Myxostiolide, Myxostiol, and Clavatoic Acid, Plant Growth Regulators from the Fungus *Myxotrichum stipitatum*

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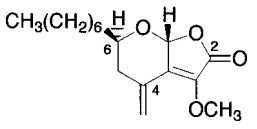
New plant growth regulators, named myxostiolide (**1**), myxostiol (**2**), and clavatoic acid (**3**), have been isolated from *Myxotrichum stipitatum*, and their structures have been established by spectroscopic methods including 2D NMR. The biological activities of **1**, **2**, and **3** have been examined using tea pollen and lettuce seedling bioassay methods. With tea pollen, compound **1** inhibited the pollen tube growth to 14% of control at a concentration of 100 mg/L. With lettuce seedlings, compound **2** accelerated the root growth from 1 mg/L to 100 mg/L and compound **3** inhibited the root growth, to 52% of control, at a concentration of 100 mg/L.

We have investigated fungal metabolites such as penienone,[1,2] penihydrone,[1] penidienone,[3] peniamidienone, penidilamine,[4] and deoxycyclopaldic acid[5] for their potential as plant growth regulators. In the course of our screening search for new plant growth regulators suitable for developing new herbicides and for new lead compounds, we investigated the cultural metabolites of *Myxotrichum stipitatum*.[6,7] The *Myxotrichum* genus belongs to the Gymnoascaceae and is found in the litter layer.[6] Bioassay-guided fractionation of the cultural metabolites of *M. stipitatum* led to the isolation of three new compounds, myxostiolide (**1**), myxostiol (**2**), and clavatoic acid (**3**) together with clavatol,[8] 6-hydroxymellein,[9] and 5-chloro-4,6-dihydroxymellein.[9] In this report, we describe the isolation, structural elucidation, and some biological activities of **1**−**3**.



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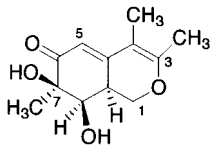

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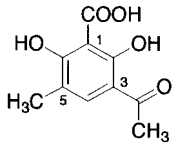

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The EtOAc extract of the culture filtrate of *M. stipitatum* exhibited plant growth activity and was fractionated by silica gel column chromatography and further separated by preparative TLC to afford compounds **1**−**3**.

The HREIMS of **1** gave $[M]^+$ at 280.1654, consistent with a molecular formula $C_{16}H_{24}O_4$. The IR absorption band at 1773 cm^{-1} and one carbon signal at δ_C 169.8 indicated the presence of an α,*β*-unsaturated *γ*-lactone. The ^1H and ^{13}C NMR spectra of **1** indicated the presence of one methyl, seven methylene, two *O*-substituted aliphatic methine, one methoxy, one olefinic methylene, one carbonyl, and three sp^2 carbons. Detailed analysis of $^1H-^1H$ and $^1H-^{13}C$ COSY, PFG-HMBC (Figure 1), ROESY, and homospin-decoupling experiments led to a planar structure of **1**. The relative stereochemistry of **1** was determined by homospin-decoupling and ROESY experiments. The values of the coupling constants between H-6 and H_2-5 in **1** (J = 5.4 and 9.0 Hz) indicated that H-6 was pseudoaxial to Hb-5. No NOE between H-6 and H-7a indicated that these two methine protons were in the *trans* relationship. These results indicated that the relative configurations at C-6 and C-7a in **1** were 6*R**and 7a*R**, respectively. From these results, **1** was established to be (6*R**,7a*R**)-6-*n*-heptyl-5, 6-dihydro-3-methoxy-4-methylene-2-oxo-2*H*,5*H*,7a*H*-furo[2,3-b]pyran, and the compound was named myxostiolide.

