Three New Phytotoxins Produced by *Pyrenochaeta terrestris*: Pyrenochaetic Acids A, B and C

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Three phytotoxins named pyrenochaetic acids A, B and C were isolated from culture filtrates of *Pyrenochaeta terrestris*, and the structures were determined to be 4-crotonoyl-3-methoxy-5-methylbenzoic acid (I), 4-(3-hydroxybutyryl)-3-methoxy-5-methylbenzoic acid (II) and 4-butyryl-3-methoxy-5-methylbenzoic acid (III), respectively. Their phytotoxicities were demonstrated in bioassays.

In the previous studies,^{1,2)} we isolated two new phytotoxins named pyrenocine A and B from culture filtrates of *Pyrenochaeta terrestris* (the causal fungus of onion pink root disease) and determined their structures to be 5-crotonoyl-4-methoxy-6-methyl-2-pyrone (V) and 5-(3-hydroxybutyryl)-4-methoxy-6-methyl-2-pyrone (VI), respectively. The phytotoxicities of these compounds were demonstrated on germination of lettuce seed and on growth of rice and onion seedlings, pyrenocine A having a higher activity.

This paper deals with the isolation and structure elucidation of a further three new phytotoxins designated as pyrenochaetic acids A (I), B (II) and C (III), the inhibitory activ-

ities of which were investigated in the same way as in the cases of pyrenocine A and B. The fungus was grown on a corn-extract medium. Monitoring by the lettuce seed germination test and TLC, phytotoxic materials other than pyrenocine A and B were found to occur in the culture filtrates. The ether extract from the culture filtrate was subjected to silica gel column chromatography using solvent systems of benzene-ethyl acetate with stepwise increases of ethyl acetate, and separated into five fractions from which pyrenochaetic acid C, pyrenocine A, pyrenochaetic acid A, pyrenocine B, and pyrenochaetic acid B, in that order of elution, were isolated.

FIG. 1. Alkali Fusion Products of Pyrenochaetic Acid C (III).

Pyrenochaetic acid C (III)

High resolution mass spectral analyses gave $\text{C}_{13}\text{H}_{16}\text{O}_4$ for compound (III) and the IR absorption was indicative of ketone grouping at 1690 together with a benzene ring at 1570, 880 and 765 cm^{-1} . The ^1H -NMR spectrum contained signals assigned as follows: a methyl at δ 2.35 (3H, s), a methoxy at 3.94 (3H, s), a butyryl at 1.04 (3H, t, $J=7\text{ Hz}$), 1.81 (2H, sextet) and 2.81 (2H, t, $J=7\text{ Hz}$), a carboxyl at 9.70 and two aromatic protons located at the *meta*-position at 7.44 (1H, br. s) and 7.56 (1H, br. s).

On treatment with diazomethane it gave a monomethyl ester (IV) confirming the presence of a carboxyl group. These data for compound (III) indicated a benzene derivative tetra-substituted with one each of methyl, methoxyl, butyryl and carboxyl groups. Alkali fusion of the compound led to two products which had empirical formulas of $\text{C}_{12}\text{H}_{14}\text{O}_4$ (IIIa) and $\text{C}_8\text{H}_8\text{O}_3$ (IIIb), determined by high-resolution mass spectral analyses. As for the former, a possible structure could be IIIa in which the methoxyl in III is converted to a hydroxyl function, as indicated by disappearance of the methoxyl signal in the ^1H -NMR spectrum and a newly occurring absorption of a hydroxyl at 3360 cm^{-1} in the IR spectrum.³⁾ Subsequently, IIIa was reduced with sodium borohydride to give a compound (IIIc) which was found to have a 1-hydroxybutyl and a carboxyl from the spectral data. This implied that the reduction of IIIb did not

TABLE I. ^{13}C -NMR SPECTRUM OF PYRENOCHAETIC ACID C (in CDCl_3 , ppm from TMS)

Carbon atom	Chemical shift	Multiplicity
1	135.6	s
2	109.4	d
3	156.1	s
4	130.3	s
5	136.5	s
6	125.4	d
7	207.2	s
8	46.2	t
9	16.5	t
10	13.3	q
11	55.5	q
12	18.5	q
13	171.7	s

form the lactone and strongly suggested that the parent compound (III) bore a carboxy at either the *meta* or *para*-position for the butyryl group on the benzene ring. On the other hand, the latter (IIIb) was easily identified as 3-hydroxy-5-methylbenzoic acid by comparison of the IR spectrum with the published one.⁴⁾ From these results the structure of pyrenochaetic acid C was deduced to be 4-butyryl-3-methoxy-5-methylbenzoic acid (III). The ^{13}C -NMR spectrum supported the structure (III) with assignments of the signals as shown in Table I.

