4-Hydroxykigelin and 6-Demethylkigelin, Root Growth Promoters, Produced by *Aspergillus terreus*

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Root growth promoters, 4-hydroxykigelin (**1**) and 6-demethylkigelin (**2**), together with 6-hydroxymellein (**3**) were isolated from cultures of the fungus *Aspergillus terreus* and their structures were identified by spectroscopic analysis. The biological activities of the three dihydroisocoumarins, **1**, **2**, and **3**, have been examined using a bioassay method with lettuce seedlings. Furthermore, interactions between the dihydroisocoumarins and indole-3-acetic acid against the root growth have been examined.

Key words: 4-Hydroxykigelin, 6-Demethylkigelin, Root Growth Promoter

Introduction

So far, many compounds have been isolated from various fungi as plant growth regulators (Turner and Aldridge, 1983; Stoessl, 1981), but few compounds have been shown the acceleration of plant growth with the exception of sescandelin (Kimura *et al.*, 1990) and penihydrone (Kimura *et al.*, 1997). In the course of our screening search for plant growth regulators from fungi suitable for developing new herbicides and for new lead compounds, we found the presence of root growth promoters in the culture filtrate of *Aspergillus terreus.* Aspterric acid and 6-hydroxymellein (**3**) isolated from this fungus show pollen growth inhibitory activity (Shimada *et al.*, 2002), but metabolites of this fungus have not been previously studied as root growth regulators. Hence, we investigated the plant growth regulators of *A. terreus* and isolated two dihydroisocoumarins as root growth promoters, using bioassay method with lettuce seedlings. In this report, we describe the isolation, structural identification and biological activities of the active compounds.

Materials and Methods

General

Melting points were determined using a Yanagimoto micromelting point apparatus and are reported uncorrected. Optical rotations were determined on a HORIBA SEPA-200 polarimeter. The IR spectra were recorded on a JASCO FT IR-7000 spectrophotometer and the UV spectra on a SHIMAZU UV-2200 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded with a JEOL JNM-ESP 500 NMR spectrometer at 500 and 125 MHz, respectively. EIMS and HREIMS data were obtained with a HITACHI M-80B spectrometer. Indole-3-acetic acid (IAA) was purchased from Wako Pure Chemical Industries, Japan. *p*-Chlorophenoxyisobutyric acid (PCIB) was purchased from Aldrich Chemical Company, USA.

Fungal material

The fungus *A. terreus* was isolated from the soil at Kitakyushu-shi, Fukuoka, Japan, in 1997 and is deposited at the Laboratory of Bioorganic chemistry in the Department of Environmental Chemistry, Faculty of Engineering, Kyushu Kyoritsu University.

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Fermentation and isolation of **1**, **2** *and* **3**

Aspergillus terreus was cultured stationarily in a malt extract medium at 24 °C for 28 d. The culture broth (40 l) was filtered, and the filtrate was adjusted to pH 2.0 with 2 N HCl, before being extracted twice with EtOAc. The combined solvents were concentrated *in vacuo*, and the resulting residue (28.8 g) was first fractionated by column chromatography on silica gel (*n*-hexane/acetone). Fraction 6 (172 mg), obtained by elution with 30% acetone, was further purified by preparative TLC ($CHCl_3$/MeOH, 98:2, v/v). One solid from preparative TLC was recrystallized from benzene to afford 16 mg of 6-demethylkigelin (**2**) as colorless needles, and another solid was recrystallized from EtOAc to afford 17 mg of 6-hydroxymellein (**3**) as colorless plates. Fractions 7–10 (5808 mg), obtained by elution with 30% acetone, were chromatographed on a silica gel column ($CHCl_3$/MeOH). The active fraction eluted with $CHCl_3$ (595 mg) was further purified by preparative TLC ($CHCl_3$/EtOAc/AcOH, 50:50:2, v/v/v) and the solid was recrystallized from acetone/*n*-hexane to afford 13 mg of 4-hydroxykigelin (**1**) as colorless needles.