The molecular formula of **2** was determined by EIMS and elemental analysis to be $C_{12}H_{16}O_4$. The IR absorption band at 1644 cm^{-1}, the UV absorption band at 331 nm, and one carbon signal at δ_C 200.0 indicated the presence of an α,*β*-*γ*,*δ*-unsaturated carbonyl carbon. A band at 3496 cm^{-1} and two proton signals at δ_H 2.77 and 4.16 indicated the presence of two D_2O exchangeable hydroxyl groups. The ^{13}C and ^1H NMR spectra of **2** indicated the presence of three methyl, one methylene, one aliphatic methine, one *O*-substituted aliphatic methine, one olefinic methine, one *O*-substituted quarternary, one carbonyl, and three sp^2 carbons. The value (J = 2.4 Hz) of the coupling constant between H-5 and H-8a was accounted by the zigzag W-rule. Detailed analysis of $^1H-^1H$ COSY, COLOC, and homospin-decoupling experiments led to a planar structure of **2**. The relative stereochemistry of **2** was determined by homospin-decoupling and differential NOE experiments. The values of the coupling constants between H-8a and H_2-1 in **2** (J =

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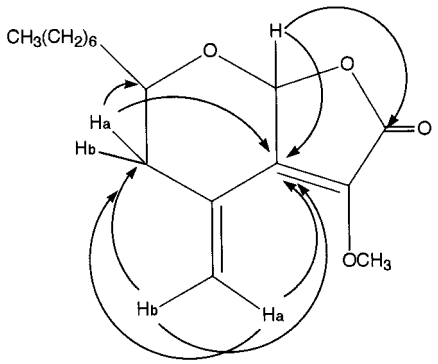

Figure 1. PFG-HMBC experimental results for **1**.

4.9 and 11.2 Hz) indicated that H-8a was pseudoaxial to Hb-1, and the value of the coupling constant between H-8a and H-8 ($J = 3.4$ Hz) indicated that OH-8 was pseudoaxial to H-8a. NOEs were observed among CH_3-7, H-8, and H-8a. These results indicated that the relative configurations at C-7, C-8, and C-8a in **2** were 7*R**, 8*R**, and 8a*S**, respectively. From these results, **2** was established to be (7*R**,8*R**, 8a*S**)-7,8-dihydroxy-3,4,7-trimethyl-1*H*,8a*H*-6-oxo-cyclohexa-[4,3-*c*]pyran, and the compound was named myxostiol.

The molecular formula of **3** was determined by EIMS and elemental analysis to be $C_{10}H_{10}O_5$. The IR absorption band at 1683 cm^{-1} and one signal at δ_C 171.3 in the ^{13}C NMR spectrum indicated the presence of a carboxyl group. The IR absorption band at 1622 cm^{-1} and two signals at δ_C 25.5 and 203.7 in the ^{13}C NMR spectrum indicated the presence of an acetyl group. The ^{13}C and ^1H NMR spectra of **3** indicated the presence of two methyl, a hydrogen-bonded hydroxyl, and a penta-substituted phenyl group. A band at 3170 cm^{-1}, positive reaction to alcoholic ferric chloride, and the remaining atoms from the molecular formula indicated the presence of another phenolic hydroxyl group. The NOEs were observed between an aromatic proton at δ_H 7.70 and two methyl protons at δ_H 2.25 and 2.65. One hydroxyl group was placed at C-2 because a hydrogen bond was formed between the hydroxyl and acetyl groups. Two signals at δ_C 164.0 and 168.7 in the ^{13}C NMR spectrum indicated that two hydroxyl groups were in the *meta* relationship.[5] From these results, **3** was established to be 3-acetyl-2,6-dihydroxy-5-methylbenzoic acid, which was an analogue of clavatol,[8] and the compound was named clavatoic acid.

Biological activities of compounds **1**−**3** were examined using tea pollen and lettuce seedling bioassay methods (Figure 2). With tea pollen, compound **1** inhibited the pollen tube growth to 14% of control at a concentration of 100 mg/L. Compound **2** showed weak inhibitory activity on the growth at the same concentration. With lettuce seedlings, compound **2** accelerated the root growth from 1 mg/L to 100 mg/L. Compound **3** inhibited that to 52% of control at a concentration of 100 mg/L, and compound **1** showed no inhibitory effect on the growth of lettuce seedlings from 1 mg/L to 100 mg/L.

