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ORIGINAL PAPER

Mycobacterium smegmatis alters the production of secondary metabolites by marine-derived *Aspergillus niger*

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Abstract

It is generally accepted that fungi have a number of dormant gene clusters for the synthesis of secondary metabolites, and the activation of these gene clusters can expand the diversity of secondary metabolites in culture. Recent studies have revealed that the mycolic acid-containing bacterium *Tsukamurella pulmonis* activates dormant gene clusters in the bacterial genus *Streptomyces*. However, it is not clear whether the mycolic acid-containing bacteria activate dormant gene clusters of fungi. We performed co-culture experiments using marine-derived *Aspergillus niger* with *Mycobacterium smegmatis,* a mycolic acid-containing bacteria. The co-cultivation resulted in the production of a pigment by *A. niger* and increased cytotoxic activity of the extract against human prostate cancer DU145 cells. An analysis of secondary metabolites in the extract of the co-culture broth revealed that the increase in cytotoxic activity was caused by the production of malformin C (**1**), and that TMC-256A1 (**2**), desmethylkotanin (**3**), and aurasperone C (**4**) were selectively produced under co-culture conditions. In addition, further study suggested that direct interaction between the two microorganisms was necessary for the production of the pigment and the cytotoxic compound malformin C (**1**) from *A. niger*. Given the biological activities of malformin C, including cytotoxic activity, our approach for increasing the production of bioactive secondary metabolites has important practical applications and may facilitate structural analyses of novel bioactive compounds.

Keywords Co-culture · *Aspergillus niger* · *Mycobacterium smegmatis* · Mycolic acid-containing bacteria · Secondary metabolite

Introduction

Secondary metabolites produced from microorganisms have a wide range of chemical properties and have made a substantial contribution to drug discovery owing to their diverse biological activities and chemical structures. Moreover, they contribute to the advancement of chemical biology research aimed at understanding such diverse biological activities at the molecular level. However, the development of medicinal seeds from microorganisms is decelerating owing to the difficulty in finding new compounds.

Metagenome and transcriptome analyses of microorganisms, actinomycetes, and fungi have indicated that a large number of unidentified gene clusters involved in the production of secondary metabolites exist, but these genes are in a dormant state under standard laboratory conditions [1, 2]. Therefore, the establishment of methods to activate dormant gene clusters in microorganisms can expand the diversity of secondary metabolites. It is also important to increase the production of bioactive secondary metabolites to facilitate drug discovery and chemical biology studies. Several studies have focused on altering the production profile of secondary metabolites from microorganisms. A combination of a histone deacetylase inhibitor and DNA methyltransferase inhibitor activates the dormant gene cluster of marine-derived

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Aspergillus spp. and alters the production profile of secondary metabolites, including a new diphenylether-*O*-glycoside [3]. Marine-derived *Penicillium purpurogenum* with acquired resistance to gentamicin produces janthinone, fructigenine A, aspterric acid methyl ester, and citrinin, whereas the wild-type strain does not produce these compounds [4]. Disruption of the non-ribosomal peptide synthetase module *aziA2* in *Streptomyces sahachiroi* activates the production of furan-2,4-dicarboxylate [5]. Recently, the application of microbial interactions has received substantial attention as a method to activate dormant gene clusters for the production of secondary metabolites. For instance, marine-derived *Aspergillus versicolor* produces a new cyclic pentapeptide, aflaquinolone, and anthraquinones by co-cultivation with *Bacillus subtilis* [6]. Cytochalasins, known to bind to actin filaments, are produced in the co-culture of marine-derived *Aspergillus flavipes* and *Streptomyces* spp. [7]. The physical direct interactions between *Aspergillus nidulans* and *Streptomyces rapamycinicus* resulted in the modification of fungal histones to produce orsellinic acid, lecanoric acid, F-9775A and B [8]. *Tsukamurella pulmonis* TP-B0596, which has a mycolic acid layer on the outside of the cells, induces the production of a red pigment by *Streptomyces lividans* TK23 [9]. Pigment production by *S. lividans* TK23 has also been observed by co-cultivation with various genera of mycolic acid-containing bacteria, such as *Dietzia*, *Rhodococcus*, *Nocardia*, *Williamsia*, *Gordonia*, *Corynebacterium,* and *Mycobacterium*. Moreover, the disruption of the *pks13* gene, which is related to mycolic acid biosynthesis, in *T. pulmonis* TP-B0596 failed to induce pigment production by *S. lividans* TK23. Therefore, mycolic acids on the cell surface are important for the induction of pigment production. *T. pulmonis* TP-B0596 also induced the production of secondary metabolites, such as new polyketide alchivermycins [9], indolocarbazole arcyriaflavin E [10], γ-butyrolactone chojalactones [11], and niizalactams [12], from some *Streptomyces* spp. However, the effects of mycolic acid-containing bacteria on the production of secondary metabolites from fungi have not been investigated to date.

