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Coprinolide, a novel antifungal tricyclic polyketide with a rare furanone-fused chromene skeleton isolated from the mushroom *Coprinus comatus*

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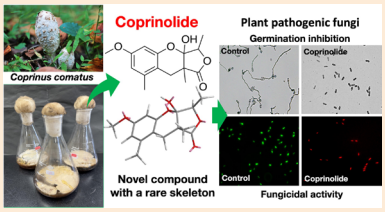
Supplementary material

A search for antifungal compounds from the mushroom *Coprinus comatus* using a bioassay-guided chromatographic fractionation approach led to the discovery of a novel polyketide harboring a rare 3,3a,9,9a-tetrahydro-1*H*-furo[3,4-*b*]chromen-1-one skeleton. The novel compound was named coprinolide. The inhibitory activity and fungicidal potential of coprinolide were evaluated against five economically important plant-pathogenic fungi. Coprinolide showed inhibitory effects on conidial germination and germ tube elongation of all tested fungi. The strongest effect was observed for *Colletotrichum orbiculare* with half-maximal inhibitory concentrations of 7.1 ppm and 8.2 ppm for conidial germination and germ tube elongation, respectively. Furthermore, coprinolide exhibited fungicidal activity against the tested fungi by inhibiting conidial germination to conidial death as confirmed by fluorescence microscopy using fluorescein diacetate and propidium iodide. These findings showed the potential of the mushroom as a source of a novel bioactive compound with promising agricultural application as an antifungal agent against different plant-pathogenic fungi.



Keywords: mushroom, polyketides, novel antifungal compound, plant pathogenic fungi, conidial germination, conidial viability.

Introduction

Diseases caused by plant pathogenic fungi are one of the main contributors to significant economic losses in the agricultural industry. The most prevalent plant pathogenic fungi include *Colletotrichum* species, which cause anthracnose disease in different fruits and crops,[1)] *Pyricularia oryzae*, the causal agent of rice blast disease,[2)] and *Fusarium oxysporum* f. sp. *cubense*, which causes Fusarium wilt and the most devastating fungal pathogen of banana, worldwide.[3)] Plant pathogenic fungi employ diverse strategies and mechanisms for a successful infection of their hosts. The attachment of fungal spores to the host plant is considered the primary requirement for spore germination, germ tube elongation, secretion of cell wall-degrading enzymes, up to the formation of specialized structure such as appressorium.[4–6)] Some plant pathogenic fungi such as *Colletotrichum* spp. and *Pyricularia oryzae* produce highly specialized melanized appressoria, which are important features for the successful penetration of their hosts.[7)] However, other plant pathogenic fungi particularly *F. oxysporum* f. sp. *cubense* do not produce appressoria or penetration pegs to invade its host successfully.[8,9)] *F. oxysporum* f. sp. *cubense* undergoes asexual reproduction, and produces three different types of spores: microconidia, macroconidia and chlamydospores.[10)] Conidia and chlamydospores are involved in the infection of host plant, leading to wilting,[11)] and

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should therefore be one the targets of any infection management strategies. However, the elimination of these plant pathogenic fungi is a huge challenge for farmers and agricultural producers. Currently, commercially available fungicides are the mainstay of treatment for the prevention of diseases caused by some plant pathogenic fungi. However, the continuous and excessive use of commercially available fungicides has disadvantages such as pollution to the natural environment and adverse effects on the other organisms associated with cultivated plants,[12] and could lead to the emergence of fungicide-resistant fungal pathogens. Therefore, the discovery of bio-eco-friendly solutions that can inhibit plant pathogenic fungi, particularly their conidial germination, is essential. The search for novel bioactive compounds from natural sources has gained considerable attention as it will lead to the discovery of new antifungal agents to combat various plant diseases caused by fungi.

Among the vast number of natural sources, mushrooms have been proven as essential producers of novel bioactive compounds. For instance, a new β-carboline alkaloid, 1-acetyl-7-hydroxy-β-carboline-3-carboxylic acid, from *Sarcomyxa edulis* exhibited an anti-inflammatory activity against lipopolysaccharide (LPS)-induced NO production in RAW 264.7 macrophage.[13] Nineteen new sesquiterpenoids, including bergamotane sesquiterpenes (craterodoratins A–R), and a victoxinine derivative (craterodoratin S), were isolated from the edible mushroom *Craterellus odoratus*. Of these new compounds, seven (craterodoratins C, J, L, M, N, O, and S) exhibited potent inhibitory activity against LPS-induced proliferation of B lymphocyte cells.[14] Additionally, eight new compounds, furanopaxins A–F, deoxybisinvolutone and coumarinvol were isolated from the wild mushroom *Paxillus involutus*. Furanopaxins E and F, deoxybisinvolutone, and coumarinvol showed significant α-glucosidase inhibitory activities.[15] In addition, cyclocircadins A and B, isolated from *Cyclocybe erebia* showed circadian rhythm-regulating activity.[16] Recently, a phenolic compound inaoside A, isolated from the edible mushroom *Laetiporus cremeiporus*, was shown to exhibit significant antioxidant activity.[17] These studies also highlighted the diverse biological activities of these novel compounds. While various species of mushrooms have been studied leading to the isolation of novel compounds, many species remain unexplored. The isolation of novel compounds from mushrooms for agricultural applications, specifically as antifungals against different plant pathogenic fungi is highly desirable.

Cop. comatus, a basidiomycete of the Agaricaceae family under the order Agaricales has been widely studied for its nutritional values, chemical composition, and functional properties.[18,19] Previous works reported that *Cop. comatus* may contain compounds with antimicrobial activities[18,19] and therefore, can be a target for the isolation of compounds for agricultural application, specifically as antifungals. Recently, we isolated a potent antifungal compound orsellinaldehyde, from the culture filtrate of *Cop. comatus* against anthracnose pathogens. Orsellinaldehyde showed significant inhibitory and fungicidal activity against the conidial germination and germ tube elongation of different *Colletotrichum* species, and suppressed anthracnose symptoms on mango and cucumber fruits infected with *Colletotrichum gloeosporioides* and *Col. orbiculare*, respectively.[20]

During the isolation of the antifungal compound from *Cop. comatus*, we detected strong antifungal activity against *Col. gloeosporioides* in another fraction. In the present study, this fraction was further purified, leading to the isolation of a novel antifungal compound, together with a known compound. The isolated novel compound was identified through spectroscopic analyses. The inhibitory activity of the isolated compound was evaluated against the conidial germination and germ tube elongation of five economically important plant pathogenic fungi. The findings of this study provide insights into the potential of mushrooms as a source of novel biologically active compounds against plant pathogenic fungi for agricultural application and will serve as additional baseline information for future studies on the development of bio-eco-friendly solutions against the problems caused by plant pathogenic microorganisms.

