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Abstract

A novel indole derivative (1) and three known compounds (2–4) were isolated from the fruiting bodies of *Tricholoma flavovirens*. Their structures were determined or identified by the interpretation of spectroscopic data. Compounds 1 and 2 promoted root growth of lettuce and inhibited hypocotyl growth at 1 μmol/paper. Compound 3 inhibited hypocotyl and root growth at 100 nmol/paper.

Key words: *Tricholoma flavovirens*; structural determination; plant growth regulating activity; mushroom

We have been continuing to search for bioactive compounds from mushrooms using various bioassays. In our previous works, we have isolated several plant growth regulators from several kinds of fruiting bodies or culture broth of higher fungi.[1-3)]

We also reported the isolation and structural determination of a novel compound and a known one from the mushroom *Tricholama flavovirens*.[4)] During the further search, we succeeded in isolation of plant growth regulating compounds from this mushroom.

Here we describe the isolation and structural determination of a novel indole derivative and three known compounds, and their activity.

Fresh fruiting bodies of *T. flavovirens* were extracted with EtOH and then with acetone. After the solutions were combined and concentrated, they were partitioned between *n*-hexane and H_2O, EtOAc and H_2O, and then *n*-BuOH and H_2O. The *n*-hexane- and EtOAc-soluble parts were fractionated by repeated chromatography. As a consequence, four compounds (**1** – **4**) were purified (Fig. 1A).

Compound **1** was isolated as a white amorphous, mp 178-180 °C (decomp.). Its molecular formula was determined as $C_{12}H_{15}NO_2$ by HRESIMS at *m/z* 206.1165 $[M + H]^+$ (calcd. for $C_{12}H_{16}NO_2$ 206.1181), indicating the presence of six degrees of unsaturation in the molecule. The structure of **1** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMQC, and HMBC (Fig. 1B). The DEPT experiment indicated the presence of four methyls, two methines and six quaternary carbons. The structure of 2,4-dimethylindole skeleton was elucidated by the HMBC correlations (H-2-Me/C-2, C-3; H-4-Me/C-3a, C-4, C-5, H-6/C-4, C-5, C-7, C-7a) and the COSY correlations (H-2-Me/H-3). The HMBC correlations (H-5-OMe/C-5; H-7-OMe/C-7) indicated the position of 5-OMe and 7-OMe on the 2, 4-dimethylindole skeleton. The complete assignment of protons and carbons of NMR was accomplished as shown in Table 1. As a result, the structure of **1** was determined to be 5,7-dimethoxy-2,4-dimethylindole.

Compound **2** was isolated as a white amorphous. It was identified as 5-methoxy-2,4-dimethylindole. This compound has been reported as a degradation product when the bitter principle of *Tricholoma lascivum*, lascivol, was treated with strong acid, and has been isolated from the same genus mushroom *Tricholoma sciodes*.[5,6)]

Compound **3** was isolated as a white crystal. Its molecular formula was determined

as $C_{10}H_9NO_3$ by HRESIMS at *m/z* 190.0510 $[M - H]^-$ (calcd. for $C_{10}H_8NO_3$ 190.0504), indicating the presence of 7 degrees of unsaturation in the molecule. The structure of **3** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMQC, and HMBC (Fig. 1B). The DEPT experiment indicated the presence of one methyl, four methines and five quaternary carbons. The structure of phthalide skeleton was elucidated by the HMBC correlations (H-3/C-1, C-3a, C-4, C-7a; H-4/C-3, C-3a, C-5, C-6, C-7, C-7a; H-5/C-3a, C-4, C-6, C-7, C-7a; H-6/C-3a, C-4, C-5, C-7, C-7a) and the COSY correlations (H-4/H-5, H-5/H-6). The HMBC correlations (H-1'/C-2'; H-3'/C-2') indicated the presence of acetamido group. The connection between acetamido group and phthalide was confirmed by the HMBC correlations (H-3'/C-6, C-7a). The complete assignment of protons and carbons of NMR was accomplished as shown in Table 1. As a result, **3** was identified to be 7-acetamidophthalide. This compound has been synthesized, but this is the first report as a natural compound.[7)]

Compound **4** was isolated as a white amorphous. It was identified as 4-methoxymethyl- 3-[(2-methyl-4-indolyl)methyl]-2-methylindole. It has also been isolated from *T. sciodes* together with **2**.[6)]

Biological activities of compounds **2** to **4** have not been reported yet.