4-Hydroxykigelin (**1**): M.p. 159–161 °C. – $[\alpha]_D^{20}$ – 9 ° (*c* 0.43, MeOH). – UV/vis (EtOH): λ_{max} (lg ε) = 220 (4.28), 272 (4.01), 307 nm (3.20). – IR (KBr): ν = 3497 (OH), 2984 (C=C), 1660 (O-C=O), 1610 (C=C), 1579, 1516, 1467, 1371, 1277, 1124, 1041, 1018 cm^{-1}. – ^1H NMR (500 MHz, CD_3OD): δ = 1.49 (d, *J* = 6.7 Hz, 3H, 3-CH_3), 3.80 (s, 3H, 6-OCH_3), 3.95 (s, 3H, 7-OCH_3), 4.50 (d, *J* = 2.1 Hz, 1H, 4-H), 4.66 (qd, *J* = 6.7, 2.1 Hz, 1H, 3-H), 6.71 (s, 1H, 5-H). – $^{13}C\{^1H\}$ NMR (125 MHz, CD_3OD): δ = 16.2 (q, CH_3-3), 56.7 (q, OCH_3-7), 60.9 (q, OCH_3-6), 67.7 (d, C-4), 79.8 (d, C-3), 102.9 (s, C-8a), 104.2 (d, C-5), 137.6 (s, C-7), 139.3 (s, C-4a), 156.6 (s, C-8), 160.2 (s, C-6), 170.9 (s, C-1). – MS (EI): *m/z* (%) = 254 (100) [M^+], 221 (13), 210 (15), 195 (13), 182 (26), 167 (23), 139 (8). – HRMS: *m/z* (M^+): calcd. for $C_{12}H_{14}O_6$: 254.0791, found: 254.0789.

6-Demethylkigelin (**2**): M.p. 137–141 °C. – $[\alpha]_D^{20}$ – 33° (*c* 0.36, MeOH). – UV/vis (EtOH): λ_{max} (lg ε) = 220 (4.29), 275 nm (4.09). – IR (KBr): ν = 3356 (OH), 2945 (C=C), 1651 (O-C=O), 1589 (C=C), 1506, 1458, 1377, 1170 cm^{-1}. – ^1H NMR (500 MHz, acetone-d_6): δ = 1.50 (d, *J* = 6.4 Hz, 3H, 3-CH_3), 2.82 (dd, *J* = 11.5, 16.8 Hz, 1H, 4a-H), 2.85 (dd, *J* = 16.8, 3.3 Hz, 1H, 4b-H), 3.98 (s, 3H, 7-OCH_3*), 4.66 (m, 1H, 3-H), 6.33 (s, 1H, 5-H), 11.38 (s, 1H, 8-OH).* – $^{13}C\{^1H\}$ NMR (125 MHz, acetone-d_6): δ = 20.6 (q, CH_3-3), 34.4 (t, C-4), 60.9 (q, OCH_3-7), 75.9 (d, C-3), 102.2 (s, C-8a), 105.2 (d, C-5), 132.9 (s, C-7), 135.6 (s, C-4a), 154.9 (s, C-8), 155.4 (s, C-6), 170.1 (s, C-1). – MS (EI): *m/z* (%) = 224 (100) [M^+], 209 (23), 206 (24), 165 (24), 163 (20), 137 (5). – HRMS: *m/z* (M^+): calcd. for $C_{11}H_{12}O_5$: 224.0684, found: 224.0681.

6-Hydroxymellein (**3**): Spectroscopic data have been previously reported (Shimada *et al.*, 2002).

MTPA esters of **1**

1 (2 mg) was dissolved in 250 μl of dry methylene chloride. The solution was treated with *N*,*N*-dicyclohexyl carbodiimide (5.4 mg), dimethylaminopyridine (2.3 mg) and (*S*)-(+)-2-methoxy-2-trifluoromethyl-2-phenylacetic acid (MTPA acid) (4.9 mg), and was then allowed to stand at room temperature for 8 h. The reaction mixture was purified by preparative TLC (*n*-hexane/acetone, 7:3, v/v) yielding 3.1 mg (*R*)-MTPA ester of **1**. With (*R*)-(–)-MTPA acid, **1** (1 mg) was esterified in the same manner to yield 1.3 mg of the (*S*)-MTPA ester of **1** (Ohtani *et al.*, 1991).