We previously isolated fungi from marine organisms and sea sand and used these as natural resources for the isolation of bioactive secondary metabolites [13, 14]. Marine-derived *Aspergillus niger* 15F41-1-3 was isolated from an unidentified marine sponge, but the culture broth extract was not selected as a candidate for isolating bioactive compounds, such as the cytotoxic and antimicrobial substances. In addition, we have utilized *Mycobacterium smegmatis* mc^{2}155 and *Mycobacterium bovis* BCG Pasteur for the screening of anti-dormant mycobacterial substances and genetically modified *M. smegmatis* mc^{2}155 for the identification of target molecules of isolated antimicrobial substances [15, 16].

In this study, we investigated whether the mycolic acid-containing bacterium *M. smegmatis* mc^{2}155 is able to alter the production of fungal secondary metabolites using marine-derived *A. niger* 15F41-1-3 as a model fungal strain. In addition, the necessity for direct interaction between the microorganisms was investigated.

Results and discussion

Comparison of pigment production, cytotoxic activity, and production profiles of secondary metabolites from *A. niger* under axenic and co-culture conditions

To evaluate whether *M. smegmatis* mc^{2}155, which has mycolic acids on the surface of bacilli, is able to alter the production of secondary metabolites, we first compared the *A. niger* 15F41-1-3 phenotype and cytotoxic activity between axenic culture and co-culture conditions. As shown in Fig. 1, the mycelium of *A. niger* under axenic culture conditions appeared white, whereas the mycelium under co-culture conditions changed from white to orange as a result of pigment production. The culture extract of *A. niger* under axenic conditions showed growth inhibition rates of 2% and 48% against human prostate cancer DU145 cells at 30 µg/ml and 100 µg/ml, respectively (Fig. 2). The extract prepared from *A. niger* under co-culture conditions with *M. smegmatis* exhibited markedly higher cytotoxic activity compared with that for the axenic culture of *A. niger*. In fact, the 30 µg/ml and 100 µg/ml extracts prepared from the co-culture broth exhibited growth inhibition of 55% and 96%, respectively (Fig. 2). These results suggested that the production of cytotoxic compounds from fungus increased by *M. smegmatis*. We comprehensively analyzed the production profile of secondary metabolites in the extracts by LC–MS (Fig. 3). The production profile for the extract prepared from the axenic culture broth was significantly different from that of the extract from the co-culture broth, and the production of secondary metabolites appeared to be increased in co-culture conditions. In particular, peak A (Rt 10.53 min), peak B (Rt 10.80 min), peak C (Rt 10.94 min), peak D (Rt 11.05 min), peak E (Rt 12.70 min), peak F (Rt 13.08 min), and peak G (Rt 13.68 min) were detected only under co-culture conditions. The MS spectra are shown in supplementary material Fig. S1. These results further suggest that *M. smegmatis* can be used to alter the production of secondary metabolites by fungi.

Isolation of secondary metabolites selectively produced in co-culture conditions

We next isolated cytotoxic compounds against human prostate cancer DU145 cells with increased production under co-culture conditions, as shown in Fig. 2. The EtOAc