Materials and methods

1. *General experimental procedures*

The high-resolution mass spectra (HRMS) were measured in an Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) while electrospray ionization (ESI) mass spectra were recorded using a Quattro Micro API mass spectrometer (Waters, Milford, MA, USA) connected with an Acquity UPLC (Waters). The ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), and 2D NMR spectra (COSY, HMQC, HMBC and NOESY) were recorded using a JEOL JNM-ECZ600 (Tokyo, Japan). Preparative high-performance liquid chromatography (HPLC) was performed using a 10A HPLC system (Shimadzu, Kyoto, Japan). The specific rotation was measured using a SEPA-500 polarimeter (HORIBA, Kyoto, Japan) while the circular dichroism (CD) spectrum was measured using J-820 circular dichroism spectrometer (JASCO, Tokyo, Japan).

2. *Mushroom strain*

The strain of *Cop. comatus* (TUFC 30838) used in this study was obtained from the Fungus/Mushroom Resource and Research Center (FMRC), Tottori University, Japan. It was immediately sub-cultured on potato dextrose agar (PDA) (DB Difco, Sparks, MD, U.S.A.) slants and incubated at 25°C for further use.

3. *Plant pathogenic fungi*

The strains of *Col. gloeosporioides* MAFF 243178, *Col. orbiculare* MAFF 726522, and *P. oryzae* MAFF 101512 were obtained from the the National Agriculture and Food Research Organization (NARO) Genebank (www.gene.affrc.go.jp/index_en.php). *F. oxyporum* f. sp. *cubense* race 1 (R1) 160527 and *F. oxyporum* f. sp. *cubense* race TR4 (TR4) MSK1-1 are strains preserved in Plant Pathology Laboratory in Tokyo University of Agriculture and Technology, Tokyo. All fungal strains were sub-cultured on PDA slant and incubated at 25°C for further use.

4. Cop. comatus culture filtrate preparation and extraction

Cop. comatus was first grown on malt extract agar (MEA) (50 g malt grain, 30 g glucose, 3 g peptone, 15 g agar, and 1 L tap water) plate at 25°C for two months. To prepare the liquid culture of *Cop. comatus*, three mycelial disks approximately 6 mm in diameter from the MEA plate were inoculated to a 500-mL flask containing 200 mL of malt extract broth (MEB, 50 g malt grain, 30 g glucose, 3 g peptone and 1 L tap water). In total, we cultured *Cop. comatus* in 5 L of MEB. The culture was incubated at 25°C under stationary condition for five months. The contents of the 25 flasks were combined and filtered through layers of Whatman filter paper no. 1 and cheesecloth. The culture filtrate was extracted three times with ethyl acetate (EtOAc), and the resulting EtOAc layer was dried over Na_2SO_4 overnight and evaporated to dryness in a rotary evaporator. The EtOAc extract yielded 2.6 g.

5. Bioassay-guided isolation of antifungal compounds

The *Cop. comatus* culture filtrate extract (2.6 g) was subjected to silica gel column chromatography (130 g silica gel, Daisogel IR-60-63/210; Daiso, Osaka, Japan) using 1300 mL each of acetone and hexane mixtures as eluting solvent (0–100% acetone, 20% increment, v/v). The eluted fractions were evaporated to dryness and subjected to a bioassay for the inhibition of conidial germination against *Col. gloeosporioides*. The strongest inhibitory activity was observed in the 20% acetone fraction (479.4 mg). This fraction was applied again onto a silica gel column (24 g silica gel) using 280 mL of EtOAc and hexane mixtures (0–100% EtOAc, 20% increment, v/v). The eluted fractions were concentrated to dryness and subjected to a bioassay to inhibit the conidial germination of *Col. gloeosporioides*. The activity was observed in the 20% EtOAc fraction (67 mg), which was subjected to further fractionation by ODS column chromatography (3.3 g; Cosmosil 75C_{18}-OPN, Nacalai Tesque, Kyoto, Japan) using 60 mL mixtures of methanol (MeOH) and water as eluting solvent (20–100% MeOH, 20% increment, v/v). The strongest inhibitory activity was observed in 40% MeOH fraction from which orsellinaldehyde was previously isolated. Furthermore, another fraction (60% MeOH fraction) had also shown strong inhibitory activity against *Col. gloeosporioides* and was subjected to preparative HPLC under the following conditions: column: Cosmosil 5C_{18} AR-II 10 ID×250 mm; temperature: 40°C; flow rate: 1.5 mL/min; solvent: 55% acetonitrile; detection: 210 nm. Two peaks at retention times of 20.5 min and 22.5 min were isolated. The peak at retention time of 20.5 min was identified as 4,6-dimethoxyphthalide upon confirmation of the NMR data with the NMR data of 4,6-dimethoxyphthalide in our previous study.[21] We focused on identifying the compound corresponding to the peak at a retention time 22.5 min, and denoted as compound **1** (3.4 mg). To obtain a higher yield of **1**, *Cop. comatus* was cultured again in MEB for six months. We purified **1** following the purification process described above. Successfully, **1** was isolated again with 3.1 mg yield.

Spectroscopic data of compound **1**: $[\alpha]_D^{20} = -30.3°$ (*c* 0.08, $CHCl_3$); ECD (MeOH) λ[nm]($\Delta\varepsilon$): 234 (–2.1); ESIMS positive: *m/z* 279.3 $[M+H]^+$, negative: *m/z* 277.2 $[M-H]^-$, *m/z* 323.2 $[M+HCOO]^-$ (Fig. S1); HRESIMS positive: *m/z* 279.1223 $[M+H]^+$ (calcd for $C_{15}H_{19}O_5$, 279.1233), *m/z* 301.1042 $[M+Na]^+$ (calcd for $C_{15}H_{18}O_5Na$, 301.1052), negative: *m/z* 277.1083 $[M-H]^-$ (calcd for $C_{15}H_{17}O_5$, 277.1076), *m/z* 313.0853 $[M+^{35}Cl]^-$ (calcd for $C_{15}H_{18}O_5{}^{35}Cl$, 313.0843) and *m/z* 315.0824 $[M+^{37}Cl]^-$ (calcd

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data of coprinolide (**1**)

Position	^{1}H NMR						^{13}C NMR
	DMSO-d_6			$CDCl_3$			$CDCl_3$
	δ_H, ppm	multiplicity		δ_H, ppm	multiplicity		δ_c, ppm, type
1							177.4, C
3	4.64	q (*J*=6.6 Hz)	H	4.65	q (*J*=6.6 Hz)	H	80.0, CH
3a							102.2, C
4a							152.6, C
5	6.24	d (*J*=2.4 Hz)	H	6.32	d (*J*=2.4 Hz)	H	100.1, CH
6							159.1, C
7	6.39	d (*J*=2.4 Hz)	H	6.43	d (*J*=2.4 Hz)	H	110.4, CH
8							137.9, C
8a							110.9, C
9	2.83 2.56	d (*J*=16.2 Hz) d (*J*=15.6 Hz)	2H	2.82 2.76	d (*J*=15.6 Hz) d (*J*=16.2 Hz)	2H	30.3, CH_2
9a							44.9, C
10	1.32	d (*J*=6.6 Hz)	3H	1.49	d (*J*=6.6 Hz)	3H	12.8, CH_3
11	3.64	s	3H	3.74	s	3H	55.3, CH_3
12	2.13	s	3H	2.21	s	3H	19.3, CH_3
13	1.10	s	3H	1.23	s	3H	16.0, CH_3
OH	7.38	brs	1H				

for $C_{15}H_{18}O_5{}^{37}Cl$, 315.0819). The ^1H and ^{13}C NMR data are presented in Table 1 (Figs. S2).

