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Activation of Secondary Metabolism and Protease Activity Mechanisms in the Black Koji Mold *Aspergillus luchuensis* through Coculture with Animal Cells

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Cite This: *ACS Omega* 2024, 9, 43129−43137

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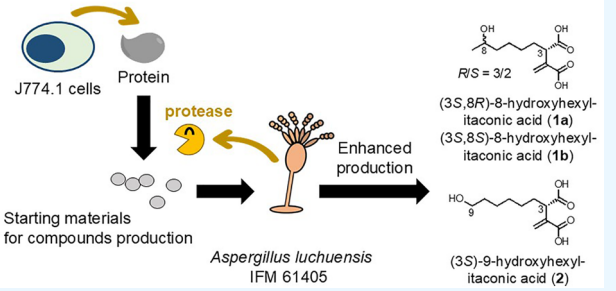
ABSTRACT: The activation of secondary metabolism plays a pivotal role in the discovery of novel natural products. We recently developed a coculture method involving actinomycetes and mouse macrophage-like cells to stimulate the production of bioactive compounds. A black koji mold, *Aspergillus luchuensis* IFM 61405, markedly enhanced the production of (3*S*,8*R*)-8-hydroxy-3-carboxy-2-methylenenonanoic acid (**1a**), (3*S*,8*S*)-8-hydroxy-3-carboxy-2-methylenenonanoic acid (**1b**), and (3*S*)-9-hydroxy-3-carboxy-2-methylenenonanoic acid (**2**) when coincubated with J774.1 mouse macrophage cells. The production of **1** and **2** increased by at least 3.5-fold and 2.7-fold, respectively, compared to monoculture after 7 days. A mechanistic investigation revealed that a protease from strain IFM 61405 plays a key role in enhancing the production of **1** and **2**. This enhancement was not replicated in *A. niger* IFM 59706, a nonkoji mold, despite the presence of biosynthetic genes for **1** and **2** in *A. niger* IFM 59706. Furthermore, the addition of protease inhibitors suppressed the production of **1** and **2**, suggesting that proteins secreted from animal cells, likely degraded by proteases secreted by strain IFM 61405, serve as precursors for **1** and **2**. The results show that the strategy of coculturing koji mold with animal cells has the potential to enhance the production of natural products.



INTRODUCTION

The use of natural products has made substantial contributions to human welfare, especially in the fields of pharmaceutical science and medicine. Notable examples include penicillin[1] and streptomycin,[2] which exhibit antibiotic activity; tacrolimus from *Streptomyces tsukubaensis*,[3] used as an immunosuppressant; avermectin from *Streptomyces avermitilis*,[4] an anthelmintic agent; and epothilones from *Sorangium cellulosum*,[5] an anticancer agent. Despite these significant contributions, discovering novel compounds produced by microorganisms is becoming increasingly challenging.[6] However, advances in the genomic sciences have revealed that many genes associated with natural product biosynthesis are underutilized, and the number of compounds discovered to date is limited relative to the potential number of compounds that could be produced.[7−9]

Numerous studies have sought to enhance the secondary metabolic capabilities of microorganisms using a variety of innovative strategies, such as by manipulating culture conditions, temperature, pH, and depleting metal components; many of these methods have been shown to increase secondary metabolism activity.[10−14] Our group has recently explored the application of heating methods to induce the production of heat-dependent secondary metabolites, referred to as heat-shock metabolites (HSMs).[15−17] These compounds, which are not produced under normal temperatures (30 °C), were synthesized at elevated temperatures (45 °C). Other approaches have also been reported, such as heterologous expression, wherein a biosynthetic gene cluster is introduced into a different organism;[18] overexpression of transcription factors;[19,20] ribosome engineering,[21,22] and the addition of specific compounds, such as histone deacetylase inhibitors[23] and high-throughput elicitor screening (HiTES).[24] In addition, coculture methods that simulate natural environments have also been employed, such as coculture with different microorganisms (e.g., *Streptomyces iranensis* and *Aspergillus nidulans*;[25] *Streptomyces coelicolor* A3(2) M145 and *Myxococcus xanthus*;[26] and *Streptomyces* with mycolic acid−containing bacteria[27]). Recently, our group developed a coculture method involving pathogenic actinomycetes and macrophage-like cells exposed to xenocidal stress from immune cells.[28−31] We hypothesized that microorganisms might activate secondary metabolism to produce defensive compounds in response to such stress. Interestingly, we successfully induced the production of nocarjamide, an immunosuppressive compound,

Received: August 2, 2024
Revised: September 7, 2024
Accepted: September 30, 2024
Published: October 10, 2024

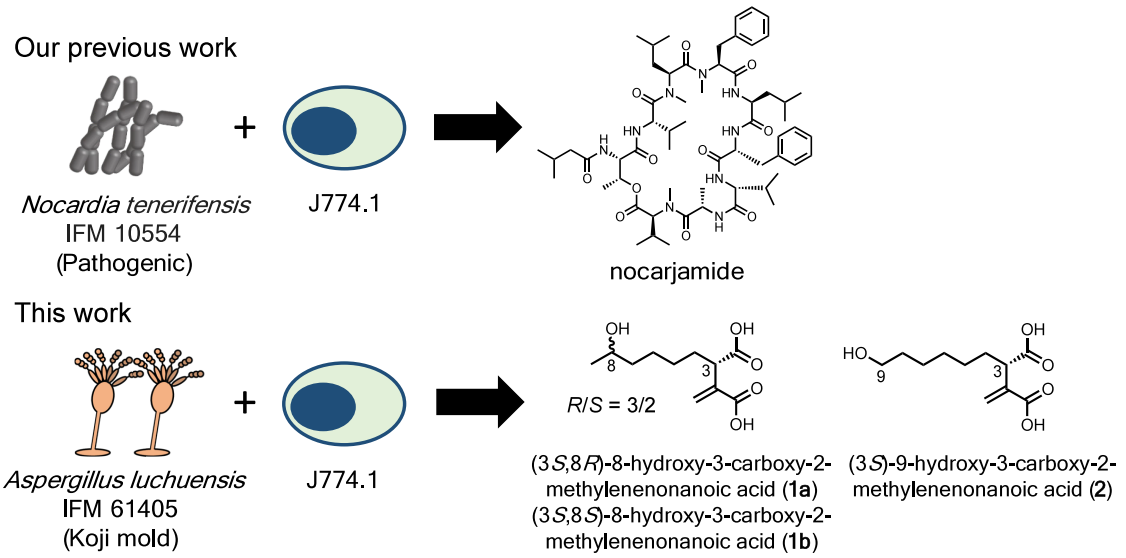


Figure 1. Coculture of microorganisms with animal cells (J774.1 mouse macrophage cells).