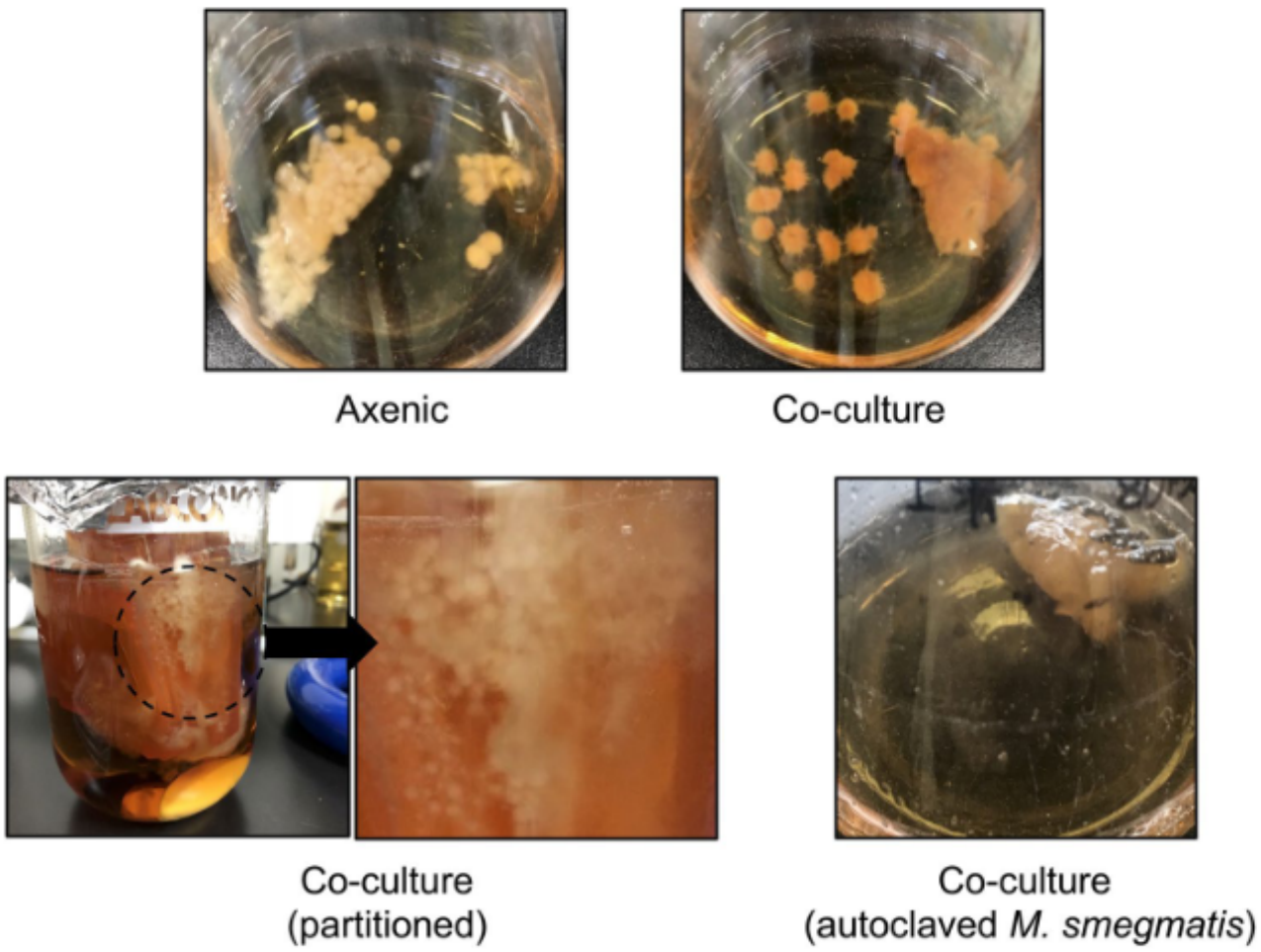

Fig. 1 Pigment production by *Aspergillus niger* 15F41-1-3 under each culture condition

