Chloromonilinic Acids A and B, Novel Catabolites of the Growth Self-inhibitor Chloromonilicin Isolated from *Monilinia fructicola*

Takeshi SASSA, Kazuo HORIGUCHI and Yoshikatsu SUZUKI*

Department of Agricultural Chemistry, Yamagata University,
Tsuruoka, Yamagata 997, Japan

*The Institute of Physical and Chemical Research,
Wako, Saitama 351, Japan

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Chloromonilinic acids A and B were newly isolated from a culture filtrate of *M. fructicola* accumulating chloromonilicin in the mycelium, and their structures were elucidated on the basis of spectroscopic evidence. The unusual catabolic route of chloromonilicin is discussed.

Chloromonilicin (**I**) has been isolated as a growth self-inhibitor from cherry rot fungus, *Monilinia fructicola*, and its novel structure with a β -chloro- $\alpha,\beta,\gamma,\delta$ -unsaturated seven-membered lactone ring has been elucidated on the basis of chemical and spectroscopic evidence.¹⁾ In the course of a biosynthetic study of **I**,²⁾ we found two strongly acidic metabolites in the culture broth of this fungus accumulating **I** in its mycelia. These metabolites containing a chlorine atom were named chloromonilinic acids A (**III**) and B (**IV**). The chlorine atom in **I** was readily eliminated as hydrogen chloride by mild alkaline methanolysis with the formation of a biologically inactive compound **II** (Fig. 1).¹⁾ On the other hand, **III** and **IV** showed a complete or almost complete lack of biological activity. These observations seemed to present an interesting clue to investigate the catabolic mechanism and structure-activity relationship of **I**. The present paper reports the isolation and structural de-

termination of **III** and **IV**. The fungal transformation of bromomonilicin (**V**, a homologue of **I**) to bromomonilinic acids A (**VI**) and B (**VII**), that was in parallel with that of **I**, was also examined in this fungus.

M. fructicola was grown in a culture medium containing 2% commercial sugar and 2% malt extract in 500-ml Sakaguchi flasks at 24°C for 14 days on a reciprocal shaker. Combined EtOAc extracts of the broth filtrate (initially pH 4.2 and then adjusted to pH 3) were chromatographed by a stepwise elution over silica gel with an *n*-hexane-EtOAc mixture containing 1% AcOH to give the strongly acidic fractions 5 (*n*-hexane-EtOAc=3:1) and 8 (*n*-hexane-EtOAc=3:2~2:3). Rechromatography of fraction 8 over silica gel with 18:1 CHCl₃-2-propanol containing 1% AcOH and its subsequent purification by preparative silica gel TLC, developing with EtOAc containing 0.5% HCOOH, afforded **III** as pale yellow needles; mp 181.5~182°C. Frac-

Fig. 1. Structures of Chloromonilicin (**I**) and Bromomonilicin (**V**), and of their Methanolysis Products (**II** and **VIII**).

Fig. 2. Catabolic Route of Halomonilicins (**I** and **V**) to Halomonilinic Acids **A** (**III** and **VI**) and **B** (**IV** and **VII**), and Structures of Chloromonilinic Acids **A** (**III**) and **B** (**IV**), and of Bromomonilinic Acids **A** (**VI**) and **B** (**VII**).

tion **5** was purified by preparative silica gel TLC, developing with 2:1 benzene–acetone containing 0.5% HCOOH, to afford **IV** as pale yellow prisms; mp 171.5–172.5°C.

III gave a pale violet color with alcoholic ferric chloride. Its molecular formula, $C_{16}H_{13}O_8Cl$, was determined by high-resolution mass spectrometry. The NMR spectrum of **III** showed the presence of a hydrogen-bonded phenolic hydroxyl (12.5 ppm) and a methoxy group (3.65 ppm). **III** gave a methyl ester on treatment with MeOH–conc. H_2SO_4 . It showed a strong IR absorption at 1731 cm^{-1} and two NMR signals of a three-proton singlet at 3.70 and 3.63 ppm, indicating the presence of two carbomethoxy groups in **III** methyl ester. The methyl ester gave a diacetate having no hydroxyl IR absorptions. Its NMR spectrum showed a two-proton singlet with an acetylation shift at 5.16 ppm, together with two acetoxyl signals at 2.39 and 2.11 ppm. These observations indicated that **III** possessed a carboxyl and a carbomethoxy group, as well as a hydrogen-bonded phenolic hydroxyl and a hydroxymethyl group in the molecule.