6. Bioassays for inhibitory activities on the conidial germination and germ tube elongation

Col. gloeosporioides MAFF 243178 was grown on PDA plates, while *Col. orbiculare* MAFF 726522 was grown in rice bran agar (RBA) plates (50 g rice bran, 20 g sucrose, 20 g agar, 1 L distilled water). *F. oxyporum* f. sp. *cubense* R1 and *F. oxyporum* f. sp. *cubense* TR4 were grown on V8 juice medium plates [200 mL V8 juice (Del Monte Quality Vegetable Juice, Nagano, Japan), 3 g $CaCO_3$, 15 g agar at 1.5%, and 1 L distilled water]. The plates were incubated at 25–27°C for 7–10 days. Meanwhile, *P. oryzae* MAFF 101512 was grown on oat meal agar (OMA) plates [50 g oatmeal (Melvit Secret Whole Rolled Oats, Warszawa, Poland), 20 g agar, 1 L distilled water], and incubated at 20°C for 14 days under a black light lamp (FL-15BLB, Hitachi, Tokyo, Japan). The aerial hyphae were removed by flooding the plate with distilled water containing 0.01% glucose by gently rubbing the surface with a spatula, and the plate was further incubated for another 3 days at 25°C. In order to collect conidia of all the test fungi, the surface of the fungal colony was flooded with distilled water containing 0.25% Tween 20 and gently scraped using a spatula. The collected conidial suspension was filtered through 4 layers of cheesecloth and microcentrifuged twice at 1200 rpm for 3 min. The conidia were resuspended in distilled water containing 0.25% Tween 20 and the number of conidia was adjusted to 5×10^5 conidia/mL using a cell counting chamber and used immediately for the inhibition of the conidial germination assay.

The assay for the inhibition of conidial germination and germ tube elongation was based on the study of Morimoto *et al.* (2018).[22] Initially, **1** was dissolved in DMSO and added to the conidial suspensions to achieve the following compound concentrations: 1, 3, 10, 30, 100, 300 and 1000 ppm, with a final concentration of DMSO at 1%. As a control, DMSO was added to the fungal suspension. The mixture was vortexed and three drops (25 μL) were placed on a microscope glass slide coated with collodion. The glass slides were placed in a container with a cover lined with tissue paper moistened with distilled water and incubated at 25°C for 24 hr in the dark. After incubation, the germinated and ungerminated conidia were observed under a compound microscope (Olympus BX43, Tokyo, Japan). The number of germinated and ungerminated conidia was counted at a limit of 100 conidia in each drop. Germ tube elongation was then measured using the ImageJ software (https://imagej.nih.gov/ij/index.html). The inhibition rate on conidial germination was calculated using the following formula:

$$\%\text{Inhibition rate} = \frac{Gc - Gt}{Gc} \times 100$$

where *Gc* is the number of germinated conidia in the control, and *Gt* is the number of germinated conidia in the sample treated with the compound. Moreover, the inhibition rate on germ tube elongation was computed using the same formula using the length (μm) of the germ tube.

For the determination of the half maximal inhibitory concentration (IC_{50}) of **1** on each fungus, a dose-response curve was generated using the log (conc.) of the compound on the *x* axis and % inhibition on the *y* axis. Then, the IC_{50} value was calculated from a linear line drawn between the two points that encompass 50% inhibition.

7. Fungal conidia viability staining assay

The fungicidal activity of **1** was determined by a fungal conidia viability staining assay using fluorescein diacetate (FDA) and propidium iodide (PI) (Wako Pure Chemical Industries, Osaka, Japan) according to Tang *et al.* (2020)[23] with some modifications. A fungal conidial suspension adjusted to 5×10^5 conidia/mL was added with the test compound dissolved in DMSO to achieve the following compound concentrations: 1, 3, 10, 30, 100, 300 and 1000 ppm, with a final concentration of DMSO at 1%. As a control, DMSO was added to the fungal suspension at 1%. The mixture was vortexed and incubated for 24 hr at 25°C. The conidia were then washed twice with sterile distilled water by microcentrifugation at 1200 rpm for 3 min, and 100 μL of conidial suspension was added with 20 μL FDA and 20 μL PI solutions. The viability of fungal conidia was observed under a fluorescence microscope (Keyence BZ-X810, Osaka, Japan) within 10 min. Dead conidia exhibited red fluorescence while living conidia appeared in green when stained with PI and FDA, respectively.[24]

8. ECD Calculation

Chem3D (version 20.1, Revvity, Waltham, MA, U.S.A.) was used to generate pdb file of **1**. Conformational searches were performed using GROMACS program (version 2018.6) with the general AMBER force field (GAFF2) in the Amber Tools 21 package. Possible rotamers of **1** in terms of the methoxy group at C-6 and hydroxy group at C-3a were built using Avogadro (version 12.0). Optimization and frequency analysis were performed using Gaussian16 software[25] at the B3LYP/631G(d) level in the gas phase. The three most stable conformers (Supplemental Fig. S14, Table S1–S4) were subjected to re-optimization and frequency analysis using M06-2X/6-311G+(2d,p) in methanol solution with IEFPCM. The ECD calculations were performed using TDDFT method with the M062X/aug-cc-pVTZ level in methanol solution with IEFPCM. The final ECD spectra of the conformers were weighed according to the Boltzmann distribution theory. The generated ECD spectra for the two enantiomers of **1** were compared with the experimental CD.

9. Detection of compound 1 from the mycelia of Cop. comatus

Mycelia obtained from the liquid culture of *Cop. comatus* were dried for 72 hr in a fume hood. Dried mycelia were soaked in MeOH 3 times every 24 hr. The MeOH extracts were collected every 24 hr and extracted 3 times with EtOAc and water. The EtOAc layer was dried over Na_2SO_4 overnight and evaporated to dryness by a rotary evaporator. The resulting extract was sub-

jected to two rounds of silica gel column chromatography and ODS column chromatography under the same conditions used for the fractionation of the culture filtrate. The MeOH fraction obtained from the ODS column chromatography was subjected to HPLC analysis under the following conditions: column: Cosmosil $5C_{18}$ AR-II 4.6 ID×150 mm; temperature: 40°C; flow rate: 0.8 mL/min; solvent: MilliQ water (A), ACN (B), gradient: B/(A+B)=5–100%, 30 min; detection: 210 nm. The peaks corresponding to orsellinaldehyde was detected at ODS–MeOH 20% fraction, while peak corresponding to **1** was detected at ODS–MeOH 40% fraction. 4,6-dimethoxyphthalide was detected at both ODS–MeOH 20% and 40% fractions.