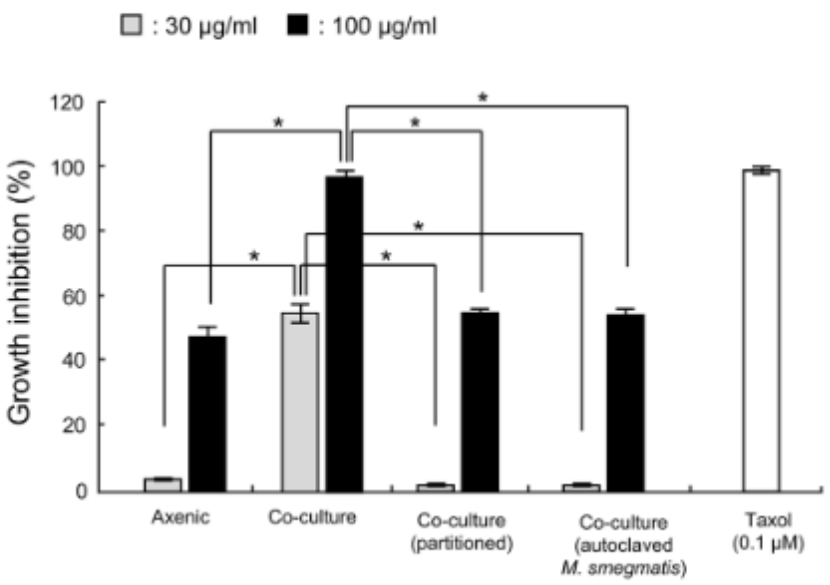

Fig. 2 Cytotoxic activity of extracts prepared from the culture broth of *A. niger* 15F41-1-3 under each culture condition. DU145 cells (1 x 10^4 cells/well/100 μl) in 96-well plates were treated with the indicated concentrations of each extract for 48 h. The growth inhibition rate was calculated as the percentage relative to parallel negative controls. Data are shown as means ± standard errors of $n = 3$ independent experiments. Differences were considered significant at $^*p < 0.05$

extract (2.3 g) prepared from 3 L of culture broth under co-culture conditions was partitioned into an *n*-hexane/90% aq MeOH mixture. In a bioassay, the 90% MeOH soluble portion (1.3 g, 55% growth inhibition at 30 μg/ml) was fractionated by silica gel column chromatography (eluted with $CHCl_3$-MeOH) to obtain three fractions (Fr. A1–A3). The active Fr. A1 (125 mg, 96% growth inhibition at 30 μg/ml) was then separated by reversed-phase HPLC [COSMOSIL $5C_{18}$-AR-II (10 mm id × 250 mm); eluted with 44% CH_3CN aq] to give three fractions (Fr. B1–B3). The active Fr. B2 (4 mg, 80% growth inhibition at 1.0 μg/ml) was further purified by reversed-phase HPLC [COSMOSIL Cholester (10 mm id × 250 mm); eluted with 37% CH_3CN aq] to obtain malformin C (**1**, 0.3 mg, $IC_{50} = 0.11$ μM) as an active compound. Malformin C (**1**) was identified by ESI-TOF-MS and 2D-NMR analyses, value of specific rotation and comparison with authentic spectral data (Fig. 4 and Supplementary material Fig. S2) [17]. We also fractionated the EtOAc extract (2.4 g) prepared from 3 L of axenic culture of *A. niger* in the same manner as the fractionation of the extract prepared from the co-culture broth. All fractions corresponding to the active fractions from the extract of co-culture origin did not show cytotoxic activity against DU145 cells at 30 μg/ml. In addition, the fraction (35 μg) corresponding to Fr. B2 did not include compound **1**, as determined by ODS-HPLC, under the same conditions used to detect the compound in 35 μg of Fr. B2 (Supplementary material Fig. S3). This result strongly suggested that cytotoxic activity in the extract of the co-culture broth would result from the production of compound **1**. Malformin C (**1**) has been isolated from the culture broth of terrestrial *A. niger* and has several known biological activities, including cytotoxic activity against cancer cells [17, 18].

In the process of analyzing the fractions from both extracts, we determined that Fr. B1 includes some secondary metabolites that were selectively produced in