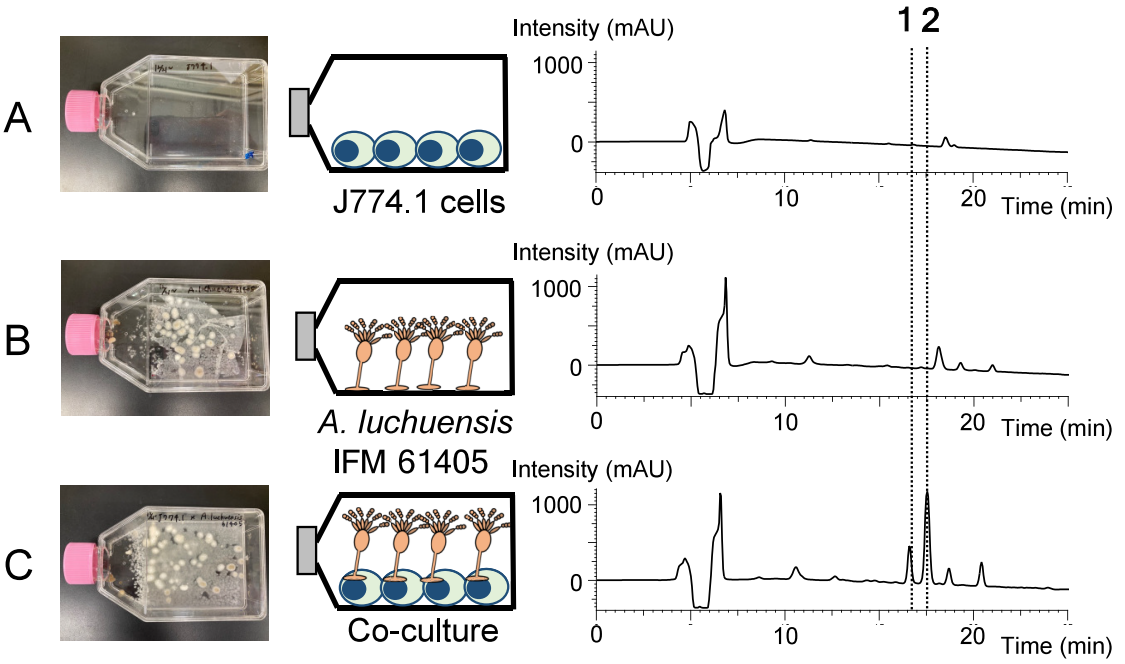


Figure 2. HPLC analysis of monoculture and coculture of *A. luchuensis* IFM 61405 with J774.1 cells: (a) monoculture of J774.1 cells, (b) monoculture of *A. luchuensis* IFM 61405, and (c) coculture of *A. luchuensis* IFM 61405 with J774.1 cells.

by coculturing *Nocardia tenerifensis* IFM 10554 and mouse macrophage-like cells (J774.1) (Figure 1). This finding raises the question of whether this phenomenon is unique to pathogenic microorganisms.

Koji molds, such as *Aspergillus oryzae*, are fungi widely used in the fermentation processes for products like sake and miso. These molds are also known for their ability to produce valuable compounds and have applications in cosmetics, such as kojic acid,[32] and in pharmaceuticals, such as lovastatin.[33] However, the number of compounds isolated from these molds to date is relatively small compared to the number of identified biosynthetic gene clusters. For example, although 75 biosynthetic gene clusters have been reported in *A. oryzae*,[34] only 27 compounds have been isolated.[35] In this study, we evaluated the potential of activating secondary metabolism in nonpathogenic microorganisms, specifically koji molds, by coincubation with animal cells.

RESULTS AND DISCUSSION

Koji molds were selected from a library maintained by the Medical Mycology Research Center, Chiba University. Screening was performed on 12 pairs of six strains of the *Aspergillus* genus and two types of immune cells: J774.1, a mouse macrophage-like cell line, and THP-1, a human monocytic cell line derived from patient with acute monocytic leukemia. Each *Aspergillus* strain was precultured for 5 days before being added to the cell culture flasks. Coculture was performed for 1 or 2 weeks at 28 °C in Czapek-Dox medium under static conditions. Subsequently, culture solutions were extracted with an organic solvent and analyzed by high-performance

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ACS Omega 2024, 9, 43129−43137