Results

1. *Identification of a novel antifungal compound from Cop. comatus*

We extracted the culture filtrate of *Cop. comatus* using ethyl acetate and performed bioassay-guided fractionation of the extract to identify a new antifungal compound. Compound **1** was purified as a pale-yellow oil by two rounds of silica gel column chromatography, ODS column chromatography and preparative high performance liquid chromatography (HPLC). It has a molecular formula of $C_{15}H_{18}O_5$ and molecular weight of 278 as determined by the exact masses of positive (*m/z* 279.1223 $[M+H]^+$ and *m/z* 301.1042 $[M+Na]^+$) and negative ions (*m/z* 277.1083 $[M-H]^-$, *m/z* 313.0853 $[M+^{35}Cl]^-$ and *m/z* 315.0824 $[M+^{37}Cl]^-$) in HR ESI-MS. Thus, the hydrogen deficiency index of **1** was seven. The ^1H NMR analysis revealed two doublet signals (δ_H 6.43 and 6.32 ppm; J=2.4 Hz) corresponding to a tetrasubstituted benzene ring, as well as the signals derived from one methine (δ_H 4.65 ppm), one methylene (δ_H 2.82 and 2.76 ppm), and four methyl groups (δ_H 3.74, 2.21, 1.49 and 1.23 ppm). On the other hand, ^{13}C NMR spectra showed signals corresponding to carbons in a benzene ring (δ_C 159.1, 152.6, 137.9, 110.9, 110.4 and 100.1 ppm), a carbonyl (δ_C 177.4 ppm), a methoxy (δ_C 55.3 ppm), an aliphatic methylene (δ_C 30.3 ppm), a hemiketal (δ_C 102.2 ppm), an oxymethine group (δ_C 80.0 ppm), a quaternary carbon (δ_C 44.9 ppm), and three methyl groups (δ_C 19.3, 16.0 and 12.8 ppm) (Table 1, Supplemental Fig. S2). The proton–carbon bonds were determined *via* HMQC analysis (Supplemental Fig. S3). ^1H–^1H COSY analysis showed a signal connecting H-3 and H-10, indicating a substructure of –CH–CH_3 (Fig. 1B, Supplemental Fig. S4). Furthermore, the presence of OH group was confirmed by ^1H NMR analysis measured using DMSO-d_6 as the solvent (Supplemental Fig. S6). The proton signal at δ_H 7.38 ppm corresponded to a hydroxyl group, shifted low field due to its attachment in a hemiacetal carbon at C-3a (δ_C 102.2 ppm). These partial structures were connected based on the HMBC correlations (Fig. 1B, Supplemental Fig. S5). The IUPAC name of **1** is 3a-hydroxy-6-methoxy-3,8,9a-trimethyl-3,3a,9,9a-tetrahydro-1*H*-furo[3,4-*b*]chromen-1-one (Fig. 1A).

The relative configurations of **1** were determined by 2D NOESY spectrum that revealed the correlations from H-10 (δ_H 1.32 ppm) to H-13 (δ_H 1.1 ppm) and OH-3a (δ_H 7.38 ppm). This indicated that the methyl groups attached to C-3 (δ_C 80.0 ppm) and C-9a (δ_C 44.9), and the OH group at C-3a (δ_C 102.2) were on the same spatial directions (Fig. 1C, Supplemental Fig. S6). The absolute configurations were determined by TDDFT calculations of the ECD spectra of the two possible enantiomers of **1**. The circular dichroism (CD) spectrum of **1** showed a negative cotton effect at 234 nm ($-2.1\Delta\varepsilon$) (Fig. 1D). One of the calculated ECD of two possible enantiomers was in accordance with the experimental spectrum leading to the assignment of the absolute configurations of **1** as 3*R*, 3a*S*, 9a*R* (Fig. 1D). These data established the overall identification of **1**. Because **1** has not yet been reported up to date, we named it as "coprinolide."

The mycelial extracts of *Cop. comatus* was also examined for the presence of compounds isolated from the culture filtrate. The HPLC analysis showed peaks corresponding to coprinolide as well as peaks corresponding to orsellinaldehyde[20] and 4,6-dime-

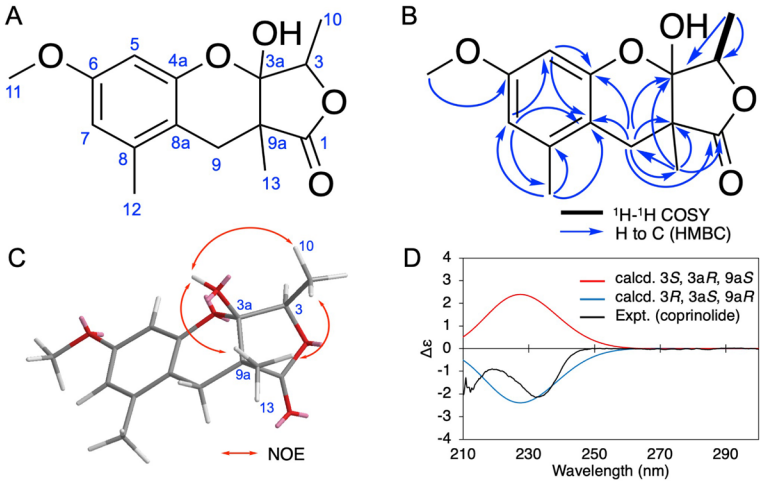

Fig. 1. The antifungal compound isolated from the culture filtrate of *Cop. comatus*. (A) Chemical structure of coprinolide (compound **1**). (B) HMBC and ^1H–^1H COSY correlations, (C) Key NOE correlations. (D) Experimental and calculated ECD spectra of coprinolide in MeOH.

