Isolation and Structure of Pestalotin, a Gibberellin Synergist from *Pestalotia cryptomeriaeicola*

By Yasuo KIMURA and Saburo TAMURA

Department of Agricultural Chemistry, The University
of Tokyo, Bunkyo-ku, Tokyo

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Pestalotin, a gibberellin synergist, was isolated from culture broth of a fungal strain, *Pestalotia cryptomeriaeicola*. The structure of the compound has been established as I on the basis of chemical and spectral data.

During the course of our screening search for plant growth regulators with novel activity among microbial metabolites, we noticed the presence of a gibberellin synergist in culture broth of *Pestalotia cryptomeriaeicola* Sawada, which is a fungus pathogenic for the Japanese cedar, *Cryptomeria japonica* (Linn. fil.) D. Don. Subsequently, we succeeded in isolating the active principle named pestalotin (I) from the broth and preliminarily reported its biological activity¹⁾ as well as its structure.²⁾ In this paper we wish to present in detail our experimental results leading to the structure elucidation.

The fungus was grown in shake culture on Czapek-Dox medium for 7 days at 27°C. Then, the cultured broth was treated by the procedure illustrated in Fig. 1. Acetone was added to the broth, and the mixture was filtered to remove mycelia. The filtrate was evaporated to a small volume and was extracted with ethyl acetate at pH 3. The

FIG. 1. Isolation Procedure of Pestalotin (I).

extract was shaken successively with dilute sodium bicarbonate solution and water and dried over anhydrous sodium sulfate. After evaporation of the solvent, the neutral residue was chromatographed on a silicic acid column, which was eluted with benzene-ethyl acetate

increasing stepwise the amount of ethyl acetate. Biological activity was detected in the fraction eluted with benzene containing 30% ethyl acetate. After evaporation of the solvent, the residue was subjected to thin-layer chromatography (TLC) by use of ethyl acetate-benzene (1:1, v/v) to give crude crystals of I. Recrystallization from *n*-hexane-benzene afforded colorless crystals melting at 88.0~88.5°C.

The molecular formula was established as $C_{11}H_{18}O_4$ through elemental analysis and mass spectrometry (M^+ 214.1210; Calcd. 214.1205). The IR spectrum of I (Fig. 2) revealed ab-

paying the disappearance of the bands at 3480 and 1090 cm^{-1} observed in I.

In the NMR spectrum of I (Fig. 4), a broad signal at δ 2.22 equivalent to one proton disappeared by the addition of D_2O , and a one-proton multiplet at δ 3.62 was deshielded to δ 5.08 (1H, m) on acetylation. These facts indicate the presence of a secondary hydroxyl group in I, and the above-mentioned one-proton multiplet at δ 3.62 is ascribable to a hydrogen attached to a carbon atom which bears the hydroxyl. A three-proton singlet at δ 3.75 is attributed to a methoxyl group, and a one-proton doublet at δ 5.14 ($J=2.0$

FIG. 2. IR Spectrum of Pestalotin (I) (Nujol).

sorption bands at 3480, 1090 (OH), 1710 ($C=O$), 1635 ($C=C$), 1255 and 1025 ($C=C-O-$) cm^{-1} . The UV spectrum (Fig. 3) showed an absorption maximum at 233 nm ($\epsilon=12,200$ in EtOH). On treatment with acetic anhydride-pyridine, I gave a monoacetate with IR absorption bands at 1745 and 1240 cm^{-1} characteristic of the ester function, accom-

FIG. 3. UV Spectrum of Pestalotin (I) (in EtOH).
— neutral or HCl; ---- NaOH

FIG. 4. NMR Spectrum of Pestalotin (I) (in $CDCl_3 + D_2O$).

Hz) to a trisubstituted double bond. Based on the chemical shift, a one-proton sextet at δ 4.28 ($J=4.0, 4.0, 12.7$ Hz) has been as-

FIG. 5. Mass Spectrum of Pestalotin (I).

signed to a hydrogen located on a carbon atom bearing an oxygen. Irradiation of the signal under consideration revealed couplings with signals at δ 2.25 ($J=4.0, 17.0$ Hz), δ 2.79 ($J=2.0, 12.7, 17.0$ Hz) and δ 3.62 mentioned earlier. The large coupling constant $J=17.0$ Hz indicates geminal relationship of methylene protons. Accordingly, the presence of a partial structure $-\text{CH}_2-\text{CH}-\text{CH}(\text{OH})-$ in the

FIG. 6. Functional Groups Constituting Pestalotin (I).

molecule of I has been proved.