(R)-MTPA ester: ^1H NMR (500 MHz, $CDCl_3$): δ = 1.5140 (3-CH_3), 3.9130 (6-OCH_3), 3.9249 (7-OCH_3), 4.8237 (3-H), 5.9411 (4-H), 6.6675 (5-H), 10.9895 (8-OH).

(S)-MTPA ester: ^1H NMR (500 MHz, $CDCl_3$): δ = 1.3665 (3-CH_3), 3.9290 (6-OCH_3), 3.9395 (7-OCH_3), 4.8099 (3-H), 5.9611 (4-H), 6.7206 (5-H), 11.1013 (8-OH).

Bioassay for the growth of lettuce seedlings

Lettuce seedlings were sown in a Petri dish (150 mm × 25 mm) lined with a filter paper containing deionized water. After one day under light at 24 °C, the seedlings were selected for uniformity (radicles; 2 mm) and transferred into a mini-Petri dish (35 mm × 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 d under continuous light (8000 lx). Hypocotyls and roots of untreated seedlings grew at the rate of about 1 mm and 4 mm a day, respectively. 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 (Kimura *et al.*, 2002).

Results and Discussion

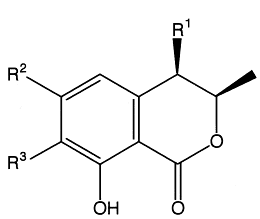
The fungus was stationarily cultured in a malt extract medium (40 l) at 24 °C for 28 d. The culture filtrate was adjusted to pH 2.0, before being extracted twice with EtOAc. The EtOAc-soluble acidic fraction (28.8 g) was purified with silica gel column chromatography and preparative TLC, and final purification by recrystallization afforded compounds **1**, **2** and **3** (Fig. 1).

Compound **1** was obtained as colorless needles. The HREIMS of **1** gave [M^+] at 254.0789, consistent with the molecular formula $C_{12}H_{14}O_6$. The IR band at 1660 cm^{-1} and a signal at δ 170.9 in the ^{13}C NMR spectrum indicated the presence of a carboxyl group. The ^1H NMR spectrum of **1** indicated the presence of one methyl, two methoxy, two *O*-substituted aliphatic methine, and one penta-substituted phenyl group. The IR band at 3497 cm^{-1} and the remaining atoms from the molecular formula indicated the presence of two hydroxy groups. The UV and IR spectra of **1** showed obvious similarities with those of kigelin (Govindachari *et al.*, 1971). Comparison of the molecular formula and ^1H NMR data of **1** with those of kigelin revealed that **1** differed from kigelin only in the presence of a hydroxy methine group at C-4 instead of a methylene group. The *cis*-configuration is allocated to 3-H and 4-H on the basis of the coupling constant (J = 2.1 Hz) (Krohn *et al.*, 1997). The absolute stereochemistry of an asymmetric center at C-4 was determined by using modified Mosher's method (Ohtani *et al.*, 1991). **1** was treated with (*R*)-(−)- and (*S*)-(+)-MTPA acid to afford the C-4-(*S*)- and -(*R*)-MTPA esters of **1**. The positive $\Delta\delta$ ($\delta_S - \delta_R$) values in the ^1H NMR spectrum were observed for 4-H, 5-H, 6-OCH_3, 7-OCH_3 and 8-OH, while the negative $\Delta\delta$ ($\delta_S - \delta_R$) values were located at 3-H and 3-CH_3. These results revealed the absolute stereochemistry of C-4 to be *R* configuration (Fig. 1). Therefore, another asymmetric center at C-3 had the *R* configuration. Thus, compound **1** was identified as 4-hydroxykigelin by comparing the physicochemical properties with those reported (Arai *et al.*, 1983).