The NMR spectra of **III** revealed clearly the presence of a pair of *meta*-coupled protons (7.07 and 6.87 ppm, $J=1.5\text{ Hz}$) on a benzene nucleus and a hydroxymethyl group (4.75 ppm) allylically coupled to each of the *meta*-coupled protons. The *meta*-coupled protons were shifted downfield (7.35 and 7.00 ppm) in the NMR spectrum of the diacetate of **III** methyl ester. These NMR data suggested the presence of a 2-acyl-3-alkoxy-5-(hydroxymethyl)-phenol moiety in **III**.³⁾

A careful comparison of the characteristic UV spectrum of **III** with those of the de-

rivatives of **I** obtained so far reveals that **III** contained a UV chromophore identical with that of **VIII**, a lactone-ring cleaved intermediate from the alkaline methanolysis of **I** (Fig. 1).¹⁾ The ^{13}C -NMR spectra (COM, INEPT, NOE and LSPD) of **III** methyl ester confirmed the structure of this UV chromophore, and further extended it to a 3-(carbomethoxymethyl)chromone substituted at 2 by a 2-carbomethoxy-1-chlorovinyl group. The structure of **III** methyl ester was justified by comparing the ^{13}C -NMR chemical shifts with those of **I**; 181.5 (C-4), 169.7 (C-1), 162.2 (C-14), 160.3 (C-6), 157.5 (C-11), 156.2 (C-10), 150.8 (C-8), 137.4 (C-12), 126.2 (C-13), 115.0 (C-3), 109.0 (C-5), 108.8 (C-9), 104.3 (C-7), 63.9 (CH_2OH), 52.3 (OCH_3), 52.1 (OCH_3) and 29.9 ppm (C-2). The ^{13}C - ^1H long-range selective proton decoupling (LSPD) analysis using an improved method of SPT difference spectrum⁴⁾ clearly indicated heteronuclear long-range couplings between the methylene protons at C-2 (3.48 ppm) and C-4 (181.5), C-1 (169.7), C-11 (157.5) and C-3 (115.0 ppm), and between the olefinic proton at C-13 (6.57 ppm) and C-11 (157.5) and C-12 (137.4 ppm).⁵⁾ Finally the ester function of C-1 in **III** was revealed by LSPD experiments and its SPT difference spectrometry on **III** in dioxane- d_8 ; long-range couplings were observed between the methoxyl group (3.61 ppm) and C-1 (170.9 ppm), and between the methylene protons at C-2 (3.46 ppm) and C-4 (183.2), C-1 (170.9), C-11 (159.2) and C-3 (117.1 ppm). Therefore, the structure of **III** was elucidated to be that shown in **III** (Fig. 2). The close similarity of the ^1H - and ^{13}C -NMR data for the C-11~C-14 moiety in **III** methyl ester to those in **I** suggested that the double bond of **III**

had the same *E* configuration as that of **I**.

IV gave the same color as that of **III** with alcoholic ferric chloride. The UV spectrum of **IV** was superimposable on that of **III**, and its IR spectrum was similar to that of **III**. The NMR spectrum of **IV** also showed many signals corresponding to those of **III**, a three-proton singlet at 2.42 ppm instead of the two-proton singlet at 4.75 ppm in **III**. These observations suggested that **IV** was a deoxy derivative of **III**. This was confirmed from the ^{13}C -NMR data for **IV**; 183.1 (C-4), 171.0 (C-1), 164.4 (C-14), 162.0 (C-6), 159.1 (C-11), 157.8 (C-10), 149.6 (C-8), 138.0 (C-12), 129.4 (C-13), 117.0 (C-3), 113.7 (C-9), 109.7 (C-5), 108.8 (C-7), 53.0 (OCH_3), 31.3 (C-2) and 23.0 ppm (CCH_3). The very weak peak of the fragment ion $\text{M}^+ - \text{COOCH}_3$ [m/z 295 and 293 (chlorine-containing ions)] in the mass spectrum of **IV** suggested that the ester function was located at C-1 in analogy with **III**. Thus, the structure of **IV** was assigned to be that shown in **IV** (Fig. 2), which is considered as

being a biosynthetic precursor of **III**.

The catabolic conversion of bromomonilicin (**V**, a homologue of **I**) produced⁶⁾ in the presence of the bromine ion was also investigated by feeding **V** and examining the products by mass analysis. **V** was fed to the fermentation broth 7 days after inoculation. After an additional 3 or 5 days, the broth filtrate (initially pH 4.3 and then adjusted to pH 3) was extracted with EtOAc. The fractions of **III** and/or **IV** obtained from the extract by preparative silica gel TLC were subjected to mass analyses. The molecular ions of the bromine-containing catabolites **VI** and **VII** (Fig. 2) were clearly detected in the mass spectra of the fractions corresponding to **III** and **IV**, respectively (Fig. 3). In addition, the incorporation of deuterated moniliphenone,^{1,7)} a biosynthetic intermediate of **I**, in **IV** was observed by mass spectrometry.