Pyrenochaetic acid A (I)

The molecular formula, $\text{C}_{13}\text{H}_{14}\text{O}_4$, obtained from high resolution mass spectral analysis corresponded to that of a dehydro-derivative of pyrenochaetic acid C (III).

Comparison of the $^1\text{H-NMR}$ data of compound (I) with those of III led us to suspect that this compound was an analog in which the butyroyl group in III was substituted with a crotonoyl group. Signals assigned to the crotonoyl appeared at δ 1.88 (3H, d, $J=5.4$ Hz), 6.13 (1H, d, $J=16$ Hz) and 6.35 (1H, dq, $J=5.4, 16$ Hz), and the other signals were comparable to those of compound III. The spectral data, together with the fact that hydrogenation of I afforded III, established the structure of pyrenochaetic acid A to be 4-crotonoyl-3-methoxy-5-methylbenzoic acid (I).

Pyrenochaetic acid B (II)

The molecular formula, $\text{C}_{13}\text{H}_{16}\text{O}_5$, corresponded to that of a hydrate of pyrenochaetic acid A (I). In the $^1\text{H-NMR}$ spectrum, the

presence of a 3-hydroxybutyroyl group was indicated by the signals appearing at δ 1.24 (3H, d, $J=6$ Hz), 2.90 (2H, m) and 4.4 (1H, m), and the other signals were comparable to those of compound III. Treatment of compound III with Ac_2O in pyridine gave pyrenochaetic acid A (I). All the data indicated the structure of pyrenochaetic acid B to be 4-(3-hydroxybutyroyl)-3-methoxy-5-methylbenzoic acid (II).

Effects on plant growth

Phytotoxic activities were investigated by the growth test using onion and rice seedlings and by the germination test using lettuce seed. The phytotoxins affected the roots more than the shoots in terms of plant growth regulation. In the growth test on rice seedlings, as shown in Table II, pyrenochaetic acid A (I)

TABLE II. EFFECTS OF PYRENOCHAETIC ACID A AND C ON THE GROWTH OF RICE SEEDLINGS

Compounds	Part of rice	Concentration (ppm)					
		0	10	50	100	250	500
Pyrenochaetic acid A	Shoot	100	91.4	98.6	102.0	76.6	74.4
	Root	100	100.8	100	74.5	39.7	0
Pyrenochaetic acid C	Shoot	100	98.3	100	102.3	80.7	73.0
	Root	100	124.5	101.3	86.8	56.7	46.0

Control (zero concentration) = 100.

TABLE III. EFFECTS OF PYRENOCHAETIC ACIDS A, B AND C ON THE GROWTH OF HYPOCOTYL AND ROOT OF LETTUCE SEEDLINGS^a

Compounds	Part of lettuce	Concentration (ppm)					
		0	10	50	100	250	500
Pyrenochaetic acid A	Hypocotyl	100	102	100	87.6	75.7	0
	Root	100	120.1	132.4	120.8	38.3	0
Pyrenochaetic acid B	Hypocotyl	100	107.4	91.5	93.6	89.4	85.2
	Root	100	100.5	114.4	107.5	189.1	211.9
Pyrenochaetic acid C	Hypocotyl	100	90.9	74.8	74.8	0	0
	Root	100	114.1	143.2	191.7	0	0

^a Using ten seeds, the length was measured five days after incubation and expressed relative to the control (zero concentration).

inhibited the root growth of onion seedlings 100% at 250 ppm, and inhibited that of rice seedlings 100% at 500 ppm. Pyrenochaetic acid C (**III**) inhibited the root growth of onion seedlings 100% at 500 ppm, and inhibited that of rice seedlings 54% at 500 ppm, and was less active biologically than pyrenochaetic acid A (**I**). Pyrenochaetic acid C (**III**) had a slight inhibitory effect on the growth of both seedlings.