In the mass spectrum of I (Fig. 5), M^+-18 ion peak is observed at m/e 196. Furthermore, fragment ions corresponding to $\text{M}^+-(\text{H}_2\text{O}+\text{CH}_3)$, $\text{M}^+-(\text{H}_2\text{O}+\text{CH}_3+\text{CH}_2)$, $\text{M}^+-(\text{CH}_3+\text{CH}_2+\text{CH}_2+\text{CH}_2)$, $\text{M}^+-(\text{H}_2\text{O}+\text{CH}_3+\text{CH}_2+\text{CH}_2)$ and $\text{M}^+-(\text{H}_2\text{O}+\text{CH}_3+\text{CH}_2+\text{CH}_2+\text{CH}_2)$ were observed at m/e 181, 167, 157, 153 and 139. Besides these mass spectral data, NMR signals at δ 0.91 (3H, t, $J=6.3$ Hz) and δ 1.30~1.60 (6H, broad) indicate the presence of a *n*-butyl group. Functions of all the carbon, hydrogen and oxygen atoms constituting I have been fully established as shown in Fig. 6.

Treatment of I with 2,4-dinitrophenylhydrazine revealed no coloration. High inten-

sity of the UV absorption at 233 nm ($\epsilon=12,200$) as well as appearance of IR bands at 1710 and 1635 cm^{-1} suggests the presence of an α,β -unsaturated lactone.³⁾ Pattern of the UV spectrum is in good accordance with those of 4-methoxy-5,6-dihydro- α -pyrone derivatives hitherto reported.^{4,5)} Thus, the structure of pestalotin has been assigned as 6-(1'-hydroxypent-1'-yl)-4-methoxy-5,6-dihydro- α -pyrone (I).

Validity of the structural assignment was further confirmed by a total synthesis as shown in Fig. 7. Ethyl acetoacetate was condensed with trimethyl orthoformate in the presence of conc. sulfuric acid at room temperature to give ethyl β -methoxycrotonate (II).⁶⁾ Treat-

FIG. 7. Synthetic Procedure of Pestalotin (I).

ment of II with N-bromosuccinimide in refluxing carbon tetrachloride yielded γ -bromo- β -methoxycrotonate (III).⁷⁾ On the other side, *n*-butylmagnesium bromide was reacted with acrolein to afford 1-helpten-3-ol (IV), which was then subjected to ozonolysis to yield 2-hydroxyhexanal (Va). The hydroxyaldehyde was converted into the corresponding pyranyl ether (Vb), and Reformatsky reaction of the ether with III was carried out. The crude product was purified by TLC to give I in a very poor yield. However, comparison of chromatographic behavior and UV and mass spectra showed a complete identity of the natural and synthetic pestalotins.

The CD and ORD curves of natural I possessing two asymmetric carbons is shown in Fig. 8. The negative Cotton effect at 246 nm (J_ϵ 12.5 in EtOH) in the CD curve suggests that the absolute configuration at C-6 is *s*. However, the stereochemistry of another asymmetric carbon C-1' remained unsettled.

FIG. 8. CD and ORD Curves of Pestalotin (I) (in EtOH).

— CD; --- ORD

EXPERIMENTAL

IR spectra were obtained with a JASCO IR-S spectrometer. NMR spectra were recorded on a JEOL-JNM-4H spectrometer at 100 Mc in CDCl_3 solutions. Chemical shifts are expressed in δ values

(ppm) with tetramethylsilane as an internal standard. The symbols 's', 'd', 't', 'q' and 'm' are used to refer to singlet, doublet, triplet, quartet and multiplet, respectively. Mass spectra were measured with a Hitachi RMU-6 at 70 eV, and high resolution mass spectra were obtained by a Hitachi RMH-2 with a direct inlet system at 70 eV. CD and ORD curves were recorded on a JASCO ORD/UV-5 spectrometer by use of ethanol as a solvent.

