3,6-diacetoxy-4-(2,4-hexadienoyl)-2,6-dimethyl-2,4-cyclohexadien-1-one (±-**4**).

Furthermore, the knowledge that generated diacetates ±-**4** and ±-**5** were hydrolyzed to 6-*O*-acetylsorbicillinol (±-**2**) by a small amount of pyridine suggested a procedure for acetylation from quinol **1** to known acetate **2** in an aqueous solution. The LH-20 aqueous fraction including quinol **1** was slowly added to an excess volume of acetic anhydride. After the acidic aqueous solution had become homogeneous, pyridine in the same volume as that of acetic anhydride was added to the aqueous solution. The reaction mixture was stirred while cooling to room temperature and then extracted with $CHCl_3$. The crude $CHCl_3$ extract was concentrated *in vacuo* to dryness and purified by preparative TLC to give a pure product (yield, 45.2%). The ^{1}H-NMR spectrum and other spectroscopic evidence for the product were identical to those of 6-*O*-acetylsorbicillinol (±-**2**). The results of these experiments supported the conclusion that the structure of the quinol (**1**) was (6*S*)-4-(2,4-hexadienoyl)-3,6-dihydroxy-2,6-dimethyl-2,4-cyclohexadien-1-one (**1**).

Chiral HPLC analysis

The investigation with a chiral HPLC column (Chiralpak AD, Daisel Chemical Ind., Japan) afforded information about the stereochemistry at C-6 of 6-*O*-acetylsorbicillinol (**2**) derived from the quinol (**1**) in the fermentation broth. Synthetic 6-*O*-acetylsorbicillinol (±-**2**) gave two separable peaks in equal parts, which had been assigned to the *S*-isomer (t_R = 9.6 min) and *R*-isomer (t_R = 11.2 min) by Barnes-Seeman and Corey,[16] while that from the quinol (**1**) revealed just one peak at 9.6 min coinciding with the *S*-isomer (Fig.3). (6*S*)-6-*O*-Acetylsorbicillinol (**2**) given from the quinol (**1**) confirmed the 6*S*-configuration of **1**, so quinol **1** was designated as sorbicillinol.

Conversion of bisorbicillinol (8) and trichodimerol (9) from sorbicillinol (1)

Solvent extraction at pH 3.0 of the fermentation broth caused rapid disappearance of the peak of sorbicillinol (**1**) at 4.3 min, while a peak at 16.8 min, which was consistent with the retention time for bisorbicillinol (**8**), was apparently heightened from the HPLC analysis. On the other hand, concentration

