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ORIGINAL PAPER

Induction of secondary metabolite production by fungal co-culture of *Talaromyces pinophilus* and *Paraphaeosphaeria* sp.

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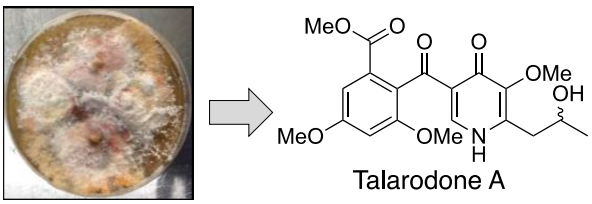
Abstract

Fungal co-culture is a strategy to induce the production of secondary metabolites by activating cryptic genes. We discovered the production of a new compound, talarodone A (**1**), along with five known compounds **2**–**6** in co-culture of *Talaromyces pinophilus* and *Paraphaeosphaeria* sp. isolated from soil collected in Miyazaki Prefecture, Japan. Among them, the productions of penicidones C (**2**) and D (**3**) were enhanced 27- and sixfold, respectively, by the co-culture. The structure of **3** should be represented as a γ-pyridol form with the reported chemical shifts, but not as a γ-pyridone form, based on DFT calculation.

Graphic abstract



Co-culture of *Talaromyces pinophilus* and *Paraphaeosphaeria* sp.

Keywords *Talaromyces pinophilus* · *Paraphaeosphaeria* sp. · Co-culture · DFT calculation

Introduction

Many medical agents have been developed from natural products as a result of their novel skeletons and various bioactivities appropriate to drugs. Although more than half of the drugs approved by the FDA were developed from natural products [1], the number of antibiotics developed from microorganisms has decreased recently. Biosynthetic gene clusters of the majority of microorganisms (> 95%) remain cryptic under standard laboratory culture conditions, as revealed by recent metagenomic studies [2–4]. Efforts such as (1) alteration of growth conditions, (2) addition of chemical elicitors, (3) modification of targeted genes, (4) expression of genes in heterologous hosts, and (5) co-culturing have been employed to activate these cryptic genes to produce microbial metabolites [5, 6]. Microorganisms in co-cultures are considered to physiologically and chemically stimulate each other, leading to activation of the cryptic genes and production of metabolites that are not produced in mono-cultures [7, 8].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11418-020-01400-1) contains supplementary material, which is available to authorized users.

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During a search for new drug candidates from cryptic fungal metabolites, we reported the isolation of a new indolinone derivative, irpexine, from the co-culture of *Irpex lacteus* (Fr.) Fr. (13S052) and *Phaeosphaeria oryzae* I. Miyake (13S070) [9]. In this study, we prepared 132 mutually co-cultured extracts using 12 fungi collected in Miyazaki Prefecture, Japan. Analysis by LC–MS indicated the presence of a new peak in the co-culture extract of *Talaromyces pinophilus* (17F4103) and *Paraphaeosphaeria* sp. (17F4110). We here report the isolation and structure determination of a new compound, talarodone A (**1**) (Fig. 1), by spectroscopic data and DFT calculation. In addition, among the five known compounds **2**–**6** (Fig. 1) isolated, the productions of penicidones C [10] (**2**) and D [11] (**3**) were enhanced by co-culture. During identification, we found that the structure of **3** should be represented as a γ-pyridol form (**3a**) with the reported chemical shifts, but not as a γ-pyridone form (**3b**), based on the DFT calculation.