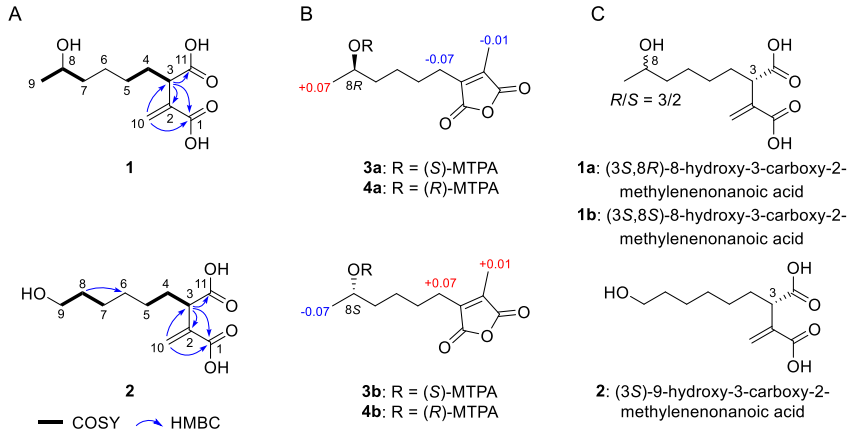
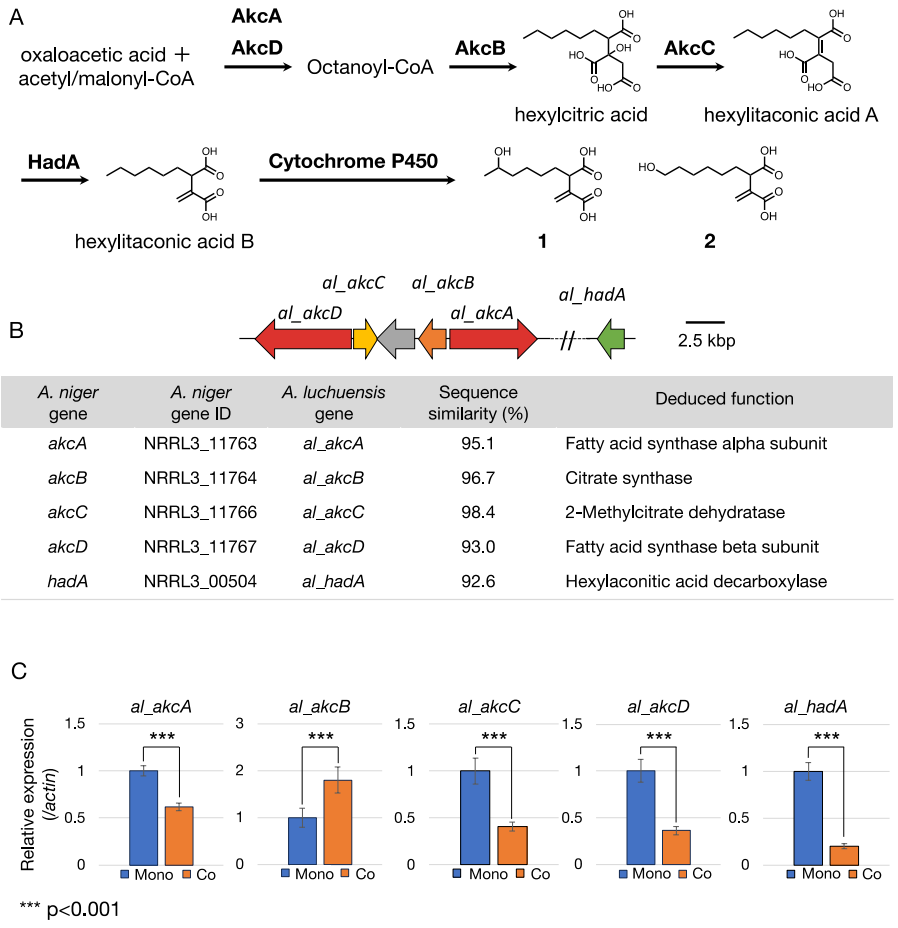


Figure 3. Comprehensive spectral analysis and structural elucidation of **1** and **2**. (A) COSY and key HMBC correlations for **1** and **2**. (B) $\Delta\delta_H$ (*S*-*R*) values in parts per million (ppm) calculated from MTPA esters. (C) Structures of **1a**, **1b**, and **2**.



A. niger gene	*A. niger* gene ID	*A. luchuensis* gene	Sequence similarity (%)	Deduced function
akcA	NRRL3_11763	*al_akcA*	95.1	Fatty acid synthase alpha subunit
akcB	NRRL3_11764	*al_akcB*	96.7	Citrate synthase
akcC	NRRL3_11766	*al_akcC*	98.4	2-Methylcitrate dehydratase
akcD	NRRL3_11767	*al_akcD*	93.0	Fatty acid synthase beta subunit
hadA	NRRL3_00504	*al_hadA*	92.6	Hexylaconitic acid decarboxylase

Figure 4. RT-qPCR analysis and biosynthetic gene mapping for **1** and **2**. (A) Biosynthetic pathway for **1** and **2** in *A. niger* NRRL3.[37] (B) Biosynthetic genes for **1** and **2** in *A. luchuensis* IFM 61405. (C) RT-qPCR results for the biosynthetic genes of **1** and **2**. RT-qPCR was repeated at least two times with different batches and one of the representative data was shown. Gene expression values are normalized relative to β-actin. Values are the means ± standard deviation (SD) (n = 3). Mono; monoculture, Co; coculture.

liquid chromatography (HPLC) to identify coculture-specific or enhanced peaks. Several combinations yielded coculture specific compounds (Table S1 and Figures S1). Of these, only *A. luchuensis* IFM 61405 and IFM 65425 reacted with both J774.1 and THP-1 cells and produced the same peaks, **1** and **2** (Figure 2). Thus, strain IFM 61405, which produced **1** and **2** exclusively in coculture, was selected for mass culture with J774.1 cells and subsequent compound isolation.

Mass culture was performed in 20 175 cm^2 flasks at 28 °C for 2 weeks. The culture solution was separated into the supernatant and insoluble components, including mycelium, which were subsequently crushed and extracted with methanol (MeOH). Following the evaporation of MeOH, the residue and the supernatant of the cell culture were combined and extracted three times with ethyl acetate (AcOEt). The AcOEt layer was concentrated and subjected to silica gel chromatog-