Compound **2** was obtained as colorless plates. The HREIMS of **2** gave [M^+] at 224.0681, consistent with the molecular formula $C_{11}H_{12}O_5$. The UV and IR spectra of **2** showed obvious similarities with those of kigelin (Govindachari *et al.*, 1971). Comparison of the molecular formula and ^1H NMR data of **2** with those of kigelin revealed that **2** differed from kigelin only in the presence of a hydroxyl group at C-6 instead of a methoxy group. The coupling constants between 3-H and 4-H_2 (J = 3.3 and 11.5 Hz) indicated that 3-H was β with axial orientation (Jolad *et al.*, 1981). The relative configulation at C-3 was 3*R*; that was the same configuration at C-3 of **1**. Thus, compound **2** was identified as 6-demethylkigelin by comparing the physicochemical properties with those reported (Govindachari *et al.*, 1971).

The structure of compound **3** had been previously reported as 6-hydroxymellein by comparing the physicochemical properties with those reported (Ayer *et al.*, 1987).

Compounds **1**, **2** and **3** are highly substituted dihydroisocoumarins and plant growth activities of these compounds are of interest because there are few published accounts of the effect of dihydroisocoumarins on plant growth with the exception of hydrangenol (Asen *et al.*, 1960). In addition, dihydroisocoumarin substituted at C-4 with a hydroxyl



	R^1	R^2	R^3
1	OH	OCH_3	OCH_3
2	H	OH	OCH_3
3	H	OH	H

Fig. 1. Structures of 4-hydroxykigelin (**1**), 6-demethylkigelin (**2**) and 6-hydroxymellein (**3**).

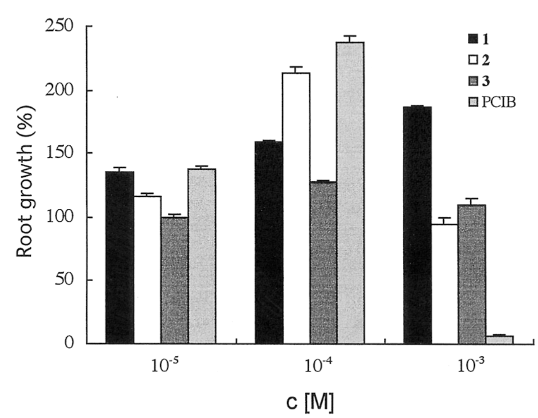


Fig. 2. Effects of **1**, **2**, **3** and *p*-chlorophenoxyisobutyric acid (PCIB) on the root growth of lettuce seedlings in percent of control. Each value represents the mean ± SD (n = 3).

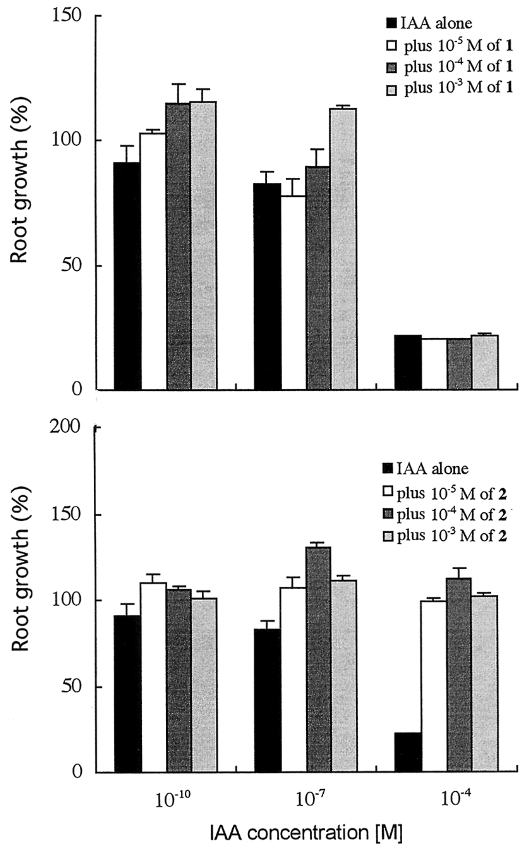


Fig. 3. Interactions between indole-3-acetic acid (IAA) and dihydroisocoumarins, **1** and **2**, on the root growth of lettuce seedlings. Each value represents the mean ± SD (n = 3).