In the germination test on lettuce seed, as shown in Table III, the phytotoxic compounds exhibited equal inhibitory actions at high concentrations, but promoted root elongation at low concentrations. Compounds **I** and **III** stimulated likewise the root elongation at concentrations of not more than 100 ppm, whereas compound **II** stimulated it at concentrations from 100 to 500 ppm, the roots lengthening two times relative to the control at 500 ppm. These stimulating effects like those observed for phytotoxins such as epoxydon,⁵⁾ phyllostine,⁶⁾ and tenuazoic acid,⁷⁾ are of interest in view of plant growth regulating activity.

EXPERIMENTAL

Mps were measured with a Yanako micro-melting point apparatus and are uncorrected. UV and IR spectra were taken on a Hitachi EPS-3T spectrophotometer and a Hitachi 285 spectrometer. ¹H-NMR and ¹³C-NMR spectra were recorded on a Hitachi R22 spectrometer and a JNM FX-100 spectrometer. Mass spectra were obtained with a JEOL TMS-D300 and a JEOL-JM-01SG-2 spectrometer. Column chromatography was performed with Kiesel gel 60 (230 mesh, Merck) and thin layer chromatography with pre-coated Kiesel gel 60 plates (0.25 mm, Merck). Compounds were visualized by spraying with anisaldehyde reagent.

Bioassay. EtOAc solution (1.5 ml) containing a sample at a definite concentration (10, 50, 100, 250 and 500 ppm) was poured on a piece of filter paper (7 cm diameter) in a Petri dish (9 cm diameter). After removal of the solvent, 3 ml of a Tween-80 solution (100 ppm) was added to the dish. Lettuce seeds were placed on the filter paper and germinated in the dark at 23°C for 5 days, while rice or onion seedlings of uniform shape and size were grown under the above conditions.

Isolation of phytotoxic metabolites. Pyrenochaeta ter-

restris was grown in 500 ml flasks containing 200 ml of a corn-extract medium at 23°C for 30 days. The corn-extract was prepared from 50 g of sweet corn (canned or frozen) by blending with water in a mixer, and subsequently filtered and made up to 1 liter with water. The culture filtrates (32 liters) were concentrated to an appropriate amount and then shaken with ether. After drying with Na₂SO₄, the ether layer was evaporated to yield 4.1 g of an oily residue. The residue dissolved in a small amount of benzene was applied on the top of a silica gel column (60 g, 3 × 23 cm) and eluted with 10 g fractions of benzene with increasing proportions of EtOAc. Individual active materials were present in the fractions: Nos. 25 ~ 28 (**III**), 29 ~ 42 (pyrenocine A), 71 ~ 130 (**I**), 131 ~ 230 (pyrenocine B) and 391 ~ 463 (**II**).

Pyrenochaetic acid C (III). The evaporated residues from fraction Nos. 25 ~ 28 crystallized from CHCl₃-*n*-hexane to give 31 mg of plates (**III**), mp, 148 ~ 152°C; TLC in benzene-EtOAc (1:1 v/v), *R_f* 0.39; Found: M⁺, *m/z*: 236.1047; Calcd for C₁₃H₁₆O₄: 236.1047. MS *m/z*: 236 (M⁺), 193 (base peak, M⁺ - C₃H₇); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 2950, 1690, 1570, 880, 765; UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ): 249 (8500), 303 (3100); ¹H-NMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$: 1.04 (3H, t, *J* = 7 Hz), 1.81 (2H, sextet, *J* = 7 Hz), 2.35 (3H, s), 2.81 (2H, t, *J* = 7 Hz), 3.94 (3H, s), 7.44 (1H, br. s), 7.56 (1H, br. s), 9.70 (1H, br. s).

