Note

Phytotoxins from Tea Gray Blight Fungi, *Pestalotiopsis longiseta* and *Pestalotiopsis theae*

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*Pestalotiopsis longiseta* (New scientific name was proposed for the fungus called *Pestalotia longiseta* by Dai et al. in 1990) and *Pestalotiopsis theae* have been known as causal fungi for tea grey blight disease. Recently we isolated a dominant phytotoxin of *P. longiseta* and then identified it as oxysporone (1). After our report, I was also identified as a phytotoxin of *Pestalotiopsis oomorae* which causes a leaf spot disease of evening primrose. In the continuation of studies on phytotoxins by causal fungi for tea grey blight disease, we isolated two phytotoxins, (2) from the culture fluid of *P. longiseta* and (3) from that of *P. theae*. In this paper, we describe the isolation, structural elucidation, biogenesis, and biological activity of phytotoxins 2 and 3.

*Pestalotiopsis longiseta* PL8301 (IFO32172) which was used for the identification of oxysporone was still-cultured on a potato broth containing 2% glucose and 0.05% peptone at pH 6.2−6.3 and 28°C for 5 days under quite dark conditions using aluminum film. The filtered culture medium (11.7 liters) was extracted with EtOAc (2 x 11.7 liters). The EtOAc extract was concentrated in vacuo, then chromatographed on silica gel and eluted with benzene-Me$_2$CO (85:15). The phytotoxic activity of each fraction was tested by a leaf-necrosis assay, using the highly susceptible cv. Yabakita. The phytotoxic fraction was separated by a silica gel column with benzene-Me$_2$CO (90:10) and then purified by HPLC using a 30 x 0.78 cm column packed with ODS (TSK gel ODS-80T) eluting with the solvent system MeOH-H$_2$O (2:8). After monitoring by TLC [silica gel and eluting with benzene-Me$_2$CO (6:4); R$_f$ = 0.38], purified phytotoxin fractions were collected. A pale yellow oil (2, 577 mg) was thus obtained.

The physicochemical properties of 2 were [α]$_{D}^{25}$ +261° (c=1.00, MeOH); M$^+$ at m/z 156.0426 (calc. for C$_7$H$_7$O$_3$N, 156.0422). UV $\lambda_{\max}$ (MeOH) nm (ε): 240 (8100); IR $\nu_{\max}$ (KBr) cm$^{-1}$: 3600−3200, 2920, 1670; EIMS m/z (rel. int.): 190 [M$^+$]$^+$ (79), 138 [M−H] (100); $\delta^{13}$-NMR (270 MHz, CDCl$_3$) δ: 2.16 (1H, t, J = 6.3 Hz, −CH$_2$OH), 2.22 (1H, d, J = 8.8 Hz, −OH), 3.51 (1H, dd, J = 3.7, 1.1 Hz, H-6), 3.82 (1H, m, H-5), 4.24 (1H, dd, J = 14.5, 6.3 Hz, −CH$_2$-1), 4.40 (1H, dd, J = 14.5, 6.3 Hz, −CH$_2$-2), 4.75 (1H, m, H-4), 6.69 (1H, m, H-3); $\delta^{13}$-NMR, see Table I.

These data of 2 were indistinguishable from those of (+)-epiepoxydon (Fig. 1), which has already been identified as a phytotoxin produced by an unidentified fungus which was separated from diseased leaves of crapemyrtle (*Lagerstroemia indica L.*) (7).

The method for isolating the phytotoxins 3 was similar to that for 2. *Pestalotiopsis theae* T-56 (IFO32237) was still-cultured on a potato broth containing 2% glucose without 0.5% peptone at pH 6.2−6.3 and 27°C for 4 days under dark conditions. The filtered culture medium (2.8 liters) was extracted with EtOAc (2 x 2.8 liters). The EtOAc extract was concentrated in vacuo, then chromatographed on silica gel, eluting with benzene-Me$_2$CO (95:5). After monitoring by TLC [silica gel, eluting with benzene-Me$_2$CO (6:4); R$_f$ = 0.78], the collected fractions were purified by HPLC using a 30 x 0.78 cm column packed with ODS (TSK gel ODS-80T) and the solvent system MeOH-H$_2$O (6:4). A yellow light oil (3, 28.4 mg) was thus obtained.

The physicochemical properties of 3 were [α]$_{D}^{25}$ +147° (c=0.82, MeOH); M$^+$ at m/z 190.0614 (calc. for C$_{13}$H$_{16}$O$_4$N$_2$, 190.0628). UV $\lambda_{\max}$ (MeOH) nm (ε): 247 (9870); IR $\nu_{\max}$ (KBr) cm$^{-1}$: 3600−3200, 2950, 1690; EIMS m/z (rel. int.): 190 [M$^+$]$^+$ (49), 162 [M−CO]$^+$ (54), 161 [M−CHO]$^+$ (65), 91 (100); $\delta^{13}$-NMR (270 MHz, CDCl$_3$) δ: 1.94 (3H, s, CH$_3$), 2.19 (1H, d, J = 8.8 Hz, −OH), 3.58 (1H, dd, J = 3.4, 1.0 Hz, H-6), 3.82 (1H, m, H-5), 4.78 (1H, m, H-4), 5.35 (1H, m, =CH$_2$), 5.44 (1H, m, =CH$_2$), 6.86 (1H, dd, J = 4.9, 2.4 Hz, H-3); $\delta^{13}$-NMR, see Table I.

