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TWELVE-MEMBERED LACTONES PRODUCED BY *CLADOSPORIUM TENUISSIMUM* AND THE PLANT GROWTH RETARDANT ACTIVITY OF CLADOSPOLIDE B

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Key Word Index—*Cladosporium tenuissimum*; fungal metabolite; plant growth retardant; 12-membered macrolide; cladospolide.

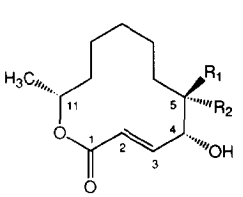
Abstract—Three 12-membered macrolides were isolated from a culture filtrate of the fungus, *Cladosporium tenuissimum*. Two were identified as cladospolides A and B, and one as a new cladospolide, which was named cladospolide C. Cladospolide C is a diastereomer of cladospolide A. Cladospolide B was inhibitory to shoot elongation of rice seedlings (*Oryza sativa* L.) without damaging the cells. Its activity was investigated in relation to gibberellin biosynthesis.

INTRODUCTION

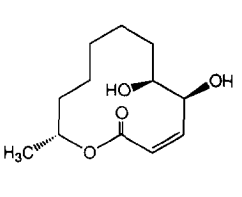
Dwarfism is a very important biological phenomenon in agriculture because it prevents crops from lying on the ground and produces a better harvest. Conversion of a normal plant into a dwarf is usually brought about by chemical plant growth retardants. In many cases, dwarfing agents act by blocking gibberellin biosynthesis; therefore, they are sometimes used in biosynthetic studies of gibberellins [1]. In the course of a screening-based search for natural plant growth retardants among the metabolites produced by soil fungi, we found a fungus, *Cladosporium tenuissimum*, whose culture filtrate showed plant growth retardant activity to rice seedlings. The culture filtrate contained three 12-membered macrolides, two of which were identified as cladospolides A and B, while the third was found to be a new cladospolide which we have named cladospolide C. Hirota and co-workers reported the isolation and structures of cladospolides A and B produced by *C. cladosporioides* FI-113 [2, 3]. They also reported that root growth of lettuce seedlings is inhibited by cladospolide A and promoted by cladospolide B. Our microdrop bioassay method [4] using rice seedings showed that cladospolide B inhibits shoot elongation without causing necrosis, although cladospolides A and C do damage the plants. To examine the relationship of its retardant activity to gibberellins, cladospolide B, together with gibberellin A_1, was applied to rice seedlings that had been converted to dwarfs by treatment with uniconazole, a gibberellin biosynthesis inhibitor. The isolation and structure of cladospolide C and the plant growth-retarding activity of cladospolide B are reported.

RESULTS AND DISCUSSION

The ethylacetate soluble neutral fraction from a culture filtrate of *C. tenuissimum* when chromatographed on a silica gel column gave three fractions that, respectively, contained compounds **1**, **2** and **3**. Each fraction was further purified by several steps, including HPLC, to afford **1**, **2** and **3** in the pure state in yields of 0.27, 0.53 and 0.13 mg l^{-1} of medium, respectively. Compounds **1**–**3** have the same molecular formula, $C_{12}H_{20}O_4$, on the basis of the HRFAB mass spectrum and ^{13}C NMR data. Compounds **1** and **2** were identified as cladospolides A (**1**) and B (**2**), both of which had been isolated previously from the culture filtrate of the fungus, *C. cladosporioides* FI-113 [2, 3]. Compound **3** was a new cladospolide which we named cladospolide C. Its IR absorption bands at 1717 and 1651 cm^{-1} and ^{13}C resonances at



1 R_1=H, R_2=OH
3 R_1=OH, R_2=H



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δ166.8, 145.3 and 124.5 indicated an α,β-unsaturated lactone. The ^{1}H NMR spectrum of **3** had a methyl doublet at δ1.31, which was transformed into a singlet signal on irradiation of the signal at δ4.98, indicative of the presence of a methyl group attached to an acylated methine. The coupling constant (16.0 Hz) between the olefinic protons at δ6.82 and 6.06 indicated that the geometry of the double bond was *E*. The ^{1}H resonances at δ3.98 and 3.56 indicated a vicinal glycol portion adjacent to the double bond. These findings suggest that **3** was a diastereomer of **1**.