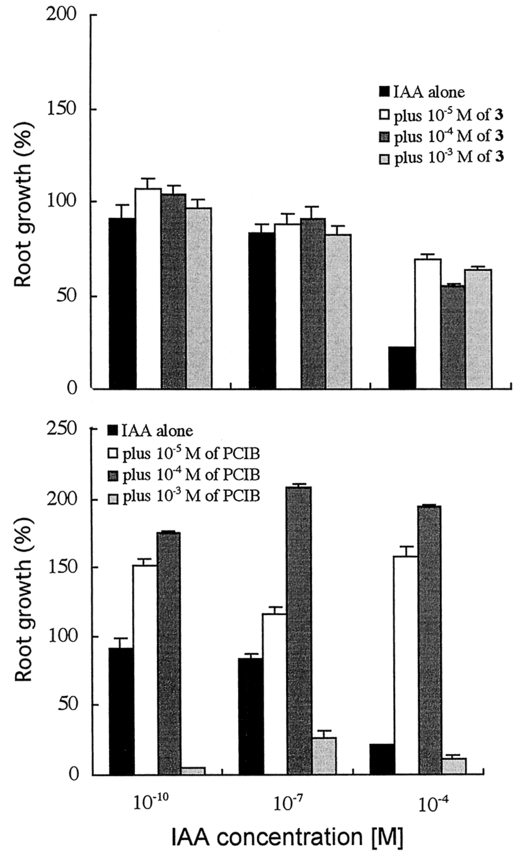


Fig. 4. Interactions between indole-3-acetic acid (IAA) and **3**, and between indole-3-acetic acid (IAA) and *p*-chlorophenoxyisobutyric ocid (PCIB) on the root growth of lettuce seedlings. Each value represents the mean ± SD (n = 3).

group is rare as fungal metabolite (Turner and Aldridge, 1983). Plant growth activities of **1**, **2** and **3** were examined using bioassay with lettuce seedlings (Fig. 2). All compounds showed no effect on the hypocotyl elongation at the concentration from 10^{-5} M to 10^{-3} M. **1** accelerated the root growth in proportion to its concentration from 10^{-5} M to 10^{-3} M. **2** accelerated the root growth to 214% of control at a concentration of 10^{-4} M, but **3** did not show any remarkable effect on that at the concentration from 10^{-5} M to 10^{-3} M.

Fig. 3 and 4 showed the interaction between IAA and the dihydroisocoumarin analogues **1**, **2** and **3** on root growth of lettuce seedlings. IAA showed no effect on the root growth at concentrations of 10^{-10} M and 10^{-7} M, but IAA inhibited the root growth to 22% of control at a concentration of 10^{-4} M. **2** prevented the inhibition of the root growth treated with 10^{-4} M of IAA at the concentrations of 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively. **3** showed the preventive effect weaker than that of **2** at the same concentrations. On the other hand, the inhibition treated with 10^{-4} M of IAA was not reduced by applying 10^{-5} M, 10^{-4} M and 10^{-3} M of **1**, respectively. *p*-Chlorophenoxyisobutyric acid (PCIB), a synthetic anti-auxin (Burström, 1950), accelerated the root growth to 237% of control at a concentration of 10^{-4} M but inhibited that to 6% of control at a concentration of 10^{-3} M (Fig. 2). The root growth treated with 10^{-4} M of IAA plus 10^{-5} M or 10^{-4} M of PCIB was promoted, but applying 10^{-4} M of IAA plus 10^{-3} M of PCIB inhibited the root growth stronger than that by 10^{-4} M of IAA alone (Fig. 4).

These results suggested that the root growth promoting activities of dihydroisocoumarins **1**, **2** and **3** were different from that of PCIB, and that **2** and **3** prevented the inhibitory effect of IAA against root growth of lettuce seedlings.

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