Cochliospicin A: A New Metabolite Related to Spiciferinone, a Phytotoxin of the Fungus *Cochliobolus spicifer*

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The structure of cochliospicin A, a new metabolite biogenetically related to a phytotoxin, spiciferinone, produced by *Cochliobolus spicifer* was determined to be (3*R**,4*S**,6*S**,7*R**,3′*R**)-7-ethyl-5,6,7,8-tetrahydro-3,4,7-trimethylisochroman-5,8-dione-6-spiro-5′-(3′-hydroxytetrahydrofuran-2′-one) (**1**) by X-ray analysis.

An isolate (D-5) of *Cochliobolus spicifer* Nelson (Pyrenophoraceae), which causes leaf spot disease in wheat, produces several phytotoxins and a plant-growth promoter. The phytotoxins spiciferones A, B, and C, spiciferinone, and the plant growth promoter spicifernin have been isolated and characterized in our laboratory.[1−4] Their biosynthesis has been studied using labeled HOAc and methionine, and a unique biosynthetic pathway in *C. spicifer* has been proposed.[5−7] To confirm this pathway, we undertook a search for metabolites biogenetically related to the phytotoxins produced by this fungus. We found a new metabolite that is biogenetically related to spiciferinone and named it cochliospicin A. In this paper, we report the isolation and structure of cochliospicin A and discuss the role of the "C_3 unit addition reaction" in the metabolism of phytotoxins in phytopathogenic fungi.

Cochliospicin A (**1**) was isolated from the culture filtrate of the fungus, strain D-5, by several chromatographies in the yield of 0.43 mg/L of medium. ^{13}C-NMR data and HREIMS showed the molecular formula $C_{17}H_{22}O_6$ (7 unsaturations). The DEPT data disclosed that one proton in the molecule was bound to an oxygen. Acetylation of cochliospicin A afforded a monoacetate derivative (**2**), confirming that the exchangeable proton was associated with an alcoholic hydroxy group. Two carbon signals at δ 148.2 and 143.5 indicated one fully substituted double bond. A γ-lactone ring was indicated by a carbon signal at δ 175.3 and IR carbonyl absorption at 1794 cm^{-1}. The two carbon signals at δ 197.4 and 192.8, together with two IR carbonyl absorptions at 1707 and 1680 cm^{-1}, indicated two conjugated ketones and thereby three rings in the structure.

The structural fragments in **1**, shown in Figure 1, were deduced by ^{1}H−^{1}H COSY, HETCOR and LR-HETCOR analyses, and chemical shift considerations. In the ^{1}H-NMR spectrum of the monoacetate, a proton signal associated with an oxygenated methine appeared at δ 5.44, indicating that the carbon (C-14) in the natural product bears a free hydroxyl group. The combination of structural fragments A and B was suggested by the correlation between the carbon signal of C-1 and the proton signal of H-2 and that between the carbon signal of C-6 and the proton signal of H-2 in

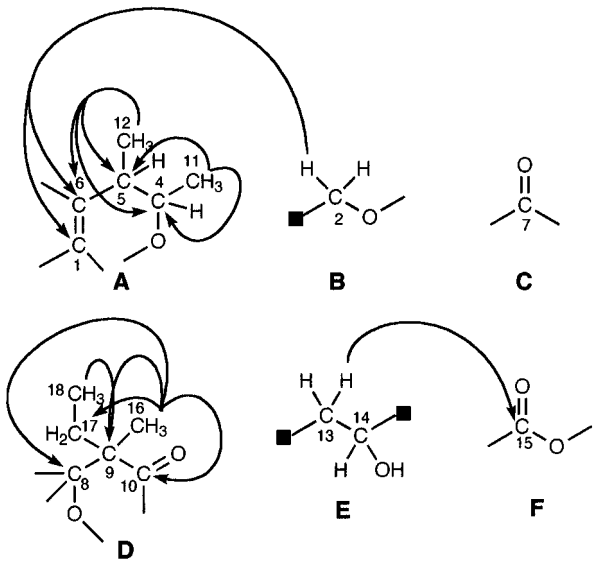

Figure 1. LR-HETCOR correlations for structural fragments of cochliospicin A (**1**).