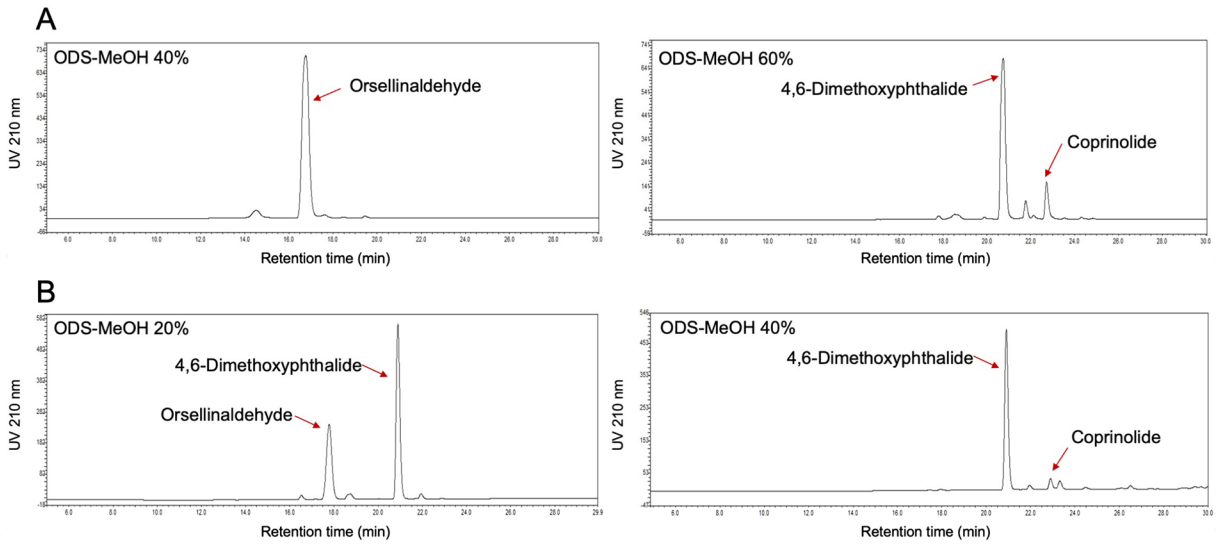

Fig. 2. HPLC analyses of the fractions showing the peaks corresponding to the isolated compounds from *Cop. comatus*. Fractions obtained from the culture filtrate (A) and mycelia (B) were analyzed by gradient HPLC.

thoxyphthalide.[21] The ODS–MeOH 20% fraction of the mycelial extracts showed peaks corresponding to orsellinaldehyde and 4,6-dimethoxyphthalide, while ODS–MeOH 40% fraction showed peaks corresponding to 4,6-dimethoxyphthalide and coprinolide. In comparison with the culture filtrate extracts, orsellinaldehyde was isolated from ODS–MeOH 40% fraction, while 4,6-dimethoxyphthalide and coprinolide were isolated from ODS–MeOH 60% fractions (Fig. 2A and 2B). The difference of the fraction containing these compounds between the culture filtrate and mycelia extracts may be because the differences in other substances present in the culture filtrate and mycelial extracts affected the timing of elution of the compounds. The presence of these compounds in both culture filtrate and mycelial extracts of *Cop. comatus* suggest intracellular production and eventually extracellular release of the compounds as well as their possible biosynthetic relationship.

2. Coprinolide inhibits conidial germination and germ tube elongation of plant pathogenic fungi

The antifungal activity of coprinolide was determined through the inhibition of the conidial germination and germ tube elongation assay against *Col. gloeosporioides*, *Col. orbiculare*, *F. oxysporum* f. sp. *cubense* R1, *F. oxyporum* f. sp. *cubense* TR4, and *P. oryzae*. All tested fungi showed sensitivity to coprinolide in a dose-dependent manner starting from 1 ppm. Complete inhibition of conidial germination and germ tube elongation of *Col. gloeosporioides*, *F. oxyporum* f. sp. *cubense* R1 and *F. oxyporum* f. sp. *cubense* TR4 was observed at 1000 ppm of coprinolide (Figs.

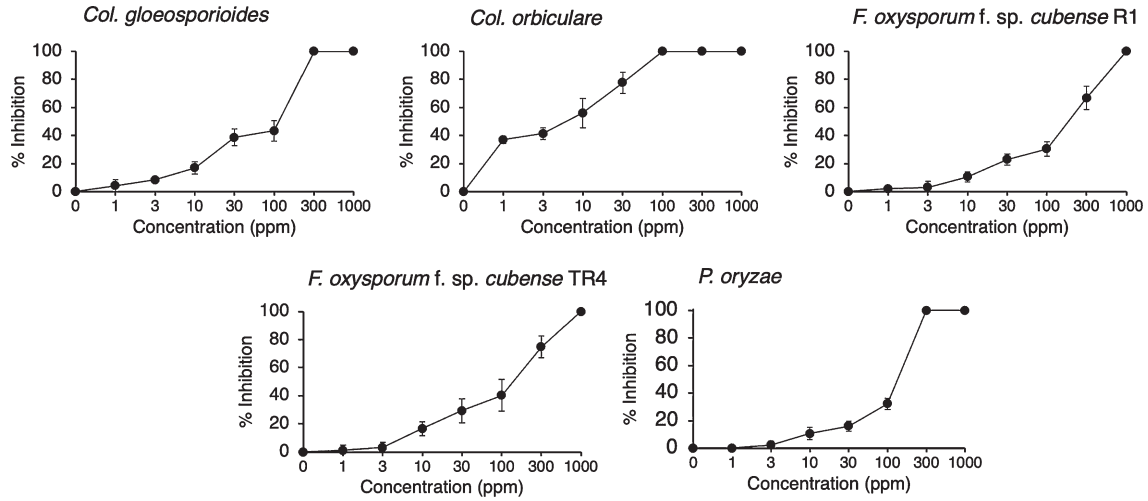

Fig. 3. Inhibitory activity of coprinolide on conidial germination of different plant pathogenic fungi. DMSO solutions of compounds were added to the fungal conidial suspension (5×10^5 conidia/mL). As control, DMSO was added to the conidial suspension at 1%. After 24 hr, the germinated conidia were counted. Data presented are mean ($n=3$)±SD.

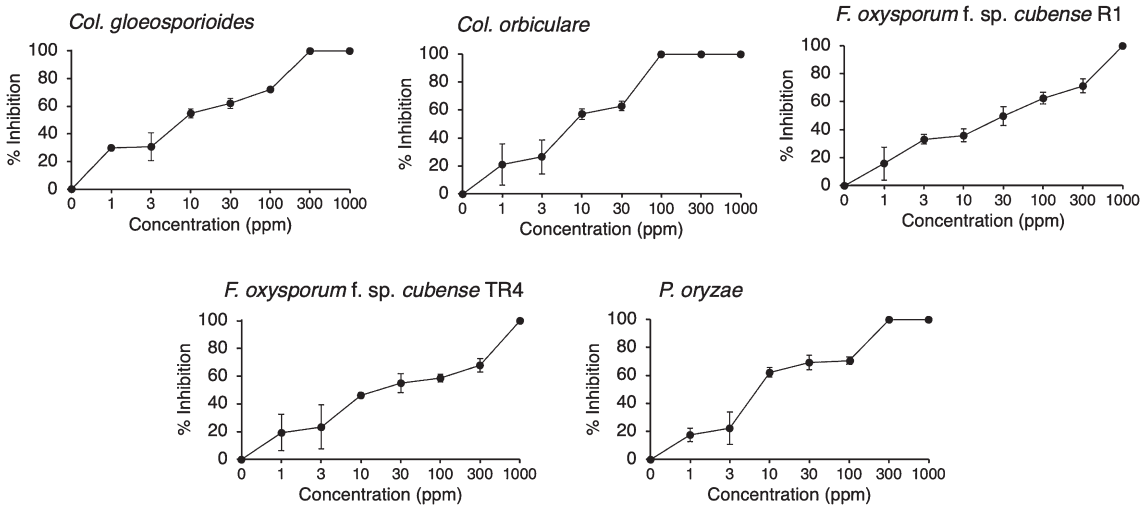

Fig. 4. Inhibitory activity of coprinolide on germ tube elongation of different plant pathogenic fungi. DMSO solutions of compounds were added to the fungal conidial suspension (5×10^5 conidia/mL). As control, DMSO was added to the conidial suspension at 1%. After 24 hr, the lengths of germ tubes were measured using ImageJ software. Data presented are mean ($n=3$)±SD.