Compounds **1** to **3** were evaluated in the plant growth regulatory assay using lettuce (Fig. 2). 2,4-Dichlorophenoxyacetic acid was used as positive control, which inhibited the hypocotyl and root growth of lettuce dose-dependently. In order to know structure-activity relationship, **3** was compared with phthalide. As a result, **1** and **2** promoted the root growth and inhibited hypocotyl growth at 1 μmol/paper. **3** and phthalide inhibited the root growth dose-dependently. In addition, phthalide inhibited the hypocotyl growth at 1 μmol/paper, while **3** showed inhibition activity at 100 nmol/paper.

Experimental

General experiments. ^{1}H-NMR spectra (one-and two-dimensional) were recorded on a Jeol lambda-500 spectrometer (Jeol Ltd., Tokyo, Japan) at 500 MHz, while ^{13}C-NMR spectra were recorded by the same instrument at 125 MHz. HRESIMS data were measured by a JMS-T100LC mass spectrometer (Jeol Ltd., Tokyo, Japan). HPLC separation was performed with a Jasco Gulliver system (Jasco Co., Tokyo,

Japan) using a reverse-phase HPLC column (Cosmosil πNAP Waters, 10×250 mm, Nacalai tesque, Kyoto, Japan) and two normal phase HPLC columns (YMC-pack Diol-60-NP, 20×250 mm, YMC Co., Ltd., Kyoto, Japan; Senshu Pak AQ, 20×250 mm, Senshu Scientific Co., Ltd., Tokyo, Japan). Silica cartridges and C18 cartridges (Nihon Waters K.K., Tokyo, Japan) were used in the pro-processing of the samples. Silica gel plate (TLC Silica gel 60 F_{254}, Merck KGaA, Darmstadt, Germany) and silica gel 60N (Kanto Chemical Co.,Inc., Tokyo, Japan) were used for analytical TLC and for flash column chromatography, respectively.

Fungal strain and plant materials. Fresh fruiting bodies of *T. flavovirens* were collected at Narusawa village, Yamanashi Prefecture in Japan. Lettuce seeds (*Lactuca sativa* L. cv. Great Lakes 366; Takii Co., Ltd., Tokyo, Japan) were used in this study.

Extraction and isolation. The fresh fruiting bodies of *T. flavovirens* (20.6 kg) were extracted with EtOH (42 L, 3 times) and then with acetone (20 L, 3 times). After the solutions were combined and concentrated under reduced pressure, the concentrate was partitioned between *n*-hexane and H_2O, EtOAc and H_2O, and then *n*-BuOH and H_2O. The *n*-hexane-soluble part (39.8 g) was fractionated by silica gel flash column chromatography (CH_2Cl_2; 90/10, 80/20 CH_2Cl_2/acetone; 90/10, 80/20 CH_2Cl_2/MeOH; MeOH; 95/5 MeOH/H_2O; 2.0 L each) to obtain 20 fractions (fractions 1 to 20). Fraction 7 (6.78 g) was further separated by silica gel flash column chromatography (CH_2Cl_2; 95/5, 90/10, 80/20 CH_2Cl_2/acetone; MeOH; 2 L each) to give 14 fractions (fractions 7-1 to 7-14). Fraction 7-4 (23.3 mg) was further separated by normal-phase HPLC (YMC-pack Diol-60-NP, UV 245 nm, 5 mL/min, 30/70 hexane/$CHCl_3$) to afford **1** (1.5 mg). Fraction 7-3 (28.5 mg) was separated by reverse-phase HPLC (Cosmosil πNAP Waters, UV 254 nm, 2 mL/min, 80/20 MeOH/H_2O) to afford **2** (1.5 mg).

The EtOAc soluble part (16.4 g) was fractionated by silica gel flash column chromatography (CH_2Cl_2; 95/5, 90/10, 80/20, 70/30, 50/50 CH_2Cl_2/EtOAc; MeOH; 2L each) to obtain 17 fractions (fractions 1 to 17). Fraction 8 (426 mg) was fractionated by C18 cartridges to give two fractions (fractions 8-1 and 8-2). Fraction 8-1 (214 mg) was separated by silica gel flash column chromatography (CH_2Cl_2; 95/5, 90/10, 80/20 CH_2Cl_2/acetone; MeOH; 500 mL each) to give eight fractions (fractions 8-1-1 to 8-1-8). Fraction 8-1-5 was further fractionated by preparative TLC to give ten fractions (fractions 8-1-5-1 to 8-1-5-10). Fraction 8-1-5-1-5 (10.9 mg) was separated by reverse-phase HPLC (Cosmosil πNAP Waters, UV 255nm, 2 mL/min, 75/25

MeOH/H_2O) to afford **3** (7.7 mg). Fraction 10 (740 mg) was fractionated by silica gel flash column chromatography (CH_2Cl_2; 90/10 CH_2Cl_2/acetone; MeOH; 500 mL each) to give 11 fractions (fractions 10-1 to 10-11). Fractions 10-4 (5.5 mg), 10-5 (7.2 mg) and 10-6 (20.5 mg) were separated by normal-phase HPLC (Senshu Pak AQ, UV 270 nm, 5 mL/min, 70/30 hexane/$CHCl_3$) respectively to afford **4** (0.8 mg) in total.