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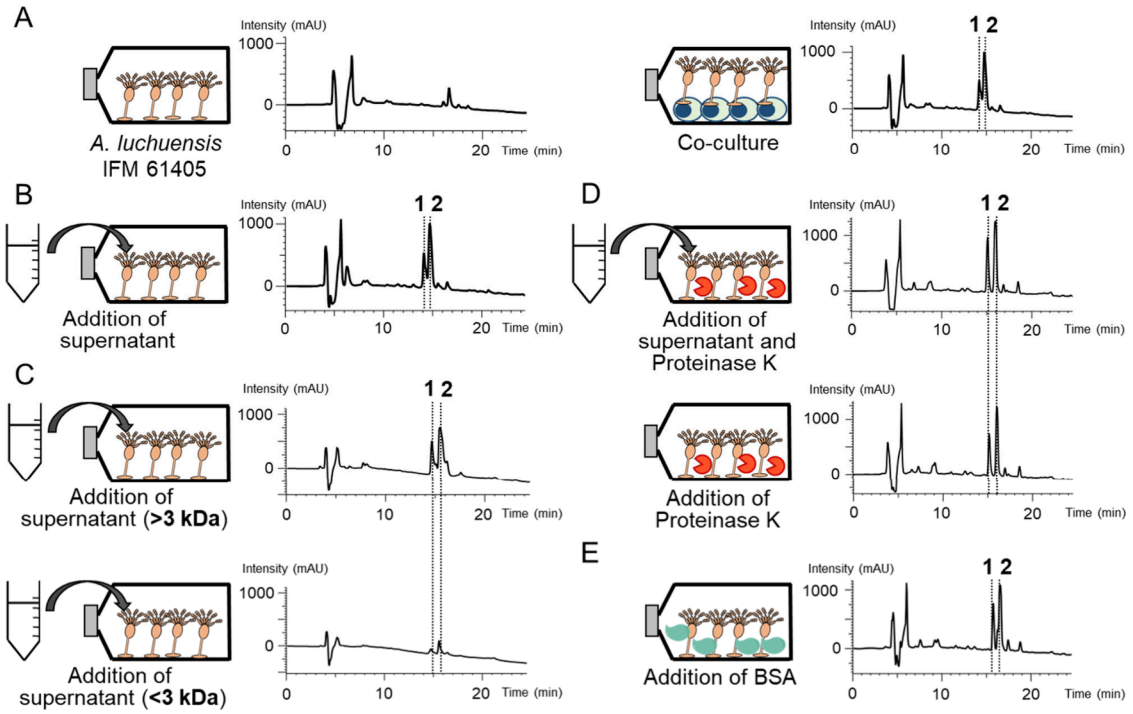

Figure 5. Experiments for elucidating the mechanism of secondary metabolism activation (A) Monoculture of *A. luchuensis* IFM 61405 and coculture with J774.1 cells. (B) Addition of the supernatant from J774.1 cell-only cultures. (C) Separation of the supernatant from the J774.1 cell-only culture using a dialysis filter (>3 kDa or <3 kDa). (D) Addition of the supernatant from the J774.1 cell-only culture treated with Proteinase K to *A. luchuensis* IFM 61405 and the effect of Proteinase K alone. (E) Addition of bovine serum albumin (BSA) to *A. luchuensis* IFM 61405.

raphy, followed by purification using reverse-phase HPLC, resulting in the isolation of **1** (1.9 mg) and **2** (1.3 mg) (Figure S2). Structural analyses were performed using one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy, and optical rotation measurements. **1** and **2** were identified as 8-hydroxy-3-carboxy-2-methylenenonanoic acid and (3*S*)-9-hydroxy-3-carboxy-2-methylenenonanoic acid, respectively, and showed good agreement with previously reported data (Figure 3A and Table S2).[36] The absolute configuration of the C-3 position in **2** was determined to be *S* by comparing the observed specific rotation, $[\alpha]^{16}_{D}$ (+9.8, *c* 0.09, MeOH), with the reported value of (+11, *c* 1.0, MeOH).[36] To elucidate the absolute configuration of **1** at the C-3 and C-8 positions, initial focus was placed on the C-3 position. The biosynthetic pathway of 8-hydroxy-3-carboxy-2-methylenenonanoic acid[37] suggests that the hydroxy group is introduced by cytochrome P450 after the stereochemistry at C-3 is established. Consequently, the stereochemistry of C-3 in **1** is also expected to be *S*, as in **2**. This was corroborated by the circular dichroism (CD) spectrum of **1**, which resembled that of the structurally related talarocyclopenta C,[38] thereby confirming the *S* configuration at the C-3 position of **1** (Figure S11). To further elucidate the stereochemistry at the C-8 position of **1**, a modified Mosher's method was applied. High-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESITOFMS) and 2D NMR spectra indicated the formation of (*S*)-2-methoxy-2-(trifluoromethyl)phenylacetic acid (MTPA) ester **3** and (*R*)-MTPA ester **4**, resulting from intramolecular condensation between two carboxylic acids (Figure 3B). The ^{1}H NMR spectra of **3** and **4** were largely identical but exhibited several duplicated signals with contrasting peak intensities, suggesting that **1** was a nonequimolar diastereomeric mixture (Figure S13). Detailed analysis of the ^{1}H NMR data revealed two ^{1}H resonances with slightly different chemical shifts. Careful interpretation of the correlated spectroscopy (COSY) spectra for **3** and **4** revealed them to be a pair of MTPA acylation diastereomers (**3a** and **3b**; **4a** and **4b**). Furthermore, by recursively applying Mosher's chiral anisotropy rule to the ^{1}H chemical shift differences between the diastereomeric pairs, the slight excesses of **3a** and **4a** were identified as (*R*)-**1** MTPA derivatives and hence, the nonexcesses of **3b** and **4b** were identified as (*S*)-**1** derivatives. The diastereomeric ratio of **1** was estimated to be *R*/*S* = 3:2 based on the integral values of each chemical shift. Thus, **1** was concluded to be an inseparable diastereomeric mixture of (3*S*,8*R*) and (3*S*,8*S*)-8-hydroxy-3-carboxy-2-methylenenonanoic acids (**1a**, **1b**), with an approximate diastereomeric ratio of *R*/*S* = 3:2 (Figure 3C). This report marks the first identification of compound **1b**. Although there have been reports of the isolation of (3S,8*R*)-8-hydroxy-3-carboxy-2-methylenenonanoic acid (**1a**) and (3*S*)-9-hydroxy-3-carboxy-2-methylenenonanoic acid (**2**) from *A. niger*[39] and *Penicillium* sp.,[36] these processes required extended incubation periods, such as 9 weeks for *A. niger*[39] and 4 weeks for *Penicillium* sp.[36] In contrast, the production of these compounds in *A. luchuensis* IFM 61405 under standard conditions was minimal; however, the interaction with cells significantly enhanced the production rate of **1** and **2**.