Isolation of pestalotin. A strain of *Pestalotia cryptomeriacecola* on an agar slant of potato-sucrose medium was transferred into a 500 ml-shaking flask containing 100 ml of Czapek-Dox medium and was grown in shake culture at 27°C for 2 days. Twenty ml of the content in the flask was further transferred into a 5 liters-shaking flask charged with 1.2 liters of the same medium. Cultivation was continued at 27°C for 7 days on a rotary shaker.

After addition of an equal volume of acetone, the culture broth was filtered to remove mycelia and was evaporated under reduced pressure. The residual aqueous solution was extracted repeatedly with ethyl acetate at pH 3. The combined extracts were washed successively with 5% sodium bicarbonate solution and water. The ethyl acetate was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The residue (130 mg) was dissolved in benzene and applied to column chromatography using 5 g of silicic acid. The column was eluted stepwise with 100 ml each of benzene and benzene containing 10, 20, 30, 40 and 50% ethyl acetate. The eluate with benzene containing 30% ethyl acetate afforded 20 mg of crude pestalotin, which was subjected to preparative TLC by use of silica gel GF₂₅₄ and benzene-ethyl acetate (1:1, v/v). The zone containing I was extracted with ethyl acetate. The extract was evaporated to dryness under reduced pressure to yield 3 mg of I.

Physico-chemical properties of pestalotin (I). Recrystallization from *n*-hexane-benzene gave I as colorless plates melting at 88.0~88.5°C. C₁₁H₁₈O₄. M⁺ 214.1210 (Calcd. 214.1205). UV λ_{max} nm (ε): 233 (12200 in 95% EtOH). IR ν_{max}^{Nujol} cm⁻¹: 3480, 1710, 1635, 1255, 1090 and 1025. NMR δ: 0.91 (3H, t, J=6.3 Hz), 1.30~1.60 (6H, broad), 2.22 (1H, broad), 2.25 (1H, q, J=4.0, 17.0 Hz), 2.79 (1H, J=2.0, 12.7, 17.0 Hz), 3.62 (1H, broad), 3.75 (3H, s), 4.28 (1H, m, J=4.0, 4.0, 12.7 Hz) and 5.14 (1H, d, J=2.0 Hz). MS m/e: 214 (M⁺), 196, 183, 181, 170, 167, 157, 153, 139 and 127 (base peak). I is soluble in most of organic solvents such as benzene, chloroform, ethyl acetate, ether, acetone, and alcohols, but sparingly

soluble in *n*-hexane. R_f value on TLC being developed with benzene-ethyl acetate (1:1, v/v) is 0.35.

Pestalotin monoacetate. I was treated with acetic anhydride-pyridine at room temperature and was allowed to stand overnight. The reaction mixture was poured onto sulfuric acid-ice and extracted with ether. The extract was washed with aqueous sodium bicarbonate successively and was evaporated under reduced pressure. The residual yellow oil was subjected to preparative TLC by use of silica gel GF₂₅₄ and benzene-ethyl acetate (1:1, v/v). The zone containing the product was extracted with ethyl acetate. Evaporation of the solvent gave the acetate as a colorless oil. C₁₃H₂₀O₅, M⁺ 256. IR ν_{max}^{Nujol} cm⁻¹: 1745, 1710, 1635 and 1240. NMR δ: 0.91 (3H, t, J=6.3 Hz), 1.25~1.50 (4H, broad), 1.60~1.85 (2H, broad), 2.14 (3H, s), 2.25 (1H, q, J=4.0, 17.0 Hz), 2.53 (1H, m, J=4.0, 12.7, 17.0 Hz), 3.74 (3H, s), 4.48 (1H, m, J=4.0, 4.0, 12.7 Hz), 5.08 (1H, q, J=4.0, 6.0 Hz) and 5.15 (1H, d, J=2.0 Hz).

Ethyl β-methoxy-cis-crotonate (II). This compound was prepared according to the method of Smismann and Voldeng.⁶¹ Bp 188~190°C. MS m/e: 144 (M⁺).

Ethyl γ-bromo-β-methoxy-cis-crotonate (III). This compound was prepared according to the method of Piantadosi and Skulason.⁷¹ Bp 115°C (18 mmHg). MS m/e: 224 and 222 (M⁺).