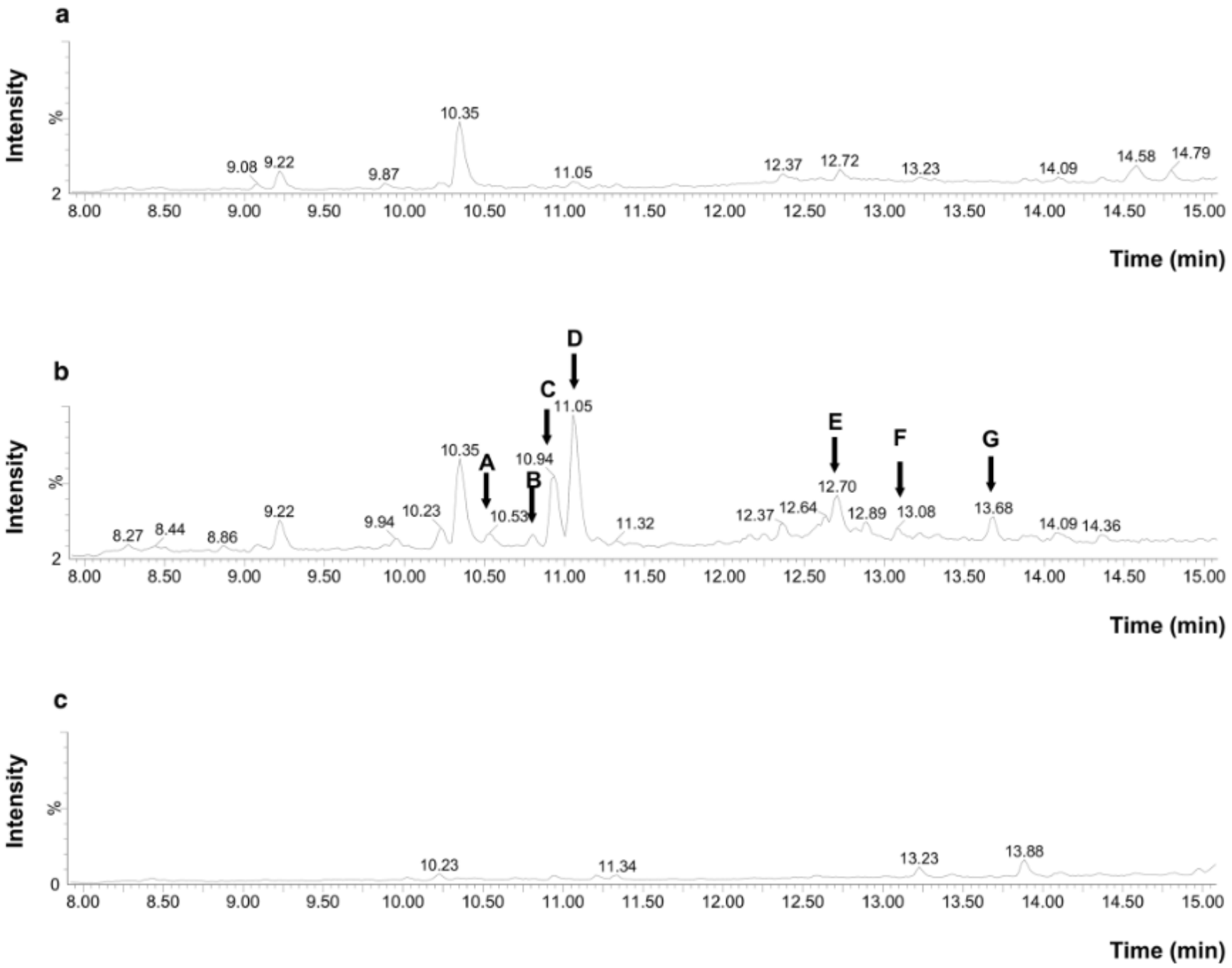

Fig. 3 Comparison of total ion current chromatograms (TICs) in each extract by LC-MS.The conditions for LC-MS are described in the experimental section. MS detection was ESI (positive ion, *m/z* 100–1500). **a** EtOAc extract from the axenic culture of *A. niger* (5.0 μg/injection). **b** EtOAc extract from the co-culture broth (5.0 μg/injection). **c** EtOAc extract from the axenic culture of *M. smegmatis* (5.0 μg/injection). Peak F was identified as compound **2**