III showed no inhibitory activity at less than 500 $\mu\text{g}/\text{disc}$ in a growth self-inhibiting assay,⁸⁾ while the growth self-inhibiting activity of **IV** (MID *ca.* 250 $\mu\text{g}/\text{disc}$) was about one-hundredth of that of **I**. **III** also showed no antifungal activity against several fungi by the agar dilution method.

The combined results indicate that, for both **I** and **V**, the fungus reductively cleaved the 7-membered lactone ring to give **IV** (or **VII**), and that the product was oxidized at the methyl group on the benzene ring to give **III** (or **VI**). The catabolic route of **I** to **IV** was not the usual hydrolytic cleavage of the lactone ring, but an unusual reductive cleavage. This biodegradation makes an interesting contrast with the ease of chemical dehydrochlorination by mild alkaline methanolysis and, further, is considered to be significant as one of the catabolic mechanisms for biologically active lactones.

Experimental

Melting points (mp) are uncorrected. The following spectroscopic instruments were used: MS, ZAB-SE (HR-MS) and JEOL JMS-Q10A; UV, Hitachi 124; IR, Jasco UVIDEC-510; ^1H -NMR, Hitachi R-24 (60 MHz) and JEOL FX-90Q (90 MHz); ^{13}C -NMR, JEOL FX-90Q (22.5 MHz). BW-820 MH and CQ-3 silica gel (Fuji

Fig. 3. MS Data for the Fractions of Chloromonilinic Acids A (**III**) and B (**IV**) Obtained by the Feeding Experiments.

(a): The fraction of **III** from the additional 5-day incubation.

(b): The fraction of **IV** from the additional 3-day incubation (**III** was not found in the culture broth by TLC).

Division Chemical Company) were used for column chromatography, and 60 F₂₅₄S silica gel TLC plates (Merck, 0.5 mm thickness) for preparative TLC.

Isolation of chloromonilinic acids A (III) and B (IV). The spore suspension of *M. fructicola* was inoculated into a 100 ml culture medium containing 2% commercial sugar and 2% malt extract in a 500-ml Sakaguchi flask, before the flasks were incubated at 24°C for 14 days on a reciprocal shaker. The broth filtrate obtained (5 l; initially pH 4.2 and then adjusted to pH 3) was saturated with NaCl and then extracted 3 times with EtOAc. The combined EtOAc extracts (1.43 g) were chromatographed by a stepwise elution over BW-820MH silica gel with an *n*-hexane-EtOAc mixture containing 1% AcOH to give the strongly acidic fractions 5 (*n*-hexane-EtOAc = 3:1; 67 mg) and 8 (*n*-hexane-EtOAc = 3:2~2:3; 330 mg). Rechromatography of fraction 8 over CQ-3 silica gel with 18:1 CHCl₃-2-propanol containing 1% AcOH and its subsequent purification by preparative silica gel TLC, developing with EtOAc containing 0.5% HCOOH, afforded **III** (155 mg); its recrystallization with EtOAc-benzene gave pale yellow needles, mp 181.5~182°. Fraction 5 was purified by preparative silica gel TLC, developing with 2:1 benzene-acetone containing 0.5% HCOOH, to afford **IV** (39 mg); its recrystallization with acetone-benzene gave pale yellow needles, mp 171.5~172.5°C. Chloromonilicin (63 mg) was also isolated from the EtOAc extracts just mentioned, and 307 mg, from the mycelial extracts.

III. EI-MS m/z : 368 (M^+), 337, 323, 309 and 292 (^{35}Cl -containing ions), 333, 263, 257, 229, 166 and 121; HR-EIMS: m/z 368.0299380 (M^+ , calcd. for C₁₆H₁₃O₇Cl: 368.0298957); UV λ_{max} (MeOH) nm (ϵ): 331 (3,900), 260 (sh. 16,000) and 244 (18,000); IR ν_{max} (KBr) cm⁻¹: 3420, 2700, 2590, 1718 (br.), 1652, 1635, 1603, 1437, 1327, 1163, 1061, 946, 840, 745 and 660; $^1\text{H-NMR}$ δ (acetone-*d*₆): 12.5 (1H, OH), 7.07 (1H, m), 6.87 (1H, m), 6.78 (1H, s), 4.75 (2H, br. s), 3.65 (3H, s) and 3.58 (2H, s); $^{13}\text{C-NMR}$ δ (dioxane-*d*₈): 183.1 (s), 170.9 (s), 164.3 (s), 162.2 (s), 159.2 (s), 158.0 (s), 154.1 (s), 138.1 (s), 129.3 (d), 117.1 (s), 110.5 (s), 110.1 (d), 105.5 (d), 64.8 (t), 53.0 (q) and 31.3 (t). **IV.** EI-MS m/z : 352, 321, 307, 293 and 276 (^{35}Cl -containing ions), 317, 248, 213, 185, 151 and 123; UV λ_{max} (MeOH) nm (ϵ): 331 (3,900), 260 (sh. 16,000) and 244 (18,000); IR ν_{max} (KBr) cm⁻¹: 2800~2400 (br.), 1738, 1703, 1657, 1601, 1435, 1344, 1291, 1163, 1083, 1000, 842 and 733; $^1\text{H-NMR}$ δ (acetone-*d*₆): 12.6~12.1 and 8.6~8.0 (each 1H, br. OH), 6.86 (1H, m), 6.75 (1H, s), 6.70 (1H, m), 3.63 (3H, s), 3.50 (2H, s) and 2.42 (3H, s); $^{13}\text{C-NMR}$ δ (dioxane-*d*₈): see the text.