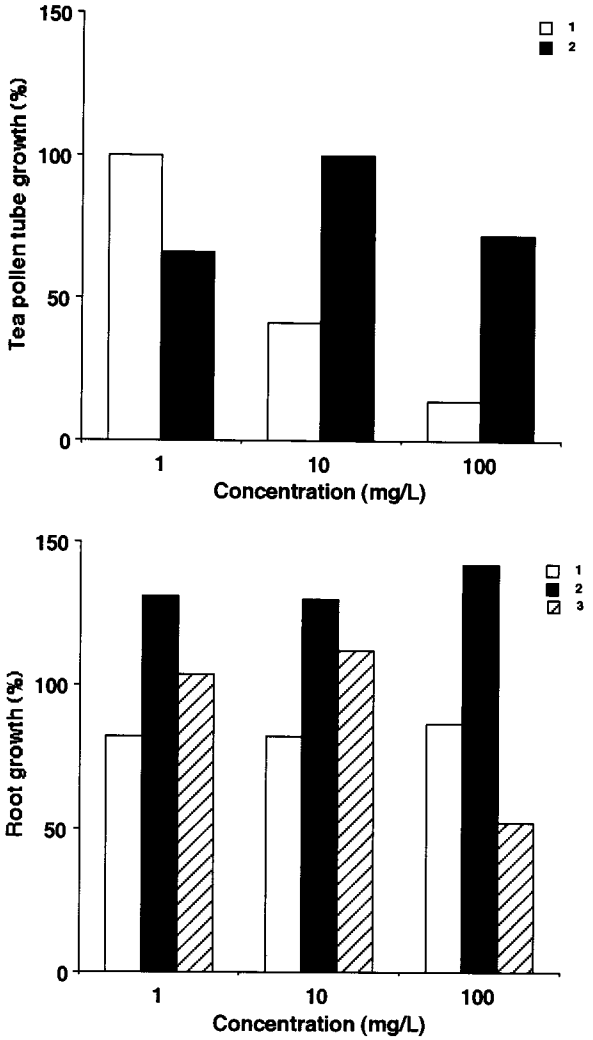

Figure 2. Effects of compounds **1**−**3** on tea pollen tube growth and the root growth of lettuce seedlings.

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were determined on a HORIBA SEPA-200 polarimeter. The IR spectra were recorded on a JASCO FT IR-7000 spectrometer and the UV spectra on a SHIMAZU UV-2200 spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded with a JEOL JNM-GX 270 and a JEOL JNM-ESP 500 NMR spectrometers at 270 and 68 MHz, and 500 and 125 MHz, respectively. Chemical shifts are expressed in δ values with solvents as internal standards. EIMS and HREIMS data were obtained with a HITACHI M80 and a M-2000 mass spectrometer, respectively. Silica gel (Wako, 75−150 mm) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F254, 0.2 mm) were used for preparative TLC.

Fungal Material and Fermentation. *Myxotrichum stipitatum* is deposited at the Laboratory of Bioorganic Chemistry in the Department of Biological and Environmental Chemistry, Faculty of Agriculture, Tottori University. One hundred and sixty 500-mL Erlenmeyer flasks, each containing 250 mL of potato dextrose broth, were individually inoculated with one 1-cm^2 agar plug taken from a stock culture of *M. stipitatum* maintained at 20 °C on potato dextrose agar. The fungus was stationarily grown at 25 °C for 21 days.

Extraction and Isolation. The culture broth (40 L) was filtered, and the filtrate was adjusted to pH 2.0 with 2 N HCl solution. The filtrate was successively extracted with EtOAc and evaporated to dryness under reduced pressure. The resulting residue (12.0 g) was first fractionated by column chromatography on silica gel with hexane and Me_2CO (500-mL fractions).