LR-HETCOR. However, there were still two possibilities, the connection of C-2 with C-1 and that of C-2 with C-6, which could not be distinguished by the available NMR data. Moreover, the combination of structural fragments E and F was suggested by the correlation of the carbon signal of C-15 to the proton signal of H-13 in LR-HETCOR. How to connect fragment E to F was not clear. The nondecoupled ^{13}C-NMR spectrum of **1** gave no information on the connectivity of these fragments.

Fortunately, a crystal suitable for X-ray analysis was obtained by crystallization from Me_2CO−*n*-hexane, and an X-ray analysis was made of a single crystal of **1**. The molecular structure of **1** is shown in Figure 2. The carbon skeleton of **1** indicates that it is probably derived from spiciferinone (**3**). If this is true, the biosynthetic origin of **1** would be a polyketide and a C_3 unit. In a bioassay of protoplasts from wheat cotyledons,[3] **3** showed phytotoxicity, whereas **1** did not. Conversion of **3** to **1** by biological reactions, including a C_3 unit addition reaction, therefore means loss of the phytotoxicity of the metabolite. Very recently, a new phytotoxin, sorokinianin (**4**), was isolated from *Bipolaris sorokiniana* and its structure determined.[8] Its carbon skeleton and results of a preliminary incorporation experiment suggest that **4** is derived from prehelminthosporol and a C_3 unit. In a bioassay using protoplasts from first leaves of barley, **4** showed more phytotoxicity than

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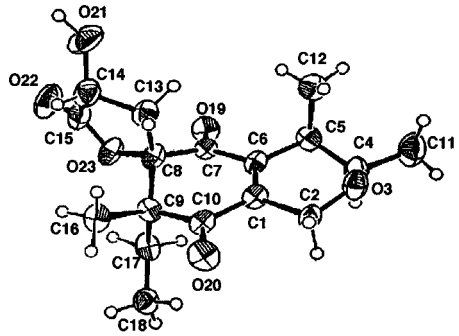
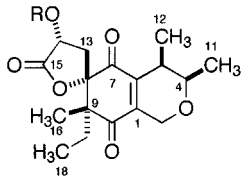
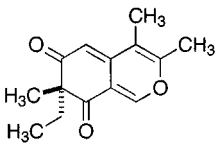


Figure 2. ORTEP drawing of **1**.

prehelminthosporol (data not shown). In the case of *B. sorokiniana*, conversion of prehelminthosporol to **4** by a C_3 unit addition reaction therefore means an increase in the phytotoxicity of the metabolite. Addition of C_3 units to fungal polyketide metabolites was reported in marticin[9] from *Fusarium martii* and canescin[10] from *Aspergillus malignus*. In both cases, the origin of the C_3 unit was considered to be from members of the TCA cycle.[9,10] The C_3 unit addition reaction is an interesting topic in phytotoxin metabolism, and studies on the biosynthetic origin of the C_3 unit in **1** and **4** are now under way.



1 R=H
2 R=Ac



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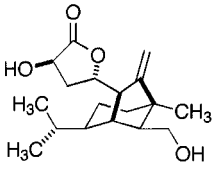

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Experimental Section

General Experimental Procedures. UV spectra were recorded on a Shimadzu UV-2200 UV-vis recording spectrophotometer and IR spectra on a JASCO FT/IR-7000 spectrometer. Optical rotation was measured with a Horiba SEPA-200 high-sensitive polarimeter. EIMS were obtained with a JEOL AX505HA spectrometer (direct probe, 70 eV). NMR spectra were measured with a JEOL JNM GX-270 FT NMR spectrometer. Chemical shifts were referenced against $CDCl_3$ (δ_H 7.26, δ_C 77.20). Daisogel IR-60 was used as the Si gel for column chromatography. Preparative TLC was carried out on Merck Kieselgel $60HF_{254}$ glass plates (20 × 20 × 0.05 cm).

Fungal Material. *Cochliobolus spicifer* Nelson D-5 was isolated from diseased wheat growing in Kagawa, Japan, and identified by Dr. Masakatsu Ichinoe of the National Institute of Hygienic Sciences, Tokyo (Present address: Tokyo Kasei University, Itabashi-ku, Tokyo 173, Japan). It has been maintained on potato dextrose agar.