co-culture conditions (Supplementary material Fig. S4). To elucidate the chemical structures of the compounds, Fr. B1 (40 mg) was separated by reversed-phase HPLC [COSMOSIL $5C_{18}$-AR-II (10 mm id × 250 mm); eluted with 35% CH_3CN aq containing 0.1% HCOOH] to give three fractions (Fr. C1–C3). Further purification of Fr. C1, C2, and C3 by reversed-phase HPLC [COSMOSIL Cholester (10 mm id × 250 mm); eluted with 40% CH_3CN aq containing 0.1% HCOOH] yielded TMC-256A1 (**2**, 1.5 mg), desmethylkotanin (**3**, 1.6 mg), and aurasperone C (**4**, 4.2 mg) (Fig. 4). Compounds **2**–**4** were identified by ^{1}H-NMR analysis, and ESI-TOF-MS or MALDI-TOF-MS analyses and comparisons with authentic spectral data (Supplementary material Figs. S5-S7) [19–21]. In addition, the selective production of **2** was supported by the detection of peak F (Rt 13.08, *m/z* 273 $[M+H]^+$) in the LC-MS analysis (Fig. 3 and Supplementary material Fig. S1). However, the peaks corresponding to compounds **1**, **3**, and **4** were not observed in the extract prepared from the co-cultured broth. Thus, the isolation and structural elucidation of compounds corresponding to peaks A–F and G is under consideration. Moreover, although the mRNA expression levels of the genes related to the biosynthesis of compounds **1**–**4** which were reported to be isolated from the *A. niger* group [22] under different axenic culture conditions have to be confirmed, our results strongly suggested that *M. smegmatis* mc^2155 activated the fungal biosynthetic gene clusters corresponding to compounds **1**–**4**, which were in a dormant state under our axenic culture conditions.

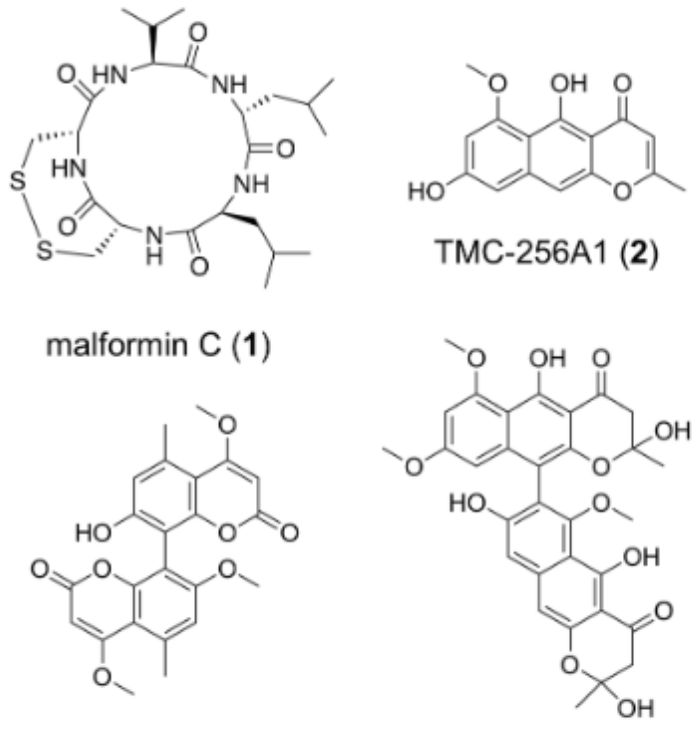

Fig. 4 Chemical structures of compounds **1–4** produced by *A. niger* under co-culture conditions with *M. smegmatis*

Importance of the direct interaction between *A. niger* and *M. smegmatis* for pigment and cytotoxic compound production

Direct interactions between *A. nidulans* and *S. rapamycinicus* have been shown to alter secondary metabolite production [8]. We investigated whether the direct interaction between *A. niger* and *M. smegmatis* was necessary or not. *A. niger* and *M. smegmatis* were cultured for 2 weeks at 30 °C in two compartments partitioned with a dialysis membrane using dialysis membrane cassettes (Slide-A-Lyzer G2 Dialysis Cassettes 10 K MWCO, 15 ml). As shown in Fig. 1, pigment production by *A. niger* was not observed when each microorganism was cultured in separate compartments. The cytotoxic activity of the extract was similar to that in axenic culture conditions for *A. niger* (Fig. 2). This result indicated that the direct interaction between *M. smegmatis* and *A. niger* was necessary for the production of pigment and increased the production of cytotoxic malformin C (**1**). In addition, autoclaved *M. smegmatis* was used for co-culture with *A. niger*, and pigment production and cytotoxic activity in extracts were investigated. Because autoclaved *M. smegmatis* did not induce pigment production and did not effectively increase cytotoxic activity, the living state of *M. smegmatis* was important for altering the production of secondary metabolites under co-culture conditions (Figs. 1 and 2). These results indicated that *M. smegmatis* interacted with *A. niger* directly, thereby activating or promoting the transcription of dormant gene clusters related to the biosynthesis of the pigment and isolated secondary metabolites (**1–4**).