3 and 4, Supplemental Figs. S7 and S8) whereas the complete inhibition of *P. oryzae* was observed starting at 300 ppm. Among the tested fungi, *Col. orbiculare* was the most sensitive in conidial germination with IC_{50} value 7.1 ppm and completely inhibited at concentration of 100 ppm or higher. For germ tube elongation, *P. oryzae*, *Col. orbiculare* and *Col. gloeosporioides* were the most sensitive with IC_{50} values of 7.7 ppm, 8.2 ppm and 8.6 ppm, respectively (Table 2). Furthermore, judging from the IC_{50} values, coprinolide showed a relatively stronger effect on the germ tube elongation of all the tested pathogenic fungi than on their conidial germination.

3. Coprinolide exhibits fungicidal activity against various plant pathogenic fungi

The fungicidal activity of coprinolide was assessed by viability staining using FDA and PI. Conidia of the *Col. gloeosporioides*, *F. oxyporum* f. sp. *cubense* R1 and *F. oxyporum* f. sp. *cubense* TR4 treated with 1–300 ppm of coprinolide showed bright green fluorescence, indicative of live conidia. However, treatment of the compound at 1000 ppm resulted in the emission of red fluorescence indicating the death of the conidia (Fig. 5A, C–E, Supplemental Figs. S9, and S11–S13). On the other hand, coprinolide at 300 ppm showed fungicidal activity against *P. oryzae*. In agreement with the inhibition of conidial germination, where in *Col. orbiculare* was completely inhibited by coprinolide at 100 ppm or higher, fluorescence microscopy confirmed that coprinolide at these concentrations was fungicidal against *Col. orbiculare* (Fig. 5B and Supplemental Fig. S10).

Table 2. IC_{50} values of coprinolide on conidial germination and germ tube elongation of different plant pathogenic fungi

Plant pathogenic fungi	Conidial germination	Germ tube elongation
	*IC_{50} (ppm)	*IC_{50} (ppm)
Colletotrichum gloeosporioides	123.5^{b}	8.6^{a}
Colletotrichum orbiculare	7.1^{a}	8.2^{a}
Fusarium oxysporum f. sp. *cubense* R1	207.7^{c}	35.6^{b}
Fusarium oxysporum f. sp. *cubense* TR4	157.6^{bc}	25.8^{ab}
Pyricularia oryzae	152.2^{bc}	7.7^{a}

*The IC_{50} values of coprinolide were calculated from the data for Figs. 3 and 4, and Supplemental Figs. S7 and S8. Different letters indicate significant differences among fungi in the same column ($p<0.05$; Tukey–Kramer test).

Discussion

Mushrooms are essential producers of compounds for agricultural applications. For instance, the first natural strobilurin, strobilurin A was isolated from the mushroom *Strobilurus tenacellus*.[26] Strobilurins have been practically used as fungicides globally to control different plant diseases caused by fungi. They are considered as quinone outside inhibitors (QoI) fungicides that inhibit mitochondrial respiration by targeting the quinol oxidation site of cytochrome *b*.[27] The unique mechanism of action of strobilurins of targeting one specific site of fungal pathogens made them susceptible to resistance. There are studies that reported the resistance of some fungi to strobilurin fungicides.[28,29] Due to these, the discovery of novel compounds for agricultural applications specifically as antifungals against plant pathogenic fungi is highly desirable as it will lead to the development of new fungicide classes against plant pathogenic fungi.

In our previous study, we successfully isolated and identified orsellinaldehyde from the culture filtrate of *Cop. comatus* which can suppress anthracnose disease.[20] In the present study, we found that another fraction of *Cop. comatus* culture filtrate showed strong antifungal activity against *Col. gloeosporioides*. We then subjected the fraction to further fractionation, leading to the identification of a novel antifungal compound together

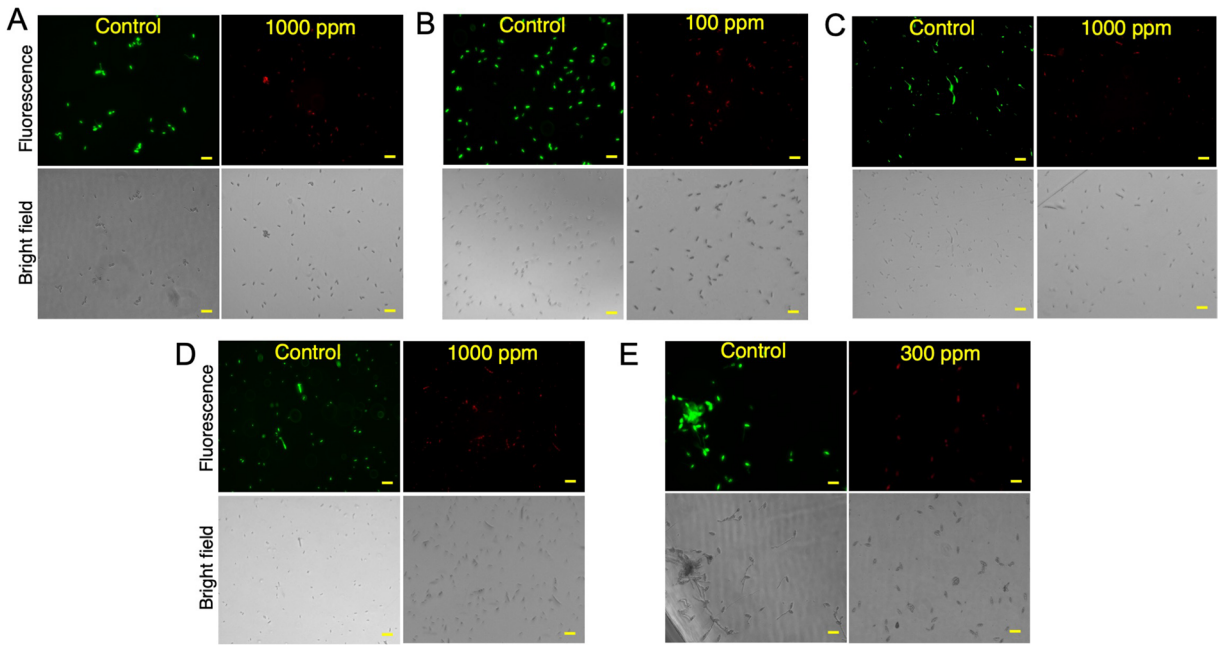

Fig. 5. Effect of coprinolide on conidial viability of different plant pathogenic fungi. (A) *Col. gloeosporioides*, (B) *Col. orbiculare*, (C) *F. oxysporum* f. sp. *cubense* R1, (D) *F. oxysporum* f. sp. *cubense* TR4, (E) *P. oryzae*. DMSO solutions of compounds were added to the fungal conidial suspension (5×10^5 conidia/mL). As control, DMSO was added to the conidial suspension at 1%. After 24 hr, conidia were washed with sterile distilled water, added with equal volume of FDA and PI, and immediately observed in fluorescence microscope (scale bar = 10 μm).