Cladospolides have three chiral centres and one double bond in their structures. Therefore, cladospolide C had to have one of the following stereochemistries: (4*R**, 5*R**, 11*R**), (4*S**, 5*R**, 11*R**), (4*R**, 5*S**, 11*R**) and (4*S**, 5*S**, 11*R**). Cladospolides A and B not only differ in the geometry of the double bond, but also in the stereochemistry of the chiral centres, because hydrogenation of **1** and **2** gives different dihydroproducts [3]. Hydrogenation of **2** and **3** on Pd–C in methanol gave two different dihydroproducts that were diastereomers to each other on the basis of their ^{1}H NMR spectra. The absolute configuration of **1** has been established as 4*R*, 5*S* and 11*R* by X-ray analysis and Mosher's method [5, 6] and that of **2** as 4*S*, 5*S* and 11*R* by synthesis studies [7]. The (2*E*, 4*R*, 5*S*, 11*S*)-4,5-dihydroxy-2-dodecene-11-olide synthesized [8] differs from **3**, the relative configuration of **3** being 4*R**, 5*R** and 11*R**. Patulolides A, B and C, 12-membered lactones from *Penicillium urticae* [9–11], have the same *R* configuration at the carbon atom attached to the exocyclic methyl group that **1** and **2** have. So far, *R* configuration has been reported at the terminus of the 10-membered lactone ring in the fungal metabolites diplodialides A, B and C [12–14], pyrenolides A, B and C [15, 16] and thiobiscephalosporolide A [17]. Consequently, the configuration at C-11 in **3** is probably *R*.

In the microdrop bioassay method using rice (*Oryza sativa* L. cv. Yamabiko) seedlings [4], shoot elongation of the second leaf sheath was inhibited by compounds **1** (respectively, 89 and 67% of the control at 10 and 50 μg/plant), **2** (respectively, 80 and 72% of the control at 1 and 10 μg per plant) and **3** (respectively, 88 and 70% of the control at 5 and 50 μg per plant). It is noteworthy that with **2** there was no visual damage to the rice plants, whereas **1** and **3** caused necrosis. This suggests that gibberellins are involved in the mode of action of **2**. Therefore, in the microdrop bioassay, gibberellin A_1 was applied after **2** to rice seedings that had been treated with uniconazole before germination [18, 19]. Uniconazole reduced the endogenous gibberellin levels in the rice seedings and caused dwarfism. Exogenously supplied GA_1 stimulated elongation of the rice shoots and restored the rice seedlings to the normal type. Compound **2** did not inhibit the elongation stimulated by GA_1 even when applied at 5 μg per plant (Table 1). These results suggest that **2** retards plant growth not by an antagonistic effect against gibberellins, but by inhibition of gibberellin biosynthesis.

EXPERIMENTAL

General. Mp: uncorr.; MS: Jeol DX-300 spectrometer; NMR: 270.05 (^{1}H) and 67.8 MHz (^{13}C): HPLC: Daisopak SP-120-5-ODS-A; solvent, 70% MeOH in H_2O; flow rate, 1.0 ml min^{-1}, detection, UV 220 nm. Analyt. TLC: Merck Kieselgel 60F_{254}, and visualization by placing the plate in a chamber containing I_2 vapour.

Fungus. The isolate of *C. tenuissimum* used was obtained from a soil collected at Karo-cho in Tottori Prefecture, Japan, in 1990. It was identified by Dr A. Tsuneda of the Tottori Mycological Institute and maintained on potato dextrose agar.

Isolation of cladospolides A (**1**), *B* (**2**) *and C* (**3**). The fungus was grown in 500 ml conical flasks containing 200 ml of a medium consisting of glucose (30 g l^{-1}), peptone (3 g l^{-1}), the extract from 50 g l^{-1} of malt, and water. The cultures were grown without shaking at 24° for 14 days. The cultures (40 l) were filtered, and the filtrates extracted with EtOAc (3 × 0.5 vol.) at pH 2.0. The EtOAc extracts were concd *in vacuo*, washed with 1 M $NaHCO_3$, dried over Na_2SO_4 and evapd to afford an EtOAc-soluble neutral fr. (2.82 g). Subsequent sepn procedures were monitored by the microdrop bioassay method described below. The EtOAc-soluble neutral fr. was chromatographed on a column of silica gel (70 g of Wakogel C-200) developed with 450 ml each of 20, 30 and 40% Me_2CO in *n*-hexane. Each 90 ml of eluate was collected as one fr. The first fr. (111 mg) eluted with 30%

Table 1. Effects of cladospolide B (**2**) on the GA-stimulated elongation of the second leaf sheath of rice seedlings treated with uniconazole

	Cladospolide B (μg/plant)			
GA_1 (ng/plant)	0	1	3	5
0	38.7*	37.8	33.1	30.2
10	100	98.5	119.6	89.5
50	197.1	180.4	194.5	183.3

* The length of the second leaf sheath is expressed as a percentage of that (27.4 mm) of the seedling treated with 0 μg/plant cladospolide B and 10 ng/plant GA_1.