1-Hepten-3-ol (IV). To the Grignard reagent prepared from 25 g of *n*-butyl bromide, 4.5 g of magnesium and a trace amount of iodine in 100 ml of ether was added a solution of 10 g of acrolein in 50 ml of ether with stirring at a rate to maintain vigorous reflux. The reaction mixture was further refluxed for 2 hr and was poured into a cold 25% ammonium chloride solution. The ether layer was separated, and the aqueous layer was repeatedly extracted with ether. The combined extracts were washed with water and dried over anhydrous sodium sulfate. After removal of the solvent, the residue was distilled under reduced pressure to give 16 g of IV. Bp 60~62°C (17 mmHg). IR ν_{max}^{Film} cm⁻¹: 3420, 1650, 1430, 1000 and 930. NMR δ: 0.93 (3H, t, J=6.0 Hz), 1.20~1.70 (6H, broad), 2.24 (1H, s), 4.07 (1H, q, J=5.0, 14.0 Hz), 5.05 (1H, q, 2.0, 12.0 Hz), 5.20 (1H, q, J=2.0, 14.0 Hz) and 5.85 (1H, m, J=5.0, 12.0, 14.0 Hz). MS m/e: 114 (M⁺), 97, 81 and 67.

2-Hydroxyhexanal (Va). A stream of ozonized oxygen was passed through a solution of IV in 30 ml

of glacial acetic acid. The ozonization was continued until the test of a small portion of the reaction mixture with bromine in the same solvent became negative. Then, 100 ml of ether was added to the reaction mixture, and the ozonide was decomposed with 10 g of zinc. After filtration, the ether layer was separated and washed successively with ice-cold 10% potassium carbonate solution and water. The solvent was dried over anhydrous sodium sulfate, filtered and evaporated. The residue was distilled under reduced pressure to give 4.5 g of Va. Bp 60~68°C (1 mmHg). Mp 78.5~79.5°C. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm^{-1} : 3470, 1725, 1090 and 940. NMR δ : 0.93 (3H, t, $J=6.3$ Hz), 1.17~1.75 (6H, broad), 3.60 (1H, broad), 5.19 (1H, broad), and 9.97 (1H, d, $J=2.5$ Hz). MS m/e : 116 (M^+), 87, 78, 69, and 57.

2-Pyranoxyhexanal (Vb). To a mixture of 2.3 g of freshly distilled Va and 2.0 g of 2,3-dihydropyran was added 10 mg of *p*-toluenesulfonic acid with stirring. After 2 hr, 0.5 g of anhydrous potassium carbonate was added to the reaction mixture, which was then filtered. The filtrate was distilled under reduced pressure to give 2.2 g of the desired pyranyl ether (Vb). Bp 78°C (5 mmHg). IR $\nu_{\text{max}}^{\text{Film}}$ cm^{-1} : 2940, 1738, 1440, 1130, 1080, 1030 and 920. NMR δ : 0.92 (3H, t, $J=6.3$ Hz), 1.25~1.90 (12H, broad), 3.50 (1H, broad), 3.84 (2H, broad), 4.95 (1H, q, $J=2.5, 5.8$ Hz) and 9.64 (1H, d, $J=2.0$). MS m/e : 200 (M^+), 185, 170, 143, 129, 85 and 67.

Reformatsky reaction of Vb with III. A mixture of 2.0 g of Vb, 2.3 g of III and 0.6 g of activated zinc dust, 16 ml of tetrahydrofuran and 4 ml of ether was heated under reflux for 6 hr. The reaction mixture was cooled to room temperature and poured into 100 ml of saturated ammonium chloride solution. After repeated extraction with ethyl acetate, the combined extracts were washed with water, dried over anhydrous

sodium sulfate and concentrated under reduced pressure. The residue was dissolved in methanol containing 70 mg of *p*-toluenesulfonic acid and was allowed to stand overnight at room temperature. After neutralization with potassium carbonate, the solvent was removed by vacuum distillation, and the residue was extracted repeatedly with ether. The combined extracts were concentrated to a small volume and subjected to preparative TLC on silica gel GF₂₅₄ by use of benzene-ethyl acetate (1:1, v/v). The spot of the *R_f* value corresponding to that of I was extracted with ethyl acetate. The residue obtained after evaporation of the solvent showed the following data characteristic of I. UV λ_{max} nm: 233. MS m/e : 214 (M^+), 196, 181, 167, 157, 153, 139 and 127 (base peak).

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