Clavatol,[8] 6-hydroxymellein,[9] and 5-chloro-4,6-dihydroxymellein[9] were identified by comparing the physicochemical properties with those reported.

(a) Fractions 7 and 8 (1300 mg), obtained by elution with 15% Me_2CO, were further fractionated by column chromatography on silica gel with hexane and Me_2CO (100-mL fractions). Fractions 7−9 (208 mg; 2% Me_2CO) were recrystallized from Me_2CO to afford **3** (154 mg). Fractions 10−12 (378 mg; 2% Me_2-

CO) were purified by preparative TLC developing with benzene−Me_2CO (7:3) to afford clavatol (212 mg).[8]

(b) Fraction 9 (100 mg), obtained by elution with 20% Me_2CO, was purified by preparative TLC developing with hexane−Me_2CO−AcOH (70:30:1) to afford 6-hydroxymellein (20 mg).[9]

(c) Fraction 10 (952 mg), obtained by elution with 20% Me_2CO, was further fractionated by column chromatography on silica gel with hexane and EtOAc (300-mL fractions). Fraction 20 (65 mg; 30% EtOAc) was purified by preparative TLC developing with benzene−EtOAc (1:1) to afford 5-chloro-4,6-dihydroxymellein (14 mg). [9]

(d) Fraction 11 (416 mg), obtained by elution with 20% Me_2CO, was further fractionated by column chromatography on silica gel with benzene and Me_2CO (300-mL fractions). Fraction 2 (27 mg; 2% Me_2CO) was purified by preparative TLC developing with hexane−Me_2CO (9:1) to afford **1** (18 mg). Fraction 4 (155 mg; 5% Me_2CO) was purified by preparative TLC developing with hexane−Me_2CO (7:3) and recrystallized from hexane−Me_2CO to afford **2** (24 mg).

Myxostiolide (1): pale yellow oil; $[\alpha]^{25}_D$ −4.0° (*c* 0.5, EtOH); UV (EtOH) λ_{max} (log ϵ) 224 (4.41), 285 (2.94) nm; IR (KBr) ν_{max} 2934, 2860, 1773, 1649, 1470, 1369, 1209, 907 cm^{-1}; ^1H NMR (CD_3OD, 270 MHz) δ 5.30 (1H, dd, J = 2.7,2.0 Hz, =C*Hb*), 5.17 (1H, s, H-7a), 5.14 (1H, dd, J = 2.7, 1.6 Hz, =C*Ha*), 4.38 (1H, m, H-6), 3.93 (3H, s, 3-OCH_3), 2.90 (1H, ddd, 14.2, 5.4, 2.0 Hz, Ha-5), 2.40 (1H, ddd, 14.2, 9.0, 1.6 Hz, Hb-5), 1.70 (2H, m, H-1′), 1.31 (10H, br s, H-2′−6′), 0.90 (3H, t, J = 8.1 Hz, H-7′); ^{13}C NMR (CD_3OD, 68 MHz) δ 176.8 (s, C-3), 169.8 (s, C-2), 144.9 (s, C-4), 110.1 (t, =CH_2), 108.4 (s, C-3a), 90.1 (d, C-7a), 80.6 (d, C-6), 59.6 (q, OCH_3), 37.5 (t, C-5), 34.9 (t, C-1′), 31.7 (t, C-2′−6′), 29.4 (t, C-2′−6′), 29.1 (t, C-2′−6′), 25.4 (t, C-2′−6′), 22.6 (t, C-2′−6′), 14.0 (q, C-7′); HREIMS *m*/*z* 280.1654 (calcd for $C_{16}H_{24}O_4$, 280.1675); EIMS *m*/*z* (%), 280 (18), 181 (100), 152 (59), 69 (70).