Me_2CO in *n*-hexane was purified by repeated Sephadex LH-20 CC (100 × 2.2 cm, MeOH) and HPLC to give 21 mg of **2** as a powder. The second fr. (364 mg) eluted with 30% Me_2CO in *n*-hexane was purified by Sephadex LH-20 CC (100 × 2.2 cm, MeOH) and HPLC, yielding 11 mg of **1** as needles. The third fr. (129 mg) eluted with 30% Me_2CO in *n*-hexane was purified by silica gel flash CC (13 g of Wakogel FC-40) with 250 ml each of 30 and 40% EtOAc in C_6H_6. Each 50 ml of eluate was collected as one fr. Frs 5, 6 and 7 were combined and concd *in vacuo*. The residue (18 mg) was purified further by HPLC to afford 5 mg of **3** as a solid.

Cladospolide A (**1**). Mp 90–91°; $[\alpha]_D^{22}$ − 27.5° (MeOH; *c* 0.4); UV λ_{max} (MeOH) nm (log ε): 217 (4.00); IR ν_{max} (KBr) cm^{-1}: 3488, 3368, 2942, 2868, 1713, 1644, 1462, 1276, 1168; ^1H NMR ($CDCl_3$): δ6.81 (1H, *dd*, *J* = 16.0, 6.0 Hz, H-3), 6.20 (1H, *dd J* = 16.0, 1.5 Hz, H-2), 5.12 (1H, *ddq*, *J* = 6.3, 3.3, 6.3 Hz, H-11), 4.55 (1H, *ddd*, *J* = 6.0, 3.5, 1.5 Hz, H-4), 3.66 (1H, *ddd*, *J* = 10.0, 3.5, 1.5 Hz, H-5), 0.89–1.84 (10H), 1.28 (3H, *d*, *J* = 6.3 Hz, H-12); ^{13}C NMR ($CDCl_3$): δ167.9, 145.8, 122.2, 74.7, 73.0, 73.0, 32.5, 30.6, 28.1, 25.1, 22.6, 19.0; HRFABMS *m/z*; 229.1329 ($[M + H]^+$, calcd for $C_{12}H_{21}O_4$ 229.1438).

Cladospolide B (**2**). Mp 109–110°; $[\alpha]_D^{22}$ + 45.0 (MeOH; *c* 0.4); UV λ_{max} (MeOH) nm (log ε): 211 (3.84); IR ν_{max} (KBr) cm^{-1}: 3312, 2934, 1715, 1632, 1454, 1381, 1294, 1077; ^1H NMR ($CDCl_3$): δ6.24 (1H, *dd*, *J* = 12.0, 8.0 Hz, H-3), 5.78 (1H, *dd*, *J* = 12.0, 1.3 Hz, H-2), 5.26 (1H, *ddd*, *J* = 8.0, 4.0, 1.3 Hz, H-4), 4.89 (1H, *ddq*, *J* = 10.5, 1.5, 6.0 Hz, H-11), 3.77 (1H, *ddd*, *J* = 9.0, 4.0, 2.5 Hz, H-5), 1.27–1.88 (10H), 1.29 (3H, *d*, *J* = 6.0 Hz, H-12); ^{13}C NMR ($CDCl_3$): δ165.9, 148.7, 121.6, 74.4, 73.8, 67.4, 32.0, 30.6, 25.7, 24.1, 21.2, 19.7; HRFABMS *m/z*; 229.1464 ($[M + H]^+$, calcd for $C_{12}H_{21}O_4$ 229.1438).