The next question addressed the mechanism underlying the activation of secondary metabolism during the coculture of koji molds with immune cells. Initially, our focus was on biosynthetic genes. In *A. niger*, **1** and **2** are synthesized by biosynthetic genes such as *akc A* to *D* and *hadA* (Figure 4A).[37] Genome mining confirmed that *A. luchuensis* IFM 61405 also possesses these biosynthetic genes, named *al_akcA* to *D* and *al_hadA* (Figure 4B). Metabolite analysis revealed that the

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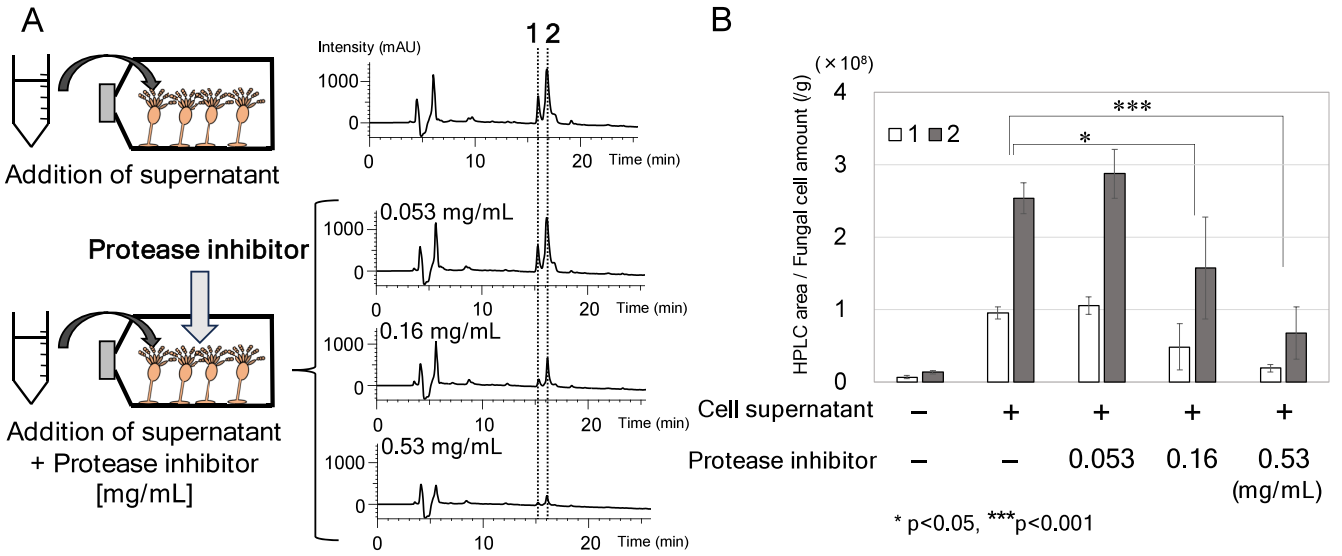

Figure 6. Impact of protease inhibitors on the production of compounds **1** and **2**. (A) HPLC analyses of *A. luchuensis* IFM 61405 incubated with the supernatant of a cell-only culture, with and without of protease inhibitors. (B) Dose-dependent decrease in the intensity of peaks for **1** and **2** due to protease inhibitors. Quantified production of **1** and **2** normalized to the bacterial cell amount. Error bars represent the standard deviation of three independent experiments.

production of **1** and **2** is activated from day 4 of coculture (Figure S14). After 4 days of coculture, total RNA was extracted from frozen fungal samples, and quantitative reverse transcription PCR (RT-qPCR) was performed. However, the expression of these genes was not upregulated by coculture, rather, expression decreased with the exception of *al_akcB* (Figure 4C). These results suggest that the enhanced production of **1** and **2** is not due to the activation of biosynthetic genes at the transcriptional level.

To clarify how *A. luchuensis* IFM 61405 responds to J774.1 cells to activate the production of **1** and **2**, the supernatant from cell-only cultures was added to the fungal culture medium. This approach was based on the hypothesis that secreted protein or compounds from cells might influence *A. luchuensis* IFM 61405. Interestingly, treatment of the fungal strain with the cell culture supernatant activated the production of **1** and **2** (Figure 5A, 5B), suggesting that strain IFM 61405 responds to substances secreted by the cells. To further elucidate this response, the supernatant was separated using a dialysis membrane, and the resulting fractions were added to the culture medium. The IFM 61405 strain responded to the fraction larger than 3 kDa, enhancing the production of **1** and **2** (Figure 5C). In addition, evaluations of cell selectivity in compound production revealed that coculture with HEK293, human fetal kidney cells, also activated the production of **1** and **2** (data not shown). This led us to hypothesize that a universal protein secreted by cells could activate fungal secondary metabolism. Accordingly, the supernatant from cell cultures was treated with Proteinase K at 37 °C for 1 h to degrade all proteins. Contrary to our expectations, the production of **1** and **2** was still observed when this treated supernatant was added to the culture strain IFM 61405 and cultivated for 7 days (Figure 5D). Furthermore, the addition of Proteinase K alone also enhanced the production of **1** and **2**. To confirm the hypothesis that strain IFM 61405 responds to proteins in general, bovine serum albumin (BSA) was added to the culture, and **1** and **2** were once again detected (Figure 5E).

These results suggest that strain IFM 61405 produces **1** and **2** in response to proteins secreted by cells.