Myxostiol (2): colorless needles (benzene−EtOAc); mp 217−219 °C; $[\alpha]^{25}_D$ −134.7° (*c* 1.0, MeOH); UV (EtOH) λ_{max} (log ϵ) 219 (3.57), 332 (4.23) nm; IR (KBr) ν_{max} 3496, 2984, 2946, 1644, 1600, 1575, 1460, 1326, 1297 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ 5.80 (1H, d, J = 2.4 Hz, H-5), 4.33 (1H, dd, J = 13.7, 4.9 Hz, Ha-1), 4.16 (1H, s, OH), 4.15 (1H, dd, J = 13.7, 11.2 Hz, Hb-1), 4.09 (1H, d, J = 3.4 Hz, H-8), 2.95 (1H, dddd, J = 11.2, 4.9, 3.4, 2.4 Hz, H-8a), 2.77 (1H, s, OH), 1.80 (3H, s, 4-CH_3), 1.67 (3H, s, 3-CH_3), 1.34 (3H, s, 7-CH_3); ^{13}C NMR ($CDCl_3$, 68 MHz) δ 200.0 (s, C-6), 160.3 (s, C-3), 152.1 (s, C-4a), 110.8 (d, C-5), 105.6 (s, C-4), 76.1 (s, C-7), 74.7 (d, C-8), 67.8 (t, C-1), 36.6 (d, C-8a), 23.1 (q, 7-CH_3), 18.6 (q, 3-CH_3), 12.7 (q, 4-CH_3); EIMS *m*/*z* (%), 224 $[M]^+$ (56), 192 (100), 177 (52), 164 (96); *anal*. C 64.29%, H 7.13%, calcd for $C_{12}H_{16}O_4$, C 64.29%, H 7.14%.

Clavatoic acid (3): pale yellow needles (Me_2CO); mp 135−137 °C; UV (EtOH) λ_{max} (log ϵ) 234 (4.28), 253 (4.02), 280 (3.86), 335 (3.93) nm; IR (KBr) ν_{max} 3170, 2928, 2862, 1683, 1622, 1590, 1444, 1381, 1277 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ 13.29 (1H, s, OH), 7.70 (1H, s, Ar-*H*), 2.65 (3H, s, Ar-COCH_3), 2.25 (3H, s, Ar-CH_3); ^{13}C NMR ($CDCl_3$, 68 MHz) δ 203.7 (s, Ar-*C*OCH$_3$), 171.3 (s, Ar-*C*OOH), 168.7 (s, C-2 or C-6), 164.0 (s, C-2 or C-6), 136.5 (d, C-4), 119.5 (s, C-5), 110.7 (s, C-1), 99.9 (s, C-3), 25.5 (q, Ar-CO*C*H$_3$), 15.2 (q, Ar-*C*H$_3$); EIMS *m*/*z* (%), 210 $[M]^+$ (33), 192 (74), 177 (54), 164 (100), 121 (24), 108 (26), 94 (17), 77 (22); *anal*. C 57.20%, H 4.80%, calcd for $C_{10}H_{10}O_5$, C 57.14%, H 4.76%.

Bioassay for Tea Pollen Tube Growth. Pollen grains of *Camellia sinensis* O. Kuntze were collected from an open flower, dried in a desiccator over silica gel, and stored in a refrigerator. The grains were sown with the edge of a cover glass on a 1.5% agar medium containing 10% sucrose, 10 ppm boric acid, and the compound to be tested at various concentrations on a microscopic slide and then incubated in a moist chamber at 24 °C in the dark. After cultivation for 12 h, the length of the pollen tube was measured and compared with that of an untreated control.[10]

Bioassay for the Growth of Lettuce Seedlings. Lettuce seedlings were sown in a Petri dish (150 × 25 mm) lined with a filter paper containing deionized water. After 1 day under light at 24 °C, seedlings were selected for uniformity (radicles; 2 mm) and transferred into a mini-Petri dish (35 × 15 mm) lined with filter paper containing 1 mL of deionized water and a defined amount of the test compound. The Petri dishes were kept at 24 °C for 4 days under continuous light. The length of the hypocotyls and roots treated with the compounds was measured, and the mean value of the length was compared with an untreated control.[11]

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