Bioassay.[2,3] Lettuce seeds were put on filter paper (Advantec No. 2, ϕ55 mm; Toyo Roshi Kaisha, Ltd., Japan), soaked in distilled water in a Petri dish (ϕ60×20 mm) and incubated in a growth chamber under dark at 25˚C for 1 day. Each sample was dissolved in 1 mL of methanol (1, 10, 10^2 and 10^3 nmol/mL) and then poured on filter paper (ϕ55 mm) in a Petri dish (ϕ60×20 mm). After the solvent was air-dried, 1mL of distilled water was poured on the sample-loaded paper or intact filter paper (control). The pre-incubated lettuces (n = 9 in each petri dish) were transferred onto the filter paper and incubated in a growth chamber under dark at 25˚C for 3 days. The lengths of the hypocotyl and the root were measured using a ruler.

Author contribution

H. Ka. designed the experiments. W. Q., H. Ko. and J. W. performed the experiments. J. C., H. H. and H. Ka. contributed to discussions. W. Q. and H. Ka. wrote the manuscript.

Disclosure statements

No potential conflict of interest was reported by authors.

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Legend to figure

Fig. 1. Structures of compounds **1**–**4** (A) and COSY and HMBC correlations in **1** and **3** (B).

Fig. 2. Growth regulating activity against lettuce of compounds **1** to **3**.
Notes: white and black columns indicate the length of the hypocotyl and the root, respectively. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as positive control. Results are the mean ± standard deviation (n = 9). [$^{*}p < 0.05$, $^{**}p < 0.01$ (growth inhibition); $^{+}p < 0.05$, $^{++}p < 0.01$ (growth promotion)].

Table 1. ^{1}H and ^{13}C NMR Data for **1** and **3** (in $CDCl_3$)

Position	Compound **1**		Compound **3**	
	^{1}H (δ; multiplicity; *J* in Hz)	^{13}C δ	^{1}H (δ; multiplicity; *J* in Hz)	^{13}C δ
1	7.29 (br. s)			172.1
2		135.0		
3	6.14 (br.s)	99.4	5.28 (s)	69.9
3a		130.6		146.6
4		109.2	7.08 (d; 7.6)	115.9
5		151.1	7.60 (dd; 7.6, 8.2)	136.3
6	6.36 (s)	92.3	8.50 (d; 8.2)	118.3
7		143.5		138.8
7a		121.7		111.4
2-Me	2.41 (s)	13.7		
4-Me	2.31 (s)	11.4		
5-OMe	3.83 (s)	58.6		
7-OMe	3.91 (s)	55.5		
1’			2.24 (s)	24.9
2’				169.1
3’			9.56 (br. s)	