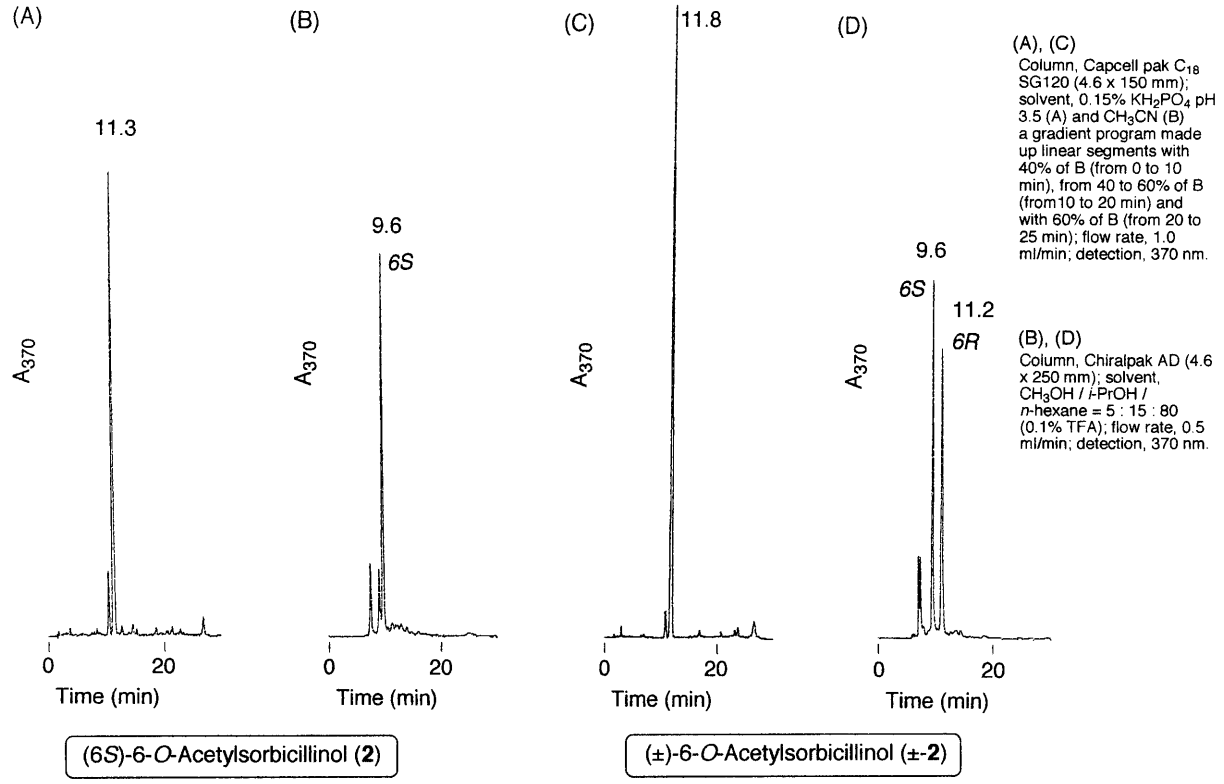

Fig. 3. HPLC Profiles of (6*S*)-6-*O*-Acetylsorbicillinol (**2**) (A and B) and (±)-6-*O*-Acetylsorbicillinol (±-**2**) (C and D).

with water to dryness, including lyophilization, increased the peak at 22.7 min for trichodimerol (**9**), with a concomitant decrease in that of sorbicillinol (**1**). These results strongly suggested that sorbicillinol (**1**) was a common precursor of these compounds.

The LH-20 aqueous sorbicillinol (**1**) fraction (0.083 mg/ml × 50 ml) was adjusted to pH 3.0 and then extracted with ethyl acetate. The solvent extract was concentrated *in vacuo* to dryness to give 4.2 mg of bisorbicillinol (**8**, 100% yield). (±)-Bisorbicillinol (±-**8**) was generated from the hydrolysis of ±-**2** with 0.05 M KOH and subsequent ethyl acetate extraction at pH 3.0 (93.5% yield). This simple procedure for the final step from ±-**2** to ±-**8** for the bisorbicillinol synthesis resulted in a higher yield than that in the literature (40%).[17]

Based on our observation of the characteristics of sorbicillinol (**1**), (−)- and (±)-trichodimerols (**9**) were expected to be synthesized from naturally occurring **1** and synthetic 6-*O*-acetylsorbicillinol (±-**2**), respectively, by a simple treatment. The aqueous sorbicillinol (**1**) solution (0.14 mg/ml × 50 ml) was concentrated *in vacuo* to dryness at 50°C and then subjected to preparative HPLC to give 1.3 mg of (−)-trichodimerol (**9**, 18.6% yield). Next, an aqueous (±)-sorbicillinol (±-**1**) solution (0.057 mg/ml), which had been prepared from 5.0 mg of (±)-6-*O*-acetylsorbicillinol (±-**2**), was evaporated *in vacuo* at 50°C to dryness. Over 50% of **1**, which might be stable as a potassium salt, remained; therefore, the residue redissolved in CH_3OH was stirred for 20 hr, and the resulting methanolic solution was concentrated *in vacuo* to dryness to give crude (±)-trichodimerol (±-**9**, 37.8% yield by HPLC). The crude material was purified by preparative HPLC, giving 0.7 mg of pure ±-**9** (16.3% yield). A summary of the chemical conversion between sorbicillinol (**1**) and the related compounds is shown in Fig. 4.