The 13C-NMR data (CH$_3$×1, CH$_2$×1, CH×4, quaternary carbon ×5) and the molecular formula (C$_{13}$H$_{16}$O$_4$) indicated that 3 had a hydroxyl group, which was also supported by the disappearance of the signal at δ 2.19 (OH) in the $\delta^{13}$-NMR spectrum by the addition of D$_2$O, and the presence of a ketone group was shown by the signal at δ 191.0 (s) in the 13C-NMR spectrum. Accordingly, the residual oxygen in 3 should be an ethereal oxygen.

The 13C-NMR spectrum demonstrated that 3 had two double bonds and one of them included an exomethylene group; the other was a trisubstituted bond. The $\delta^{13}$-NMR spectrum also indicated that the sole methyl group (δ 1.94 (br. s)) was connected with an olefinic carbon. The coupling constants and chemical shifts in the $\delta^{13}$-NMR spectrum aided in deriving the partial structure shown in Fig. 2. The etheral oxygen

**Table 1.** 13C-NMR Spectral Data of Two Toxins in CDCl$_3$

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<tr>
<th>Carbon no.</th>
<th>Chemical shift (ppm)</th>
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<th>J$_{C-\alpha}$ (Hz)</th>
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Fig. 1. The Structure of Phytotoxins of Two Tea Gray Blight Fungi. 1. oxysporone; 2. (+)-epiepoxydon; 3. PT-toxin.

Fig. 2. The Partial Structure of 3.
mentioned above was assigned as an epoxide one.

The $^{1}{H}$ and $^{13}{C}$-NMR spectral data of the partial structure in Fig. 2 are quite similar to those of the ring structure of 2 (Table I). Furthermore, the dextrorotatory nature of 3 ($^{+}147^\circ$, $c=0.82$, MeOH) shows that the ring must be either $^{(+)}$-epiepoxyd (2) or $^{(+)}$-epoxyd. It has been reported that $J_{3-5}$ of both compounds are 5.0 and 2.65 Hz, respectively. Since that value of 3 is 4.9 Hz, its ring is identical with $^{(+)}$-epiepoxyd. In other words, 3 not only had the same ring structure but also the same conformation as 2. The remaining carbons ($\delta 81.2$ (s) and $\delta 95.9$ (s)) of 3 should form a triple bond. Thus the absolute stereostructure of 3 was identified as shown in Fig. 1.

The planar structure of 3 was identical to that of harveynone which was isolated from Curvularia harveyi as an inhibitor of spindle formation. However the stereochemistry of harveynone was of the $^{(-)}$-epiepoxyd type.

2 has been reported as an precursor$^{9,11}$ of patulin, which is a potent antibiotic produced by a variety of fungi, Penicillium, Aspergillus, Nectria, and Byssosphilamys species. As shown in Fig. 3, a biosynthetic route for patulin has been proposed via 2, phyllostine, a 7-membered lactone, an acyclic intermediate, and rearranged bicyclic compounds.$^{12,13}$ Phyllostine was identified as an intermediate through an experiment using a labeled compound$^{15}$ but the 7-membered lactone was a hypothetical one. Based on the biosynthetic route of patulin, we propose that of oxysporone as shown in Fig. 3. It is the same as that of patulin as far as the steps in which a 7-membered lactone is cleaved by hydrolysis. The following acyclic compound is a geometrical isomer of the compound on the patulin route, and then it is converted into 1 by formation of a heterocyclic ring and rearrangement.

It was reported that 2 is formed from gentisaldehyde, as shown in Fig. 3. We have not yet isolated 2 from P. thrice, but 2 may also be an intermediate in the biosynthetic route for 3 because of having the same ring structure. Since the 3-methyl-3-buten-1-inyl group in 3 appears to be derived from the isoprene unit, elimination of a hydroxysmethyl group in 2 and the subsequent addition of an isoprene unit are possible in the pathway from 2 to 3. Thus both major phytotoxins, 1 and 3, having a different ring structure, are proposed to be biosynthetically derived from gentisaldehyde or phytotoxin 2.

The threshold concentration of induced leaf necrosis for cv. Yabukita by 3, 2, and 1 was found to be about 4 ug/ml, 60 ug/ml, and 15 ug/ml, respectively. These figures mean that the metabolite of 2 greatly enhanced the phytotoxicity through substitution at position 2 or isomerization. The other compounds belonging to the epoxyd group, 2-substituted 2,3-epoxy-5-cyclohexenone derivatives, have been reported as candidates for fungicides and herbicides without description of the configuration.$^{14,15}$

One of them, No. 169 isolated from Gaumannomyces graminis as a pathogen, is 2-epoxy-2-(3-methyl-3-buten-1-inyl)-5-cyclohexen-4-ol-1-one. The difference between No. 169 and 3 in planar structure is only the substituted position of the 3-methyl-3-buten-1-inyl moiety. These facts suggest that phytotoxic tetraketals whose precursors are epoxides are significantly produced by many injurious fungi.

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References