Chloromonilinic acid A methyl ester (III methyl ester). **III** was methylated with MeOH in the presence of a catalytic amount of conc. H₂SO₄ at 55°C for 3 hr. The **III** methyl ester obtained was purified by preparative silica gel TLC, developing with 18:1 CHCl₃-2-propanol, as a viscous oily substance. EI-MS m/z : 382 (M^+ , ^{35}Cl -

containing ion) the fragment ion of $M^+ - \text{COOCH}_3$ (m/z 325 and 323) being observed as one of the most abundant peaks; UV λ_{max} (MeOH) nm (ϵ): 331 (3,700), 261 (sh. 16,000) and 242 (20,000); IR ν_{max} (CHCl₃) cm⁻¹: 3600, 3600~3200 (br.), 1731 (br.), 1660 and 1611; $^1\text{H-NMR}$ δ (CDCl₃): 12.1 (1H, OH), 6.93 (1H, m), 6.76 (1H, m), 6.57 (1H, s), 4.69 (2H, s), 3.70 (3H, s), 3.63 (3H, s), 3.48 (2H, s) and 2.6 (1H, br. OH); $^{13}\text{C-NMR}$ δ (CDCl₃): see the text.

Diacetate of chloromonilinic acid A methyl ester (diacetate of III methyl ester). **III** methyl ester was acetylated with acetic anhydride in pyridine at 25°C overnight. The diacetate of **III** methyl ester was purified by preparative silica gel TLC, developing with 7:1 benzene-acetone, and obtained as crystals having mp 148~148.5°C (benzene). EI-MS m/z : 466 (M^+) and 424 (^{35}Cl -containing ions); UV λ_{max} (MeOH) nm (ϵ): 307 (7,700) and 230 (27,000); IR ν_{max} (CHCl₃) cm⁻¹: 1781, 1740 (br.), 1724 and 1646; $^1\text{H-NMR}$ δ (CDCl₃): 7.35 (1H, m), 7.00 (1H, m), 6.53 (1H, s), 5.16 (2H, s), 3.63 (6H, s), 3.43 (3H, s), 2.39 (3H, s) and 2.11 (3H, s).

Fungal transformation of bromomonilicin (V) to bromomonilinic acids A (VI) and B (VII). The spore suspension of *M. fructicola* was inoculated into a 100 ml culture medium containing 2% commercial sugar and 1% malt extract in a 500-ml Sakaguchi flask, before the flask was incubated at 24°C for 7 days on a reciprocal shaker. **V** (2 mg in 0.04 ml of DMSO/0.1 ml of EtOH) was then fed to the fermentation broth. After an additional 3 or 5 days, the broth was filtered, and the filtrate (initially pH 4.3 and then adjusted to pH 3) was extracted with EtOAc. The EtOAc extract obtained was subjected to preparative silica gel TLC, developing with 9:2 CHCl₃-2-propanol containing 1% AcOH for the purification of **III** (and **VI**) and with 5:1 benzene-acetone containing 1% AcOH for **IV** (and **VII**). **III** (and **VI**) and **IV** (and **VII**) were analyzed by mass spectrometry, these MS data being shown in Fig. 3.

Incorporation of moniliphenone-*d*₅ into chloromonilinic acid B (IV). Feeding experiments with moniliphenone-*d*₅ were carried out in a similar manner to those of bromomonilicin (**V**). **IV** (and deuterated **IV**) were extracted from the broth filtrate obtained from the 3-day incubation and purified by preparative silica gel TLC. The presence of deuterated **IVs**, i.e., **IV-*d*₅** and **IV-*d*₄**,⁷⁾ was confirmed by EI-MS data for purified **IV** (and deuterated **IV**); m/z 352 [M^+ of **IV-³⁵Cl**, 100 (rel. intensity)], 353 (17), 354 (34), 355 (8), 356 ($M+4$, 8), 357 ($M+5$, 14), 358 (6), 359 (6) and 360 (2). EI-MS data for an authentic specimen of **IV**: m/z 352 [M^+ of **IV-³⁵Cl**, 100 (rel. intensity)], 353 (20), 354 (39), 355 (7) and 356 (1).

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