Methyl ester of pyrenochaetic acid C (IV). Pyrenochaetic acid C (3 mg) was treated with ethereal diazomethane at room temperature for 10 min, and water was added to the reaction mixture which was then evaporated. The resulting residue was dissolved in CHCl₃ and filtered. On evaporation, the CHCl₃ solution yielded 3 mg of solids, mp, 53 ~ 55°C; Found: M⁺, *m/z*: 250.1237; Calcd for C₁₄H₁₈O₄: 250.1206. MS *m/z*: 250 (M⁺), 207 (base peak, M⁺ - C₃H₇); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 2950, 1720, 1685, 1565, 1235, 890, 760; ¹H-NMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$: 1.10 (3H, t, *J* = 7 Hz), 1.83 (2H, sextet, *J* = 7 Hz), 2.34 (3H, s), 2.72 (2H, t, *J* = 7 Hz), 3.94 (3H, s), 4.01 (3H, s), 7.50 (1H, br. s), 7.62 (1H, br. s).

Alkali fusion of pyrenochaetic acid C (III). Pyrenochaetic acid C (25 mg) in 50% KOH (1 ml) was heated for 30 min up to a temperature of 240°C. The solution was cooled, treated with H₂O, acidified with 20% hydrochloric acid and successively extracted with CHCl₃ and EtOAc. The CHCl₃ extract was worked up to isolate 5 mg of compound **IIIa** by column chromatography using CHCl₃-MeOH (95:5 v/v). Mp of **IIIa**, 131 ~ 134°C; Found: M⁺, *m/z*: 222.0907; Calcd for C₁₂H₁₄O₄: 222.0892; MS *m/z*: 222 (M⁺), 179 (base peak, M⁺ - C₃H₇); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3360, 2950, 1690, 1665, 1575, 880, 760; ¹H-NMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$: 1.03 (3H, t, *J* = 7 Hz), 1.85 (2H, sextet, *J* = 7 Hz), 2.67 (3H, s), 3.00 (2H, t, *J* = 7 Hz), 7.46 (1H, br. s), 7.52 (1H, br. s).

On the other hand, the EtOAc extract was evaporated, and the resultant residue was chromatographed on a silica

gel column with CHCl_3 -MeOH (9:1 v/v) to yield **IIIb** (1.5 mg), mp. 198~200°C (sublimed), lit.³⁾ 210°C (sublimed); Found: M^+ , m/z 152.0468; Calcd for $\text{C}_8\text{H}_8\text{O}_3$: 152.0463. MS m/z : 152 (M^+), 135 ($\text{M}^+ - \text{OH}$), 107 ($\text{M}^+ - \text{COOH}$), 78 (base peak); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3275, 3100, 1700, 1600, 880 (lit.³⁾); $^1\text{H-NMR}$ $\delta_{\text{TMS}}^{\text{CDCl}_3}$: 2.31 (3H, s), 6.62 (1H, br. s), 7.19 (1H, br. s), 7.28 (1H, brs. s).

Reduction of IIIa to IIIc with sodium borohydride. Compound **IIIa** (5 mg) in MeOH was treated with NaBH_4 (10 mg) at room temperature for 10 min. The solution was acidified and extracted with CHCl_3 . The solvent was evaporated and the resulting residue was chromatographed on a silica gel column using CHCl_3 -MeOH (9:1 v/v) to afford a colorless plate of **IIIc** (1 mg), mp. 166~167°C; Found: M^+ , m/z 224.1052; Calcd for $\text{C}_{12}\text{H}_{16}\text{O}_4$: 224.1049; MS m/z : 224 (M^+) 206 ($\text{M}^+ - \text{H}_2\text{O}$), 181 (base peak, $\text{M}^+ - \text{C}_3\text{H}_6\text{OH}$), 165; IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400, 2950, 1690, 1575, 890, 770.