Fermentation and Extraction. The fungus was grown in 500-mL conical flasks (200) containing liquid medium (200 mL/flask) composed of glucose (30 g/L), peptone (3 g/L), L-methionine (0.3 g/L), the extract from 100 g/L of malt, and H_2O, without shaking, at 24 °C for 21 days in the dark. The metabolites were extracted from the culture filtrate with EtOAc (3 × 10L) after adjusting the pH of the filtrate to 2.0 with HCl. The EtOAc extracts were dried over Na_2SO_4, concentrated, and extracted with 1 M $NaHCO_3$ (2 × 0.5 volume). The combined $NaHCO_3$ solutions were acidified to pH 2.0 with HCl and extracted with EtOAc (3 × 1 volume).

Purification. The extract was dried over Na_2SO_4 and concentrated to dryness. The residue (4.0 g) was purified by partition column chromatography (200 g Si gel impregnated with 120 mL of 0.1 M HCO_2H, 50 × 250 mm). Elution was performed with 1 L each of 10, 20, and 30% EtOAc in *n*-hexane saturated with 0.1 M HCO_2H. The fraction eluted with 20% EtOAc in *n*-hexane (825 mg) was subjected to Sephadex LH-20 column chromatography (35 × 1350 mm, MeOH). The column was washed with 250 mL of MeOH, and then each 7 mL of eluate was collected as one fraction. Fractions 13−16 were combined and evaporated to dryness. The residue (49 mg) was purified by preparative TLC [EtOAc−benzene (2:8), triple development] to give **1** (R_f 0.55−0.59, 12 mg) as an amorphous solid.

Cochliospicin A (1): $[\alpha]^{25}_D$ −8.0° (*c* 0.1, MeOH); UV (EtOH) λ_{max} (log ϵ) 250 (2.40) nm; IR (film) ν_{max} 3295, 2974, 1794, 1707, 1680, 1389, 1208, 1117 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ 4.71 (1H, d, J = 19.4 Hz, H-2), 4.56 (1H, dd, J = 6.8, 9.0 Hz, H-14), 4.24 (1H, dd, J = 2.6, 19.4 Hz, H-2), 3.63 (1H, dq, J = 2.6, 6.5 Hz, H-4), 2.82 (1H, m, H-5), 2.50 (1H, dd, J = 9.0, 14.2 Hz, H-13), 2.06 (1H, dd, J = 6.8, 14.2 Hz, H-13), 1.87 (1H, dq, J = 15.4, 7.6 Hz, H-17), 1.58 (1H, dq, J = 15.4, 7.6 Hz, H-17), 1.27 (3H, d, J = 6.5 Hz, H-11), 1.13 (3H, s, H-16), 1.01 (3H, d, J = 7.0 Hz, H-12), 0.74 (3H, t, J = 7.6 Hz, H-18); ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 197.4 (s, C-10), 192.8 (s, C-7), 175.3 (s, C-15), 148.2 (s, C-6), 143.5 (s, C-1), 89.8 (s, C-8), 72.6 (d, C-4), 67.2 (d, C-14), 64.0 (t, C-2), 58.5 (s, C-9), 36.8 (t, C-13), 30.6 (d, C-5), 30.1 (t, C-17), 17.7 (q, C-11), 13.2 (q, C-16), 11.6 (q, C-12), 9.2 (q, C-18); EIMS (70 eV) *m*/*z* $[M]^+$ 322 (30), 278 (68), 250 (36), 223 (100), 221 (23), 206 (26), 180 (25), 179 (63), 178 (84), 177 (30), 166 (53), 163 (35), 138 (29), 135 (22); exact mass calcd for $C_{17}H_{22}O_6$ 322.1405, found 322.1406.

Monoacetyl cochliospicin A (2): Compound **1** (5 mg) was acetylated (Ac_2O−pyridine) to afford monoacetyl cochliospicin A (**2**) quantitatively; ^1H NMR ($CDCl_3$, 270 MHz) δ 5.44 (1H, dd, J = 8.1, 10.3 Hz, H-14), 4.69 (1H, d, J = 19.2 Hz, H-2), 4.24 (1H, dd, J = 2.7, 19.2 Hz, H-2), 3.63 (1H, dq, J = 2.7, 7.3 Hz, H-4), 2.83 (1H, m, H-5), 2.63 (1H, dd, J = 10.3, 14.0 Hz, H-13), 2.12 (3H, s, Me of Ac), 1.98 (1H, dd, J = 8.1, 14.0 Hz, H-13), 1.86 (1H, dq, J = 14.0, 7.8 Hz, H-17), 1.58 (1H, dq, J = 14.0, 7.8 Hz, H-17), 1.27 (3H, d, J = 7.3 Hz, H-11), 1.17 (3H, s, H-16), 0.99 (3H, d, J = 7.0 Hz, H-12), 0.75 (3H, t, J = 7.8 Hz, H-18).