with a known compound, 4,6-dimethoxyphthalide. The structure of the novel compound was elucidated and identified as 3a-hydroxy-6-methoxy-3,8,9a-trimethyl-3,3a,9,9a-tetrahydro-1*H*-furo[3,4-*b*]chromen-1-one. To the best of our knowledge, this compound has not been reported. Hence, we named it as coprinolide.

Coprinolide is a tricyclic polyketide characterized by the presence of furanone-fused chromene units. Similar furan-fused chromene units are present in some natural products isolated from fungi such as alboatrin from the fungus *Verticillium alboatrum*,[30] xyloketal B from the marine-derived fungus *Xylaria* sp.,[31] and recently isolated simpliketal A, B, C and D from the fungus *Simplicillium* sp. AHK071-01.[32] Xyloketal B and its derivatives have been extensively studied in various disease models, and have been shown to possess medicinal potentials.[33] Notably, coprinolide harbors a 3,3a,9,9a-tetrahydro-1*H*-furo[3,4-*b*]chromen-1-one skeleton. Natural compounds containing this skeleton are very rare. A literature search showed three compounds having the closest or same skeleton with coprinolide. These include penilactones A and B, isolated from the Antarctic deep-sea derived fungus *Penicillium crustosum* PRB-2[34] and pallenic acid isolated from the plant *Calyptranthes pallens*.[35] Hence, reports on the biological activities of compounds bearing such skeleton are also rare. Penilactones A and B were evaluated for their cytotoxic activities against five human cancer cell lines as well as for NF-κB inhibitory activity through transient transfection and reporter gene expression assay, but both compounds showed no cytotoxic activity against the tested human cancer cell lines with $IC_{50}>50\,\mu$M (>23 ppm for Penilactone A, >25 ppm for Penilactone B), and only penilactone A showed weak NF-κB inhibitory activity with 40% inhibition rate at 10 μM (4.7 ppm).[34] Pallenic acid had no cytotoxic effects on different human cancer cell lines.[35] Thus, the isolation of coprinolide from *Cop. comatus* is a significant finding to expand the structural diversity and biological activities of compounds with a rare 3,3a,9,9a-tetrahydro-1*H*-furo[3,4-*b*]chromen-1-one skeleton.

In this study, the inhibitory activity on conidial germination and fungicidal activity of coprinolide were evaluated against five plant fungal pathogens. Coprinolide had the most pronounced inhibitory activity based on low IC_{50} values against the conidial germination and germ tube elongation of *Col. orbiculare*, a *Colletotrichum* species responsible for anthracnose disease in cucurbits, with complete inhibition of the conidia at 100 ppm and higher. Other fungal pathogens, *P. oryzae*, the causative agent of rice blast disease was completely inhibited at 300 ppm of coprinolide, while *Col. gloeosporioides*, and the two strains of *F. oxysporum* f. sp. *cubense* (R1 and TR4), were completely inhibited at a higher concentration (1000 ppm). These findings are important as development of resistance to fungicides have been reported in *P. oryzae*[36] and some *Colletotrichum* species.[37,38] On the other hand, *F. oxysporum* f. sp. *cubense* cannot be easily eradicated despite several practices and strategies to eliminate this pathogen.[11] Inhibiting these plant pathogenic fungi would be a huge contribution to the agricultural industry, as these plant pathogenic fungi cause detrimental effects on different crops worldwide.

Coprinolide showed significant stronger activity against *Col.*

orbiculare than *Col. gloeosporioides* in the germination inhibition assay, indicating species-specific inhibition in the genus. This variation may be attributed to differences in gene sequences encoding proteins involved in the expression of coprinolide's toxicity, such as the target protein, transporters, or enzymes responsible for detoxification or activation of the compound. This seems likely because *Col. orbiculare* belongs to orbiculare clade that is outside of *Col. gloeosporioides* species complex, which is a diverse clade including a number of important plant pathogens.[39,40] In the absence of the data that identify the factors underlying the differential sensitivity of the two *Colletotrichum* species towards coprinolide, it is important to further examine the coprinolide sensitivity of other *Colletotrichum* species under the same and different clades.

The adhesion of fungal spores to the plant surface is a preliminary stage towards spore germination until successful invasion and establishment of disease on host plant.[6] Hence, the inhibitory effect of coprinolide on conidial germination led to the prevention of the possible pathogenic mechanisms of fungi. Further, the fungicidal activity of coprinolide was confirmed by fluorescence microscopy using FDA and PI. The treatment of fungal conidia with coprinolide at specific concentrations resulted in red fluorescence, indication of dead conidia. FDA and PI are used to determine the viability and non-viability of cells, respectively. Viable cells fluoresce in bright green due to the conversion of FDA to fluorescein by the cellular esterases present in the cells.[41,42] On the other hand, PI can only traverse into the membranes of dead cells or cells with a damaged membrane, leaving the cells in red fluorescence.[41] Although the higher concentrations of coprinolide induced conidial death, signs of damage or malformation on conidia were not observed. This may indicate the possible specific mechanism of coprinolide on fungal conidia of inhibiting only the conidial germination and germ tube elongation. Further investigation is needed to determine its specific mechanism on membrane integrity of fungal conidia.

The activity of coprinolide can be deduced based on its chemical structure. The presence of a furanone skeleton in coprinolide which is a characteristic structure of phthalide compounds, may be responsible for its possible antifungal mechanisms. Phthalide and its derivatives exhibit a wide range of biological activities, including antifungal activity against various plant pathogenic fungi.[21,43,44] With regards to phytotoxicity, an antifungal phthalide derivative 3-butylidene phthalide was found to be non-toxic against the plant *Lemna minor* at the same concentrations that inhibited the fungus *Macrophomina phaseolina*. However, it posed toxicity on the same plant at 1000 μg/mL (5.3 mmol/L).[45] On the other hand, a phthalide compound, 5,7-dihydroxy-3(*R*)-methylphthalide isolated from the endophytic fungus *Xylaria brevipes* was found to be phytotoxic at 4.6 μg/mL against *Brassica chinensis*.[46] Coprinolide at concentrations ranging from 100 to 1000 ppm induced conidial death to plant pathogenic fungi. Given the shared pharmacophore with phthalide compounds, these concentrations of coprinolide should be subjected for phytotoxicity testing for further development as antifungal agent against plant pathogens.