Pyrenochaetic acid A (I). Evaporation of fraction Nos. 71~130 gave an active material (**I**) which crystallized from CHCl_3 -*n*-hexane as needles (22 mg), mp. 176~179°C; TLC in benzene-EtOAc (1:1 v/v), R_f 0.35; Found: M^+ , m/z 234.0894; Calcd for $\text{C}_{13}\text{H}_{14}\text{O}_4$: 234.0892. MS m/z : 234 (M^+), 219 (base peak, $\text{M}^+ - \text{CH}_3$), 205 ($\text{M}^+ - \text{C}_2\text{H}_5$), 193 ($\text{M}^+ - \text{C}_3\text{H}_5$), 175, 160, 77; UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ): 240 sh (8900), 303 (1700); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 2900, 1680, 1657, 1580, 880, 765; $^1\text{H-NMR}$ $\delta_{\text{TMS}}^{\text{CDCl}_3}$: 1.88 (3H, d, $J=5.4$ Hz), 2.18 (3H, s), 3.73 (3H, s), 6.13 (1H, d, $J=16$ Hz), 6.35 (1H, dq, $J=5.4, 16$ Hz), 7.45 (1H, br. s), 7.57 (1H, br. s), 9.08 (1H, br. s).

Hydrogenation of Pyrenochaetic acid A (I) to Pyrenochaetic acid C (III). Pyrenochaetic acid A (2 mg) in 70% EtOH (7 ml) was stirred under a hydrogen atmosphere over PtO_2 (20 mg) for 2 hr. After filtration, the solution was concentrated and the product was purified by preparative TLC using benzene-EtOAc (1:1 v/v). The R_f value of the product was 0.39 which coincided with that of pyrenochaetic acid C (**III**). Found: M^+ , m/z 236.1049; Calcd for $\text{C}_{13}\text{H}_{16}\text{O}_4$: 236.1049.

Pyrenochaetic acid B (II). The concentrates from fraction Nos. 391~463 crystallized from CHCl_3 -*n*-hexane to give needles (1.5 mg) of **II**, mp. 139°C; TLC in benzene-EtOAc (1:1 v/v), R_f 0.08; Found: M^+ , m/z 252.1030; Calcd for $\text{C}_{13}\text{H}_{16}\text{O}_5$: 252.0998; IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400, 2960, 1695, 1580, 885, 770; $^1\text{H-NMR}$ $\delta_{\text{TMS}}^{\text{CDCl}_3}$: 1.26 (3H, d, $J=6$ Hz), 2.28 (3H, s), 2.88 (1H, d, $J=4$ Hz), 2.93 (1H, s), 3.88 (3H, s), 4.42 (1H, m), 7.47 (1H, br. s), 7.56 (1H, br. s).

Conversion of pyrenochaetic acid B (II) to pyrenochaetic acid A (I). Pyrenochaetic acid B (1 mg) in a mixture of pyridine (1 ml) and Ac_2O (1 ml) was kept overnight. The reaction mixture was diluted with water, evaporated to dryness and the product recovered in a small amount of CHCl_3 . TLC revealed two spots, R_f 0.35 and 0.26, the former was identical with that of pyrenochaetic acid A (**I**).

The CHCl_3 solution without further purification was evaporated to yield the mixed product as a solid. FD-MS revealed two molecular ion peaks at m/z 234 and 294.

The former was pyrenochaetic acid A. Found: M^+ , m/z 234.0902; Calcd for $\text{C}_{13}\text{H}_{14}\text{O}_4$: 234.0892. The latter was compatible with the mono-acetate of pyrenochaetic acid B. Found: M^+ , m/z 294.1113, Calcd for $\text{C}_{15}\text{H}_{18}\text{O}_6$: 294.1103.

REFERENCES

- 1) H. Sato, K. Konoma and S. Sakamura, *Agric. Biol. Chem.*, **43**, 2409 (1979).
- 2) H. Sato, K. Konoma, S. Sakamura, A. Furusaki, T. Matsumoto and T. Matsuzaki, *Agric. Biol. Chem.*, **45**, 795 (1981).
- 3) M. S. Kharasch and M. Kleiman, *J. Am. Chem. Soc.*, **65**, 11 (1943).
- 4) M. Syoyama and Y. Miyachi, *Eisei Kagaku*, **20**, 213 (1974).
- 5) R. Sakai, R. Sato, H. Niki and S. Sakamura, *Plant & Cell Physiol.*, **11**, 907 (1970).
- 6) R. Sakai, R. Sato, J. Ito and S. Sakamura, *Ann. Phytopath. Soc. Jpn.*, **38**, 290 (1972).
- 7) S. Iwasaki, H. Muro, S. Nozoe and S. Okuda, *Tetrahedron Lett.*, **1972**, 13.