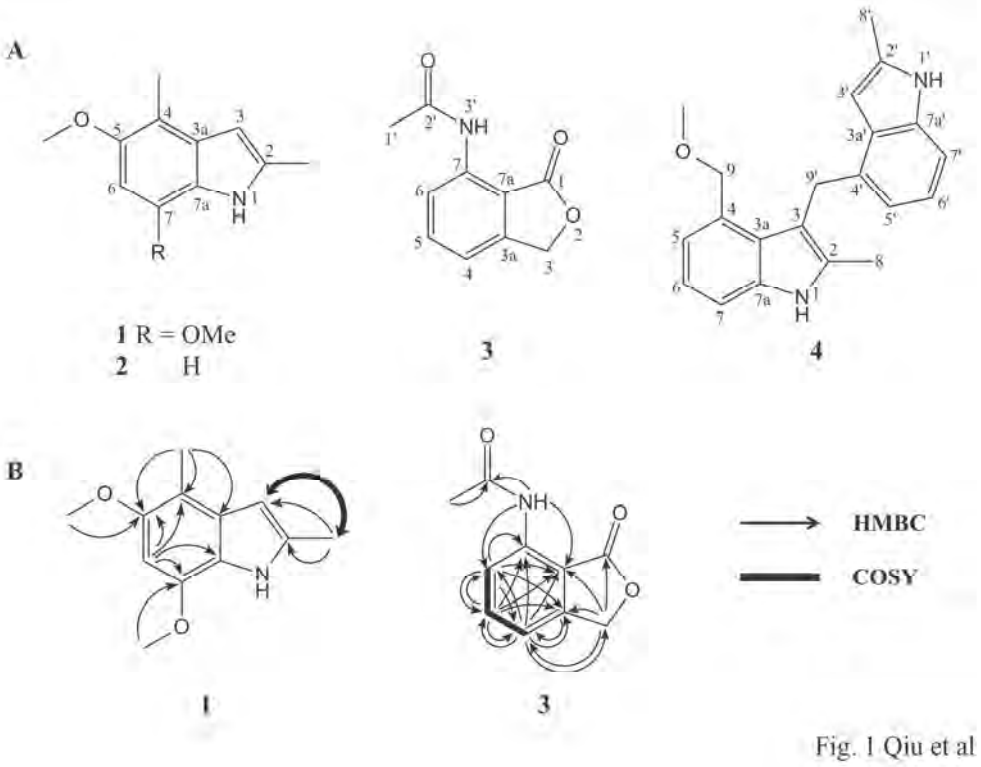


Figure 1

238x183mm (300 x 300 DPI)

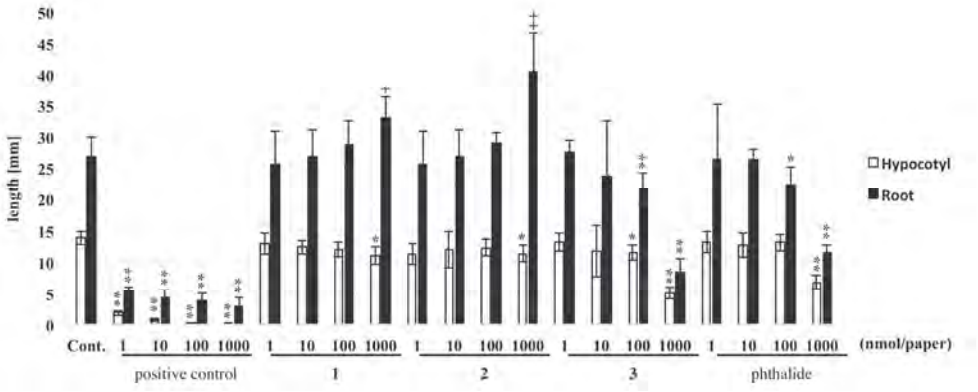
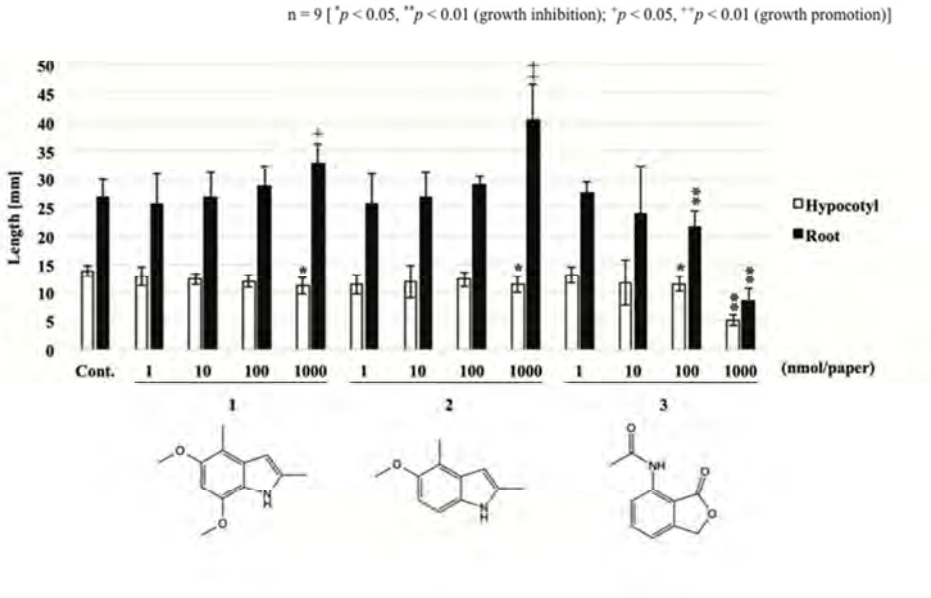


Fig. 2 Qiu et al

Figure 2

236x143mm (300 x 300 DPI)



Graphic abstract

254x190mm (72 x 72 DPI)

Plant growth regulators **1**, **2** and **3** were isolated from the mushroom *Tricholoma flavovirens*.