The isolation of orsellinaldehyde from the same mushroom prompted us to hypothesize a plausible biosynthetic pathway of coprinolide (Fig. 6). Orsellinaldehyde is the aldehyde analogue of orsellinic acid, a structural backbone of many secondary metabolites.[47] Reyes-Fernández *et al.* (2021)[48] proposed the biosynthesis of melanin in the fungus *Ustilago maydis*, which involves the production of orsellinic acid from 1 acetyl-CoA and 3 malonyl-CoA by polyketide synthases (Pks3 and Pks4) and the subsequent reduction of orsellinic acid to orsellinaldehyde by another polyketide synthase (Pks5). In our hypothetical pathway, orsellinaldehyde is further reduced and dehydrated

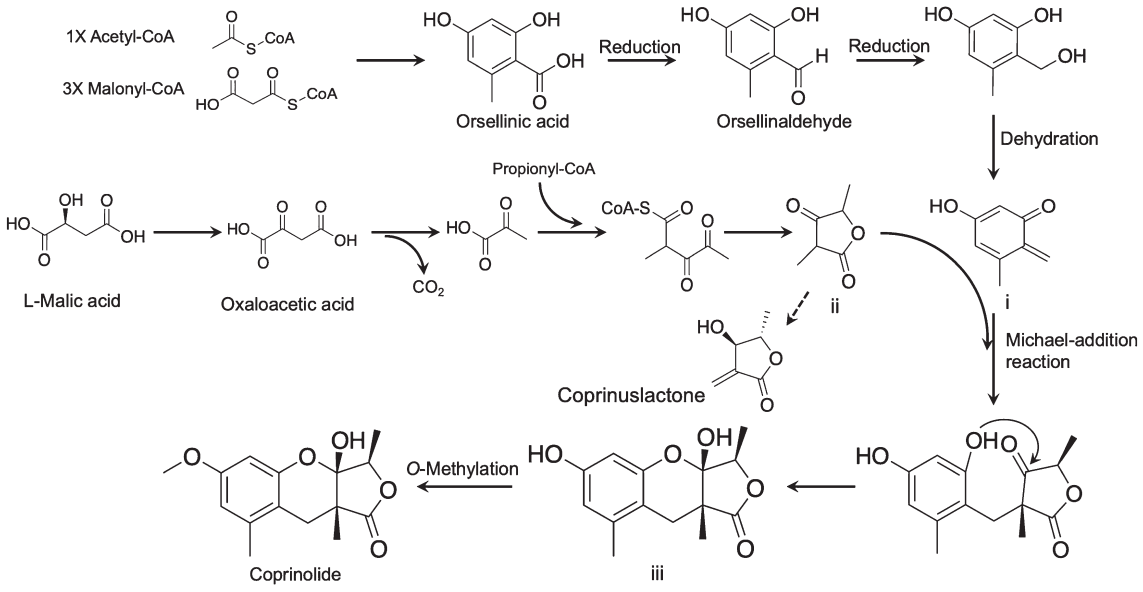

Fig. 6. Hypothetical biosynthetic pathway of coprinolide.

to a key intermediate, 3-hydroxy-5-methyl-6-methylenecyclohexa-2,4-dien-1-one (i in Fig. 6). The other key intermediate, 3,5-dimethylfuran-2,4(3*H*,5*H*)-dione (ii), is likely derived from malic acid. Malic acid is widely produced by fungi.[49] Wu *et al.* (2012)[34] suggested that the key intermediate (5-methylfuran-2,4(3*H*,5*H*)-dione) in the biosynthesis of penilactone A may be derived from L-malic acid. An analogous reaction with propionyl-CoA instead of acetyl-CoA in the biosynthesis of penilactone A would provide 3,5-dimethylfuran-2,4(3*H*,5*H*)-dione (ii). Interestingly, a structurally related compound with intermediate ii named coprinuslactone ((3*R*,4*S*)-2-methylene-3,4-dihydroxypentanoic acid 1,4-lactone) was previously isolated from *Cop. comatus*.[50] A Michael-addition reaction between intermediates (i) and (ii) may possibly be resulted in the formation of intermediate (iii). Finally, the dehydration and *O*-methylation of (iii) generate coprinolide. Orsellinaldehyde and coprinolide were simultaneously detected in the mycelial extracts of *Cop. comatus* suggesting that these compounds are biosynthetically related to each other. In addition, the phthalide derivative 4,6-dimethoxyphthalide, isolated from the culture filtrate and detected in the mycelia of *Cop. comatus* which shares a common furanone structure with coprinolide, may also be derived from orsellinic acid. In relation to this, Regueira *et al.* (2011)[51] proposed a biosynthesis of a phthalide intermediate which was originated from 5-methylorsellinic acid, a compound structurally like orsellinic acid. Both orsellinaldehyde and 4,6-dimethoxyphthalide have been reported in our previous studies as antifungals against multiple plant pathogenic fungi.[20,21]

Comparing the antifungal activity of coprinolide with orsellinaldehyde and 4,6-dimethoxyphthalide in our previous studies,[20,21] coprinolide showed weaker activity than orsellinaldehyde and 4,6-dimethoxyphthalide against *Col. gloeosporioides* and *P. oryzae*, respectively, but had stronger activity than 4,6-dimethoxyphthalide against *Col. gloeosporioides Cop. comatus* likely survives in environments inhabited by diverse microorganisms by utilizing multiple compounds with varying antifungal spectra.

The present study identified a novel compound coprinolide which possessed a rare tricyclic skeleton, isolated from the mushroom *Cop. comatus* through a bioassay-guided fractionation. Coprinolide exhibited antifungal activity against five economically important plant pathogenic fungi. The inhibitory and fungicidal activities of coprinolide on fungal conidia indicated its potential as an antifungal agent for agricultural crops. This study further supports mushrooms as promising sources of novel natural products for agricultural applications. Studying the mode of action of coprinolide and *in-vivo* experiment is needed to fully establish its promising and underlying antifungal mechanisms.

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Competing interests

There are no financial and non-financial competing interests.

Author Contributions

E.M.C.: conceptualization, performed experiment, data analysis, writing of original draft, K.S.: data analysis, review of manuscript, M.K. and T.A. prepared the fungal strains, review of manuscript. K.U.: data curation, review and editing of the manuscript, T.E.E.D.C. supervision, review and editing of the manuscript, and A.I.: conceptualization, supervision, review and editing of the manuscript.

Electronic supplementary materials

The online version of this article contains supplementary materials, which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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