DPPH radical-scavenging activity

Sorbicillinol (**1**), 6-*O*-acetylsorbicillinol (±-**2**), the diacetate (±-**4**), and BHT were submitted to an assay with DPPH.[4] After standing for 30 min, the evaluation of their ED_{50} values in this assay at less than a 100-μM dosage was as follows: sorbicillinol (**1**, 49.5 μM) and BHT (33.4 μM). Derivatives ±-**2** and ±-**4** did not give an ED_{50} value at a dosage below 100 μM; that is, the DPPH radical-scavenging activities at 100 μM were 36.9% for ±-**2** and 18.1% for ±-**4** . The results of the DPPH radical-scavenging test for **1** and the two acetates of **1** revealed that the strong DPPH radical-scavenging activity of **1** needed not only the 3-OH group of **1**, but also 6-OH as a functional group.

Time-course study on the bioconversion from bisorbicillinol (8) to bisorbibutenolide (12) and bisorbicillinolide (13).

We have recently reported that our observations from ^{13}C-labeled compound feeding studies indicated that there were biosynthetic routes from bisorbicillinol (**8**) to bisorbibutenolide (**12**) and from bisorbicillinol (**8**) to bisorbicillinolide (**13**), while additional experiments removed the possibility of the route from **8** to **12** *via* **13** or from **8** to **13** *via* **12**, and of the reverse biosynthesis from **12** or **13** to **8**.[15] In the filtered 4-day-cultivated broth, however, an HPLC analysis of the bisorbicillinoids could reveal only trace amounts of precursors **8** and **13** and 10 μM of **12**. Our bioconversion experiment in sterilized water by using the washed mycelium indicated that the quantity of bisorbicillinol (**8**) was decreasing at a rate of about 10 μM an hour. On the other hand, bisorbibutenolide (**12**) was increasing at a rate of about 5.7 μM an hour; a constant concentration of bisorbicillinolide (**13**, about 20 μM) was detected (Fig. 5). In the case of the incubation with bisorbicillinolide

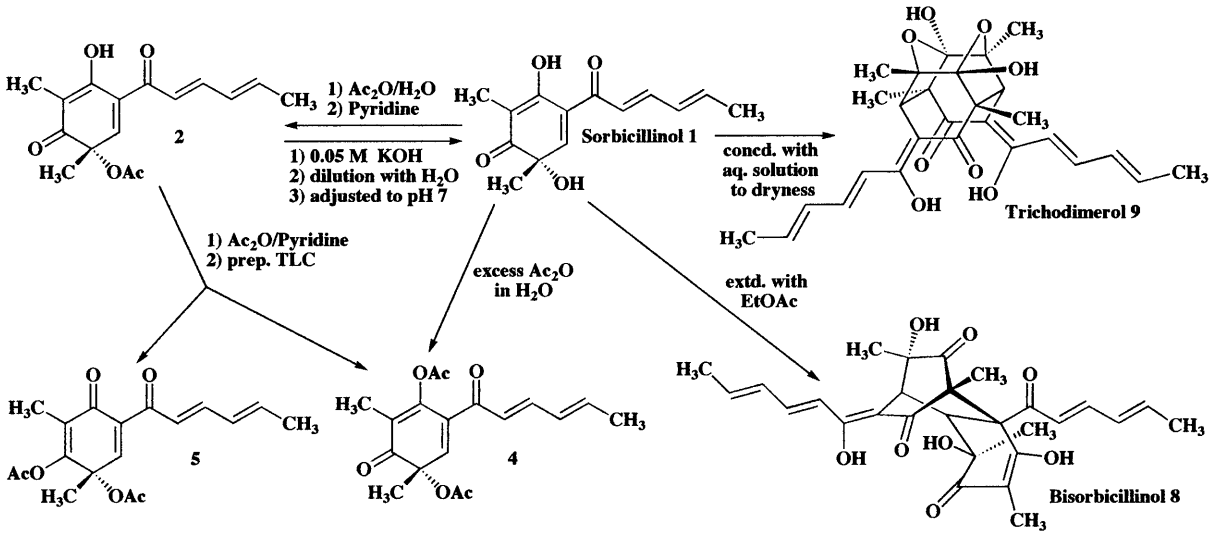

Fig. 4. Chemical Conversion for the Structural Determination of Sorbicillinol (**1**).