Among the unique characteristics of koji molds, the ability to secrete numerous proteases, i.e., enzymes that degrade proteins, is considered to be particularly interesting. For example, the enzymatic activity associated with koji molds is responsible for breaking down proteins into amino acids during the fermentation process of sake and miso. Since **1** and **2** are synthesized from oxaloacetic acid, acetyl-CoA, malonyl-CoA, and citric acid (Figure 4A), it was predicted that amino acids might serve as raw materials for their production. In this study, coculture of *A. niger* IFM 59706 with J774.1 cells did not result in the production of **1** and **2**, despite both strains possessing the relevant biosynthetic genes (Table S3 and Figure S15). This observation led us to hypothesize that proteases secreted from strain IFM 59706 might also play a role in this coculture system. To test this possibility, we added the supernatant from a cell-only culture and a protease inhibitor to the culture medium, which was then incubated with strain *A. luchuensis* IFM 61405 for 7 days. The production of **1** and **2** decreased in a dose-dependent manner with increasing concentrations of the protease inhibitor (Figure 6A). Normalization to the fungal cell amount (g) also confirmed that the production of **1** and **2** was inhibited by protease inhibitor (Figure 6B). The results were statistically significant. To further elucidate the role of the secreted protease from strain IFM 61405 in the production of **1** and **2**, we employed an alternative method (Figure S16). The protease activity in the culture supernatant was inactivated by heating, as verified by a reduction in its ability to degrade BSA over a 3-day incubation period and visualized by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE). Subsequently, these heat-treated protease BSA solutions were added to the fungal culture and incubated for 7 days. Interestingly, the production of **1** and **2** was slightly reduced with the inactivated (heated) supernatant. These findings suggest that protease from *A. luchuensis* IFM 59706 plays a key role in the production of **1** and **2**.

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Finally, we hypothesized that coculture might increase the transcription of proteases genes and assessed their expression level in *A. luchuensis* IFM 61405 by RT-qPCR (Figure 7). Our

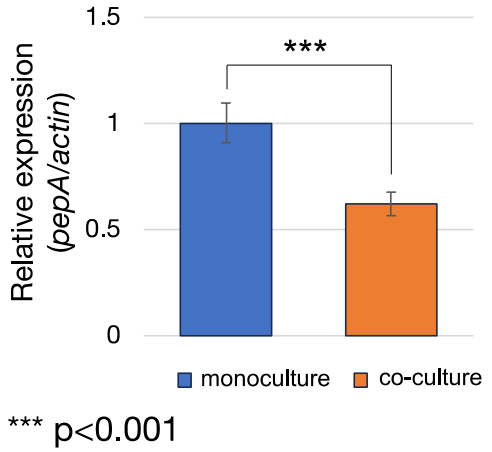

Figure 7. RT-qPCR analysis of the protease gene in *A. luchuensis* IFM 61405. The *pepA* gene encodes the major extracellular acid protease, Aspergillopepsin A. RT-qPCR was repeated at least two times with different batches and one of the representative data was shown. Gene expression values are normalized relative to *β*-actin. Values are the means ± standard deviation (SD) (n = 3).

search for proteases expressed in black koji mold led us to a report describing the clear expression of the aspartic protease Aspergillopepsin A (PEPA)-encoding gene (*pepA*) in *A. luchuensis* RIB2604, which is the same fungal strain as IFM 61405.[40] Furthermore, the authors of that study also reported that disruption of the *pepA* gene resulted in decreased acid protease activity and reduced amino acid concentrations.[40] Therefore, we have been interested in the amount of this *pepA* gene. Consequently, we focused on quantifying the transcription level of the *pepA* gene during coculture. However, the expression levels of the *pepA* gene tended to decrease compared to monoculture. The results were statistically significant. If the basal secretion level of protease is sufficient, increase in proteins secreted from macrophages, such as interleukins, as a result of the immune response may be important. Other possible reasons include the increased expression of protease genes other than *pepA*, or the activation of proteases at the translation or enzyme activity levels. Although further investigation is required to clarify the involvement of proteases under coculture conditions, it is suggested that fungal proteases degrade proteins secreted by cells and use them as starting materials for secondary metabolism.

In conclusion, this study demonstrates the activation of secondary metabolism in the black koji mold *A. luchuensis* IFM 61405 using a coculture method with mouse macrophage-like cells J774.1. The coculture of strain IFM 61405 with J774.1 cells markedly enhanced the production of **1** and **2**. Structural elucidation of **1** and **2** by NMR, mass spectroscopy, and optical rotation confirmed that **2** is (3*S*)-9-hydroxy-3-carboxy-2-methylenenonanoic acid. MTPA esterification revealed that **1** was a diastereomeric mixture of (3*S*,8*R*)-8-hydroxy-3-carboxy-2-methylenenonanoic acid (**1a**) and (3*S*,8*S*)-8-hydroxy-3-carboxy-2-methylenenonanoic acid (**1b**) in a ratio of 3:2. This is the first study to report the identification of compound **1b**. In addition, the mechanisms underlying the production of **1** and **2** were investigated. The addition of supernatant from cell-only cultures and BSA enhanced the production of these compounds, while protease inhibitor reduced the amounts of **1** and **2**. These findings suggest that the protease secreted by strain IFM 61405 plays a key role in enhancing the production of **1** and **2**. Thus, the activation of secondary metabolism by coculturing microorganisms with animal cells is demonstrated to be applicable to koji molds. This study highlights the potential of using koji molds to accelerate the production of bioactive compounds. We believe that coculture methods involving various microorganisms with animal cells will contribute considerably to natural product science.

EXPERIMENTAL SECTION

General Experimental Procedures. The following instruments were used in this study: a P-1020 polarimeter (JASCO Corp., Japan) for measuring optical rotations; a J-720WI circular dichroism spectrometer (JASCO Corp.) for measuring circular dichroism; a high-resolution electrospray ionization time-of-flight mass spectrometer (HR-ESI-TOF MS) fitted with a quadrupole mass filter and time-of-flight detector (Q-Tof Micro, Waters Corp., Milford, MA, USA); a D-2000 high-performance liquid chromatography (HPLC) system (Hitachi, Ltd., Japan); and a JNM-ECA500 spectrometer (JEOL Ltd., Japan) for obtaining nuclear magnetic resonance (NMR) spectra. Solvent chemical shifts in NMR spectra were used as internal standards (δ_H 3.31, δ_C 49.00 for methanol-d_4, and δ_H 7.26 and δ_C 77.16 for chloroform-d_1). The following adsorbents were used for purification: Silica Gel 60N (Kanto Chemical Co., Inc., Japan) for flash silica gel column chromatography and CAPCELL PAK C18 MGII (ϕ 20 × 250 mm, Osaka Soda Co., Ltd., Japan) for preparative HPLC.