X-ray Crystal Structure Analysis of 1. Intensity data were measured on a Rigaku four-circle diffractometer using Ni-filtered Cu Kα radiation (λ = 1.5418 Å) and a rotating anode generator. A crystal of dimensions 0.50 × 0.15 × 0.05 mm was used. The $\omega - 2\theta$ scan mode was employed, with background measurement at each end of the scan. Intensities of 1357 unique reflections were measured to $2\theta_{max}$ = 120° in the range $0 \le h \le$

13, $0 \le k \le 6$, $-13 \le l \le 13$, with ω scan width of 1.7° + 0.15° tan θ, a scan speed of 3° min^{-1}, and background counting time of 5 s. No significant change was observed in the intensities of the three standard reflections measured every 100 reflections. Intensity data were corrected only for Lorenz and polarization effects. The structure was determined by direct methods using SHELX 86[11] and refined by block diagonal least squares[12] on F using atomic scattering factors from *International Tables for X-ray Crystallography* (1974).

Crystal Data for 1: $C_{17}H_{22}O_6$, $M = 322.4$, monoclinic, space group $P2_1$, $a = 11.683(4)$, $b = 5.841(2)$, $c = 12.118(5)$ Å, $\beta = 99.25(5)°$, $V = 816.2(4)$ Å^3, $Z = 2$, $D_X = 1.31$ gcm^{-1}. The final R was 0.082 and R_W 0.120 for 1117 unique reflections with $F_o > 2\sigma(F_o)$.[13]

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References and Notes

(1) Nakajima, H.; Hamasaki, T.; Kimura, Y. *Agric. Biol. Chem*. **1989**, *53*, 2297−2299.
(2) Nakajima, H.; Hamasaki, T.; Kohno, M.; Kimura, Y. *Phytochemistry* **1991**, *30*, 2563−2565.
(3) Nakajima, H.; Kimura, Y.; Hamasaki, T. *Phytochemistry* **1992**, *31*, 105−107.
(4) Nakajima, H.; Hamasaki, T.; Maeta, S.; Kimura, Y.; Takeuchi, Y. *Phytochemistry* **1990**, *29*, 1739−1743.
(5) Nakajima, H.; Matsumoto, R.; Kimura, Y.; Hamasaki, T. *J. Chem. Soc. Chem. Commun*. **1992**, 1654−1656.
(6) Nakajima, H.; Fujimoto, H.; Matsumoto, R.; Hamasaki, T. *J. Org. Chem*. **1993**, *58*, 4526−4528.
(7) Nakajima, H.; Fukuyama, K.; Fujimoto, H.; Baba, T.; Hamasaki, T. *J. Chem. Soc. Perkin Trans. 1* **1994**, 1865−1869.
(8) Nakajima, H.; Isomi, K.; Hamasaki, T.; Ichinoe, M. *Tetrahedron Lett*. **1994**, *35*, 9597−9600.
(9) Holenstein, J. E.; Kern, H.; Stoessl, A.; Stothers, J. B. *Tetrahedron Lett*. **1983**, *24*, 4059−4062.
(10) Lewis, C. N.; Staunton, J.; Sunter, D. C. *J. Chem. Soc. Perkin Trans. 1* **1988**, 747−754.
(11) Sheldrick, G. M. *SHELX86, Crystallographic Computing 3*; Sheldrick, G. M., Krüger, C., Goddard, R., Eds.; Oxford: Oxford, UK, 1985.
(12) Ashida, T. *The Universal Crystallographic Computing System*; Computation Center, Osaka University: Osaka, Japan, 1974.
(13) Atomic coordinates, temperature factors, and bond lengths and angles have been deposited with the Cambridge Crystallographic Data Centre and can be obtained, upon request, from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge, CB2 1E2, UK.

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