(**13**) as a starting material, we observed that **13** was gradually degraded to many unidentified compounds in sterilized water, including the washed mycelia. These results suggested that 1) the production of bisorbicillinol (**8**) could not make up for that lost, 2) the degradation of **8** might proceed *via* bisorbicillinolide (**13**), and 3) bisorbibutenolide (**12**) was apparently increasing in the production medium.

Proposed biosynthetic pathway to bisorbicillinoids starting from sorbicillinol (1)

We propose the biosynthetic pathway to bisorbicillinoids starting from sorbicillinol (**1**) that is illustrated in Fig. 6. The biosynthetic route from bisorbicillinol (**8**) to bisorbibutenolide (**12**) and bisorbicillinolide (**13**) had already been proved by our incorporation study of ^{13}C-labeled acetates.[15] In the present study, the chemical conversion from **1** to **8** suggested that two tautomeric forms of sorbicillinol (**1**), one molecule playing the role of a diene (**1**) and the other a dienophile (**1'**) (present in the ratio of *ca.* 6:1 in each form, this being deduced from the production of **4** and **5**), reacted with each other according to the Diels-Alder reaction to biologically produce bisorbicillinol **8**. The second metabolite inferred to be biosynthesized from sorbicillinol (**1**) was trichodimerol (**9**), this being followed by our chemical conversion study. The two steps of the Michael-addition and ketalization may have occurred simultaneously between two molecules of sorbicillinol (**1**), generating **9** in the fermentation broth. In addition, we propose a third route starting from sorbicillinol (**1**) in the present paper. By this route, the nucleophilic attack of C-2 in **1** on C-5 in oxosorbicillinol (**6**) with ketalization between C-1 in **1** and C-6-OH in **6** may produce bisvertinolone (**11**), and the intramolecular SN2 reaction in **11** may give bisorbibetanone (**14**) as the next step. A biosynthetic investigation of the third route on the basis of ^{13}C-labeled compounds is underway.

In conclusion, we found an unidentified major metabolite that accumulated in the early stage of fermentation of *Trichoderma* sp. USF-2690 by an HPLC analysis of the bisorbicillinoids. This metabolite decreased gradually with bisorbicillinoid production; therefore, we expected that the metabolite occupied an important position in bisorbicillinoid

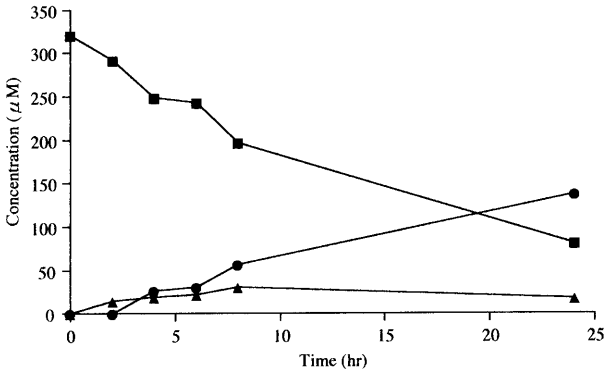

Fig. 5. Time-Course Study on the Bioconversion from Bisorbicillinol (**8**) to Bisorbibutenolide (**12**) and Bisorbicillinolide (**13**) by Using Washed Mycelia.
■ Bisorbicillinol (**8**); ● Bisorbibutenolide (**12**); ▲ Bisorbicillinolide (**13**)