Microorganisms. Strains of koji molds were obtained from the Medical Mycology Research Center, Chiba University;[41] *A. luchuensis* (IFM 61405; source: black malted rice), *A. luchuensis* (IFM 65425; source: awamori koji), *A. oryzae* (IFM 58511; source: sake, miso, soy sauce koji), *A. oryzae* (IFM 59475; source: cereal (broad bean)), *A. sojae* (IFM 47667; source: miso koji), *A. niger* (IFM 59706; source: skin of patients with severe acute pancreatitis).

Cell Culture. The mouse macrophage-like cell line J774.1 was obtained from RIKEN BioResource Research Center[42] and cultured on Dulbecco's Modified Eagle Medium (D-MEM), High Glucose (Fujifilm Wako Pure Chemical Corp., Japan) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific K.K., Japan). Human THP-1 cells were obtained from Japanese Cancer Research Resources Bank (JCRB)[43] and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Fujifilm Wako Pure Chemical Corp., Japan) supplemented with 10% FBS. Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

Coculture Method. *Seed Culture of Fungal Strains for Coculture in Czapek-Dox Medium.* Each fungal strain was grown for 7 days on dishes containing potato dextrose agar (PDA), prepared with BD *Difco Potato Dextrose Broth* (2.4 g/100 mL, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and Difco Nobel Agar (1.5 g/100 mL, Becton, Dickinson and Co.). To collect the fungal spores, 10 mL of a solution containing NaCl (0.8 g/100 mL, Fujifilm Wako Pure Chemical Corp.) and Tween 80 (0.1 mL/100 mL, Tokyo Chemical Industry Co., Ltd., Japan) was added to the dishes, and the fungal spores were collected with a disposable loop (Sansyo Co., Ltd., Japan). The number of fungal spores was counted

https://doi.org/10.1021/acsomega.4c07124
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under a microscope using a cell counter plate (Watson Co., Ltd., Japan). Fungal spores (1×10^6 spores/mL) were added to 50 mL of PD liquid medium containing *potato dextrose broth (PD)* (2.4 g/100 mL) in a 100 mL Erlenmeyer flask along with eight glass beads (BZ-5, As One Corp., Japan). The flask was incubated at 28 °C for 5 days with shaking at 160 rpm. After cultivation, the seed culture broth was collected in a 50 mL tube and the supernatant was removed after centrifugation at 2,383 *g* at 20 °C for 5 min. Then, 10 mL of Czapek-Dox (CD) medium, consisting of sucrose (3 g/100 mL, Fujifilm Wako Pure Chemical Corp.), $NaNO_3$ (0.3 g/100 mL, Fujifilm Wako Pure Chemical Corp.), K_2HPO_4 (0.1 g/100 mL, Kanto Chemical Co., Inc.), KCl (0.05 g/100 mL, Nacalai Tesque, Inc., Japan), $MgSO_4 \cdot 7H_2O$ (0.05 g/100 mL, Nacalai Tesque, Inc.), and $FeSO_4 \cdot 7H_2O$ (0.001 g/100 mL, Fujifilm Wako Pure Chemical Corp.) was added to the tube and the supernatant was removed again after centrifugation. This procedure was repeated twice, and the fungal volume was measured using a glass-stoppered test tube with an osmotic baking scale.

Coculture in CD Medium in a Cell Culture Flask. J774.1 cells cultured in 15 mL of D-MEM supplemented with 10% FBS in 75 cm^2 cell culture flask (Violamo, As One Corp., Japan) until they reached approximately 80−90% confluency. The supernatant was removed, and 5 mL of CD medium was added to the flask. After repeating this procedure three times, 25 mL of CD medium was added. A fungal suspension was then added to the flask to achieve the desired cell number ratio (J774.1: fungi = 1.5×10^7 cells: 0.4 cm^3) and the flask was incubated at 28 °C for 2 weeks.

THP-1 cells were cultured in 10 mL RPMI-1640 medium supplemented with 10% FBS in a 100 mm dish until they reached to approximately 80−90% confluency. The cells were collected into 50 mL tubes and the supernatant was removed after centrifugation at 1,507 g at 20 °C for 5 min. Subsequently, THP-1 cells (5.0×10^5 cells/1 mL) in 25 mL CD medium were added to a 75 cm^2 cell culture flask. A fungal suspension was then added to the flask to achieve the desired cell number ratio (THP-1: fungi = 1.25×10^7 cells: 0.3 cm^3) and the flask was incubated at 28 °C for 2 weeks.

Detection of Coculture-Specific Compounds by HPLC. The culture broth was separated into the supernatant and mycelial cake by filtration. The mycelial cake was then extracted with 25 mL of methanol (MeOH). The MeOH extract was combined with the supernatant and partitioned with ethyl acetate (EtOAc) in an equal volume of the supernatant; this process was repeated three times. The EtOAc extracts were analyzed by HPLC under the following conditions: a 15−85% acetonitrile/water ($MeCN/H_2O$) gradient containing 0.1% formic acid (HCOOH) for 60 min, followed by an isocratic hold at 100% MeCN for 15 min; flow rate: 0.7 mL/min; UV detection: diode array (200−600 nm); column: CAPCELL PAK C18 MGII (ϕ 4.6 × 250 mm).