Cladospolide C (**3**). Mp 90–91°; $[\alpha]_D^{22}$ + 59.7° (MeOH; *c* 0.4); UV λ_{max} (MeOH) nm (log ε): 214 (3.90); IR ν_{max} (KBr) cm^{-1}: 3380, 2940, 2866, 1717, 1651, 1462, 1263, 1166, 1038, 996; ^1H NMR ($CDCl_3$): δ6.82 (1H, *dd*, *J* = 16.0, 9.0 Hz, H-3), 6.06 (1H, *d*, *J* = 16.0 Hz, H-2), 4.98 (1H, *ddq*, *J* = 9.0, 2.5, 6.3 Hz, H-11), 3.98 (1H, *dd*, *J* = 9.0, 7.5 Hz, H-4), 3.56 (1H, *ddd*, *J* = 7.5, 7.5, 2.5 Hz, H-5), 1.02–1.80 (10H), 1.31 (3H, *d*, *J* = 6.3 Hz, H-12), ^{13}C NMR ($CDCl_3$): δ166.8, 145.3, 124.5, 77.3, 76.4, 74.4, 34.0, 32.0, 27.3, 24.5, 24.1, 20.7; HRFABMS *m/z*; 229.1334 ($[M + H]^+$, calcd for $C_{12}H_{21}O_4$ 229.1438).

Dihydrocladospolide B (**4**). Compound **2** (5.0 mg) in MeOH (1 ml) containing 10% Pd–C catalyst (5 mg) was stirred under H_2 at room temp. for 30 min. The reaction mixt. was then filtered and evapd *in vacuo* to afford dihydrocladospolide B (**4**) obtained in quantitative yield as an oil. ^1H NMR ($CDCl_3$): δ5.01 (1H, *ddq*, *J* = 6.3, 6.3, 6.3 Hz), 3.67 (1H, *ddd*, *J* = 7.2, 5.9, 4.1 Hz), 3.44 (1H, *ddd*, *J* = 7.2, 6.8, 2.8 Hz), 2.57 (1H, *ddd*, *J* = 14.8, 9.7, 2.7 Hz), 2.46 (1H, *ddd*, *J* = 14.8, 9.5, 3.1 Hz), 1.14–2.08 (12H), 1.24 (3H, *d*, *J* = 6.3 Hz).

Dihydrocladospolide C (**5**). Compound **5** was obtained as an oil from **3** (4.0 mg) by the procedure used to prepare **4**. ^1H NMR ($CDCl_3$): δ5.13 (1H, *ddq*, *J* = 6.3, 3.3, 6.7 Hz), 3.70 (1H, *ddd*, *J* = 7.8, 5.9, 3.0 Hz), 3.61 (1H, *ddd*, *J* = 7.8, 7.3, 3.8 Hz), 2.50 (1H, *dd*, *J* = 13.0, 4.9 Hz, AB), 2.49 (1H, *d*, *J* = 13.0 Hz, AB), 1.17–1.80 (11H), 1.24 (3H, *d*, *J* = 6.7 Hz).

(2E, 4R, 5S, 11S)-4,5-*Dihydroxy*-2-*dodecene*-11-*olide*. $[\alpha]_D^{24}$ − 87.3° (MeOH; *c* 0.257); IR ν_{max} (KBr) cm^{-1}: 3240, 2930, 1718, 1652, 1376, 1251, 1168, 1042, 998; ^1H NMR ($CDCl_3$): δ6.98 (1H, *dd*, *J* = 15.8, 3.1 Hz), 6.14 (1H, *dd*, *J* = 15.8, 2.1 Hz), 4.99 (1H, *m*), 4.60 (1H, *m*), 3.87 (1H, *m*), 2.76 (2H, *brs*), 1.59–1.72 (2H, *m*), 1.40–1.59 (2H, *m*), 1.10–1.40 (6H, *m*), 1.30 (3H, *d*, *J* = 6.7 Hz).

Microdrop bioassay. Seeds of rice (*O. sativa* L. cv Yamabiko) were soaked in H_2O for 48 hr at 28° under fluorescent light. Sets of six germinated seeds were planted on 1% agar medium in 30 ml beakers and incubated for 48 hr under continuous light. A 1 μl sample containing 50% aq. Me_2CO was placed as a drop between the shoot and first leaf of a seedling. After incubation for 72 hr under continuous light, the length of the second leaf sheath of the seedling was measured. In the experiments in which a reduction in the endogenous levels of gibberellins was required, seeds were first soaked in a soln of uniconazole (20 mg l^{-1}) for 24 hr at 28°, then washed with running tap water for 10 min [18]. The washed seeds were germinated by soaking them in H_2O for 24 hr at 28°. Gibberellin in 1 μl of 50% aq. Me_2CO was applied to the seedling 30 to 60 min after the application of the test sample [19]. The other experimental conditions were the same as those described above.

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