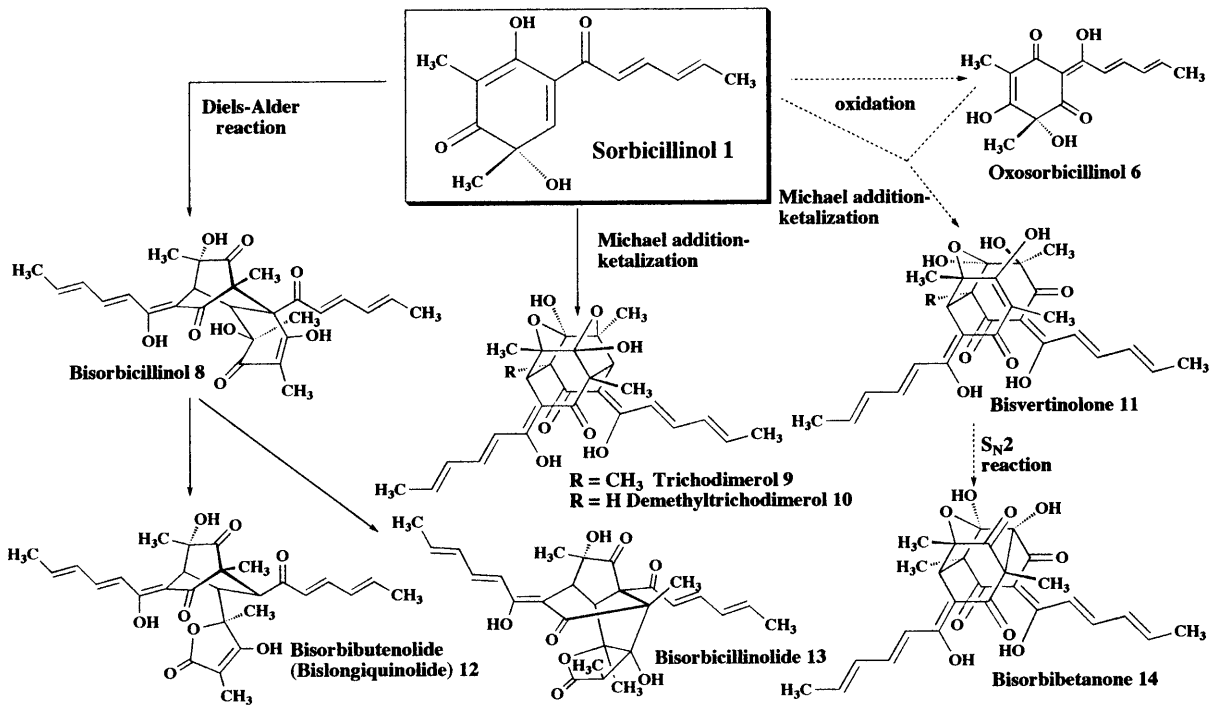

Fig. 6. Proposed Biosynthetic Pathway to Bisorbicillinoids.

biosynthesis. Solvent extraction at pH 3.0 and concentration of the aqueous solution to dryness each markedly increased the area of bisorbicillinol (**8**) and that of trichodimerol (**9**) in the HPLC chromatogram. Our careful observation of the net bisorbicillinoid production revealed that only a small amount of bisorbicillinol (**8**) could exist in the fermentation broth, because **8** tended to change easily into bisorbibutenolide (**12**) and bisorbicillinolide (**13**) and disappeared rapidly in the broth (Fig. 5). On the other hand, trichodimerol (**9**) was at *ca.* 30 μM in the broth. These results suggest that the metabolite was a quinol as had been postulated as a key intermediate of bisorbicillinoid biosynthesis by independent investigators. The LC-ESI-MS, chemical conversion, and chiral HPLC experiments established the structure of the metabolite as (6*S*)-4-(2,4-hexadienoyl)-3,6-dihydroxy-2,6-dimethyl-2,4-cyclohexadien-1-one (sorbicillinol, **1**). The results reported here provide the first definitive evidence for the reality of intermediate **1** in bisorbicillinoid biosynthesis, in answer to a long-outstanding question. The evaluation of its ED_{50} value by a DPPH radical-scavenging experiment indicated that sorbicillinol (**1**) had strong activity equal to that of BHT as a noted antioxidant.

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