Fermentation and Isolation. J774.1 cells cultured in 25 mL D-MEM supplemented with 10% FBS in 20 175 cm^2 cell culture flasks (Violamo, As One Corp.) until they reached approximately 80−90% confluency. The supernatant was removed, and 5 mL CD medium was added. After repeating this procedure three times, 50 mL CD medium was added. The strain *A. luchuensis* IFM 61405 was seed-cultured and replaced with CD medium using the method described above. A fungal suspension was then added to the flask to achieve the desired cell number ratio (J774.1: *A. luchuensis* = 1.5×10^7 cells: 0.4 cm^3) and the flask was incubated at 28 °C for 2 weeks. The total culture broth (1.0 L) was separated into supernatant and mycelium cake, which was extracted using MeOH (1.0 L). The MeOH extract and the supernatant were then combined and partitioned with EtOAc (1.0 L × 3), yielding 375 mg of EtOAc extract. This extract was subjected to silica gel column chromatography ($CHCl_3$: MeOH = 100:0, 100:1, 100:3, 100:5, 100:10, 100:30, 1-BuOH: MeOH: H_2O = 4:1:1) to produce seven fractions (1a−1g). Fractions 1d (45.6 mg) and 1e (42.6 mg) were subjected to preparative reversed-phase HPLC (40% MeOH, including 0.1% HCOOH, isocratic for 60 min; flow rate 9.9 mL/min; UV detection at 210 nm; column: CAPCELL PAK C18 MGII, ϕ 20 × 250 mm) to obtain (3*S*,8*R*) and (3*S*,8*S*)-8-hydroxy-3-carboxy-2-methylenenonanoic acid (**1**) (1.9 mg) and (3*S*)-9-hydroxy-3-carboxy-2-methylenenonanoic acid (**2**) (1.3 mg).

(3S,8R)- and (3S,8S)-8-Hydroxy-3-carboxy-2-methylenenonanoic Acid (1). Colorless oil; $[\alpha]^{16}_{D}$ +1.3 (*c* 0.06, MeOH); ^1H and ^{13}C NMR data, see Table S1; HR-ESI-TOF MS *m*/*z* 231.1232 $[M + H]^+$ (calcd. for $C_{11}H_{19}O_5$, 231.1227).

(3S)-9-Hydroxy-3-carboxy-2-methylenenonanoic Acid (2). Colorless oil; $[\alpha]^{16}_{D}$ +9.8 (*c* 0.09, MeOH); ^1H and ^{13}C NMR data, see Table S1; HR-ESITOFMS *m*/*z* 231.1224 $[M + H]^+$ (calcd. for $C_{11}H_{19}O_5$, 231.1227).

RT-qPCR Experiments. The cultivated fungi were frozen in liquid nitrogen and quickly pulverized in the frozen state using a mortar and pestle. Total RNA was purified from cultured fungi using a Plant Mini Kit (Qiagen N.V., Hilden, Germany) according to the manufacturer's instructions.[44] Extracted RNA was immediately reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant) (Promega K.K., Madison, WI, USA) or GeneAce Reverse Transcriptase (Nippon Gene Co., Ltd., Japan) using oligo dT primers. qPCR was then performed on a LightCycler 96 system (Roche Diagnostics K.K., Tokyo, Japan) using Thunderbird SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan). The thermal cycling program consisted of initial denaturation at 95 °C for 30 s, followed by 50 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. A melting curve analysis was conducted using the following steps: 95 °C for 10 s, 65 °C for 60 s, 97 °C for 1 s. Primers were designed for specific sequences using Primer3[45] (see Supporting Information).

Addition of the Supernatant of Cell Culture Medium. J774.1 cells were cultured in D-MEM supplemented with 10% FBS in a 75 cm^2 cell culture flask and then replaced with CD medium using the method described above. The cell culture was incubated at 28 °C for 5 days. After cultivation, the cells were collected using 50 mL tubes and centrifuged at 2,383 *g* at 20 °C for 5 min. The supernatant was then filtered through a 0.45 μm hydrophobic polytetrafluorethylene (PTFE) membrane (Merck & Co., Inc., Rahway, NJ, USA) to remove cells, resulting in a clear supernatant fraction. This supernatant fraction was subsequently added to a flask containing fungi and cultured at 28 °C for 1 week.

Fractionation of the Cell Supernatant with Amicon. A total of 8 mL of J774.1 cell supernatant was processed using 15 Amicon Ultra−Centrifugal Filters (Ultracel- 3K Millipore, Merck & Co.) and centrifuged at 11,741*g* for 60 min. The fraction above 3 kDa was dissolved in 8 mL of new CD medium and added directly to 0.1 cm^3 of the fungal suspension. The fraction below 3 kDa was added directly to 0.1 cm^3 of the fungal suspension. Both fractions were

https://doi.org/10.1021/acsomega.4c07124
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incubated in 25 cm^2 cell culture flasks (Violamo, As One Corp.) at 28 °C for 1 week.

Degradation of Proteins in Cell Supernatant by Proteinase K. A total of 8 mL of J774.1 cell supernatant was treated with 0.9 mg of Proteinase K (Fujifilm Wako Pure Chemical Corp.) at 37 °C for 1 h. The Proteinase K was then inactivated by heating at 90 °C for 10 min. As a control, 8 mL of CD medium was treated with 0.9 mg of Proteinase K at 37 °C for 1 h, followed by heating at 90 °C for 10 min. Samples were added to 0.1 cm^3 of the fungal suspension and incubated at 28 °C for 1 week.

Addition of BSA. A total of 1.7 mg of BSA (Sigma-Aldrich Co. LLC, Saint Louis, MO, USA) was dissolved in CD medium and added to 0.1 cm^3 of the fungal suspension. The samples were then incubated in 25 cm^2 culture flasks at 28 °C for 1 week.

Addition of Protease Inhibitor. Protease inhibitor cocktail tablets (Merck & Co.) were added to 8 mL of cell supernatant in a 25 cm^2 culture flask at final concentrations of 0.053, 0.16, and 0.53 mg/mL. Then, 0.08 cm^3 of the fungal suspension was added to the flask and the mixture was cultured at 28 °C for 1 week.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c07124.

Isolation charts, experimental procedures, spectral data, and additional information (PDF)

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Author Contributions

Y.A. isolated the compounds, performed spectroscopic analysis, and analyzed the data. Y.U. and C.I. conducted RT-qPCR and HRMS experiments and contributed to data analysis. T.Y. collected and provided the microorganisms. S.S. and M.A.A. wrote the manuscript. All authors read, discussed, and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for research in Transformative Research Areas (A) "Latent Chemical Space" to M.A.A. (Grant Nos. JP23H04880 and JP23H04884) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a grant from the Mitsubishi Foundation.

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https://doi.org/10.1021/acsomega.4c07124
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