In conclusion, we demonstrated for first time that the mycolic acid-containing bacterium *M. smegmatis* mc^2155 is able to change the production of fungal secondary metabolites under co-culture conditions using marine-derived *A. niger* 15F41-1-3. Analyses of signal transductions in *A. niger,* the molecular mechanisms underlying the role of *M. smegmatis*, and whether *M. smegmatis* is able to alter the production profiles of secondary metabolites by other fungi under co-culture conditions are currently underway.

Experimental

General Experimental Procedures

NMR spectra, with reference to tetramethylsilane (TMS) or residual solvent peaks were measured using an agilent NMR system (^{1}H: 600 MHz, ^{13}C: 150 MHz). ESI-TOF-MS and MALDI-TOF-MS were recorded using a Q-Tof Ultima API mass spectrometer (Waters Co., Milford, MA, USA) and JMS-S3000 (JEOL Ltd., Tokyo, Japan). IR spectra and specific rotations were obtained using a JASCO FT/IR-5300 (KBr pellets) and JASCO P-2200 digital polarimeter ($L = 50$ mm) (JASCO Co., Tokyo, Japan), respectively. UV spectra were obtained using a UV-2450 spectrophotometer (SHIMADZU Co., Kyoto, Japan). Column chromatography was performed on silica gel 60 N (63–210 µm, Kanto Chemical Co., Inc., Tokyo, Japan), COSMOSIL $5C_{18}$-AR-II (10 mm id × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan), and COSMOSIL Cholester (10 mm id × 250 mm, Nacalai Tesque, Inc.). HPLC was performed using a Hitachi L-6000 pump equipped with a Hitachi L-4000H UV detector (Hitachi High-Tech Science Co., Tokyo, Japan). Thin-layer chromatography (TLC) was performed using pre-coated TLC plates $60F_{254}$ (Merck KGaA, Darmstadt, Germany). Spots on the TLC plates were detected by spraying acidic *p*-anisaldehyde solution (*p*-anisaldehyde: 25 ml, *c*-H_2SO_4: 25 ml, AcOH: 5 ml, EtOH: 425 ml) or phosphomolybdic acid solution (phosphomolybdic acid: 25 g, EtOH: 500 ml), with subsequent heating. The LC-MS system consisted of ACQUITY UPLC (Waters Co.) for HPLC and quattro premier XE (Waters Co.) for MS. The conditions for the LC-MS analysis were as follows—0–100% CH_3CN in 0.1% HCOOH, 0–15 min, linear gradient, and 100% CH_3CN, isocratic 15–20 min; flow rate: 0.25 ml/min; column: Acquity UPLC BEH C18 (2.1 mm id × 150 mm, 1.7 µm, Waters Co.). MS detection conditions were ESI (positive and negative) (*m/z* 100–1500). The following reagents and materials were used for cell culture and bioassays—RPMI 1640 and cell count reagent SF (Nacalai Tesque, Inc.), fetal bovine serum (FBS; lot 30-2215) (Equitech-Bio Inc., Kerrville, TX, USA),

taxol (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), kanamycin (Sigma-Aldrich, St. Louis, MO, USA), and other chemicals (Sigma-Aldrich or Nacalai Tesque, Inc.).

Maintenance and axenic culture of microorganisms

The marine-derived fungal strain 15F41-1-3 was isolated from an unidentified marine sponge, which was collected at North Pagai Island, Indonesia in 2015. The strain was identified as *A. niger* by Techno Suruga Laboratory Co., Ltd. (Shizuoka, Japan) based on the morphology and 5.8S rDNA sequence. *A. niger* 15F41-1-3 was maintained on an MG agar plate (2.0% malt extract, 2.0% glucose, 0.1% Bacto peptone, 2.0% agar, and 3.8% Marine Art SF-1) at 30 °C. As the seed culture of *A. niger*, 1 cm^2 of the fungal mycelium on the MG agar plate was inoculated into two Erlenmeyer flasks containing 1.5 L each of MG broth (2.0% malt extract, 2.0% glucose, 0.1% Bacto peptone, and 3.8% Marine Art SF-1) and incubated for 2 days at 30 °C with shaking at 160 rpm. In the axenic culture of *A. niger*, the supernatant of the seed culture (800 ml each) was transferred into three Erlenmeyer flasks containing 200 ml each of MG7H9 broth consisting of MG broth supplemented with complete 7H9 broth [Middlebrook 7H9 broth (BD, Franklin, NJ, USA) containing 0.2% glycerol, 0.04% NaCl, 0.2% glucose, and 0.05% Tween 80] and incubated for 1 week at 30 °C with shaking at 150 rpm. The culture was then incubated statically at 30 °C for an additional 1 week.

M. smegmatis mc^2155 was grown on Middlebrook 7H10 agar (BD) supplemented with 0.2% glycerol, 0.04% NaCl, 0.2% glucose, and 0.05% Tween 80 at 37 °C and pre-cultured in Complete 7H9 broth at 37 °C for 2 days with shaking at 150 rpm. For the axenic culture of *M. smegmatis*, pre-cultured *M. smegmatis* (200 ml, 1.0×10^8 CFU/ml) was inoculated into Erlenmeyer flasks containing 800 ml of MG broth and incubated for 1 week at 30 °C with shaking at 150 rpm. The culture was then statically cultured at 30 °C for additional 1 week.

In the co-culture of *A. niger* with *M. smegmatis*, pre-cultured *M. smegmatis* (200 ml each, 1.0×10^8 CFU/ml) in complete 7H9 broth was mixed with the supernatant of the seed culture of *A. niger* (800 ml each) in the three Erlenmeyer flasks. Pre-cultured *M. smegmatis* (1.0×10^8 CFU/ml) was autoclaved at 121 °C for 20 min and used to consider the effect of dead *M. smegmatis* on the production of secondary metabolites by *A. niger*.

Preparation of extracts from each culture

After culturing under the conditions described above, culture extracts were obtained using equal volumes of MeOH by sonication for 15 min and MeOH was then evaporated under reduced pressure to obtain a crude extract. The extract was partitioned into a water/EtOAc mixture. The EtOAc soluble portion was used to evaluate cytotoxic activity against DU145 cells and to analyze secondary metabolites by LC-MS.

Fungal pigment production and cytotoxicity under two-compartment culture conditions using a dialysis membrane

Pre-cultured *M. smegmatis* (3.3×10^8 CFU/ml, 15 ml each) was placed in two dialysis membrane cassettes (Slide-A-Lyzer G2 Dialysis Cassettes 10 K MWCO, 15 ml; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and each dialysis membrane cassette was then placed in the supernatant of pre-cultured *A. niger* 15F41-1-3 (470 ml) in a glass beaker. After sealing the top of the glass beaker by gas exchange seals (Breathe-easier; Electron Microscopy Sciences, Hatfield, PA, USA), the microorganisms were cultured at 30 °C for 2 weeks with stirring. After confirming the presence or absence of pigment production from fungi, each compartment was extracted by an equal volume of MeOH, and each MeOH extract was combined and evaporated under reduced pressure. The extract was partitioned into a water/EtOAc mixture. The EtOAc soluble portion was then used for bioassays.

Cytotoxic activity on DU145 cells

Human prostate cancer DU145 cells were maintained in RPMI 1640 supplemented with heat-inactivated 10% FBS and kanamycin (50 µg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. To evaluate the cytotoxic activity of the samples, the DU145 cells were plated in 96-well plates (1×10^4 cells/100 µl/well) and incubated for 12 h in a humidified atmosphere containing 5% CO_2 at 37 °C. The test samples were later added and the plates were incubated for an additional 48 h under the same conditions. Cell proliferation was detected by cell count reagent SF according to the manufacturer's protocol. The growth inhibition rate was calculated as a percentage relative to negative controls. The IC_{50} values were determined by linear interpolation from the growth inhibition curve.

Statistical analysis

Data are shown as means ± standard errors of $n=3$ independent experiments, and the differences between data sets were assessed by Dunnett's test. Differences with p values of < 0.05 were considered significant.

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