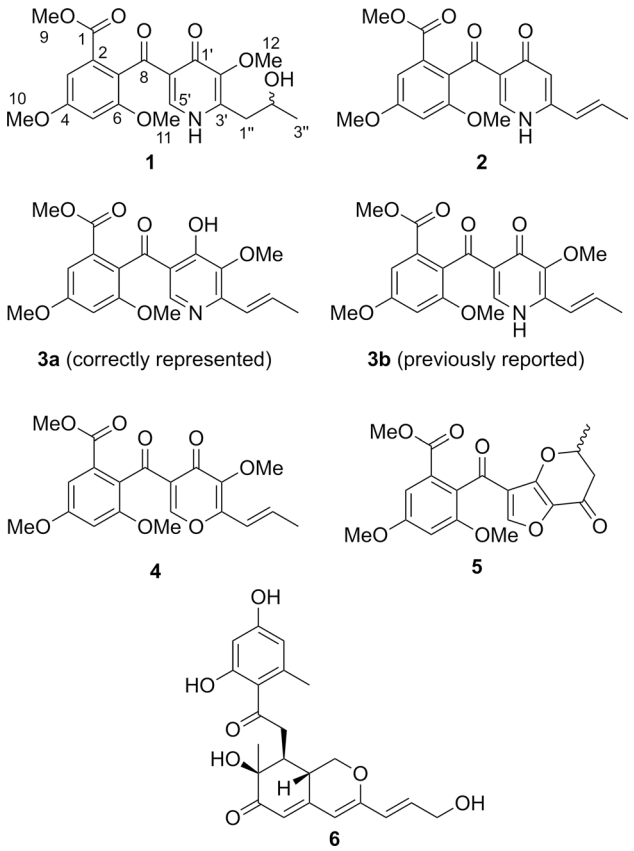

Fig. 1 Structures of compounds **1**–**6**

Results and discussion

Separation of fungal metabolites 1–6

Two fungi, *T. pinophilus* (17F4103) and *Paraphaeosphaeria* sp. (17F4110), were inoculated on malt agar medium and cultured (Fig. 2a). LC–MS analysis of the extracts of the co-culture and mono-cultures revealed the presence of a new ion peak at *m/z* 406 ($[M+H]^+$) in the co-culture that was absent in the mono-cultures (Fig. 2b). This result indicated that the co-culture activated the production of a new compound, **1**.

The MeOH extract of the co-culture was partitioned between EtOAc and H_2O, and the aqueous layer was further partitioned between *n*-BuOH and H_2O. The *n*-BuOH fraction containing the compound with *m/z* 406 was purified by ODS column chromatography and HPLC to afford **1**. LC–MS analysis of the EtOAc fraction indicated the presence of congeners of **1**. This was purified to afford penicidones C (**2**) and D (**3**), 3-*O*-methylfunicone [12] (**4**), (±)-penifupyrone [13] (**5**), and pinophilin B [14] (**6**).

Structure elucidation of new compound 1

The molecular formula of **1** was determined as $C_{20}H_{23}NO_8$ (*m/z* 406.1528 $[M+H]^+$). The ^1H and ^{13}C NMR data of **1** (Table 1) were similar to those of penicidon D (**3**), except for the presence of a 2-hydroxypropyl group (C1″–C3″) in **1** instead of a 1-propenyl group in **3**, which was confirmed

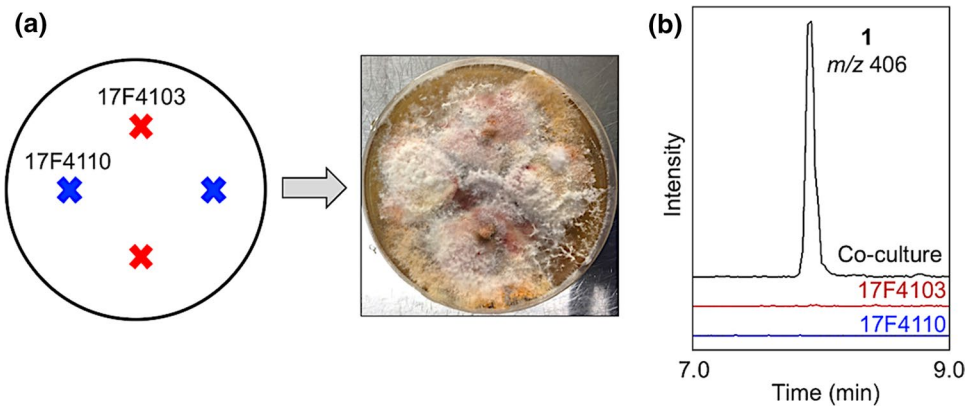

Fig. 2 **a** Co-culture of *T. pinophilus* (17F4103) and *Paraphaeosphaeria* sp. (17F4110). **b** LC–MS profiles of the extracts of the co-culture and mono-cultures for **1** extracted with *m/z* 406 $[M+H]^+$

Table 1 ^{1}H and ^{13}C NMR data of **1** (DMSO-d_6)

No.	δ_C, type	δ_H, mult (*J* in Hz)	HMBC
1	165.9, C		
2	129.2, C		
3	104.9, CH	6.92, brs	1, 2, 4, 5, 7
4	159.6, C		
5	102.7, CH	6.79, brs	3, 4, 6, 7, 8
6	157.2, C		
7	127.5, C		
8	192.0, C		
9	52.0, CH_3	3.62, s	1
10	55.7, CH_3	3.82, s	4
11	56.1, CH3	3.64, s	6
12	58.8, CH3	3.60, s	2′
1′	170.9, C		
2′	148.9, C		
3′	139.6, C		
5′	138.9, CH	8.00, s	8, 1′, 3′, 6′
6′	124.6, C		
1″	36.9, CH_2	2.59, t (6.3)	2′, 3′, 2″
2″	65.0, CH	3.91, m	
3″	23.6, CH3	1.10, d (6.0)	1″, 2″

Chemical shifts were assigned by HSQC or HMBC spectra. Measured at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR

by COSY and HMBC correlations (Fig. 3). DFT calculation was applied for the γ-pyridone and γ-pyridol forms of **1** (Table 2). The standard deviations (SDs) of the differences between the observed and calculated shifts for the γ-pyridone and γ-pyridol forms were 2.5 and 7.0, respectively, which indicated **1** to be a γ-pyridone form. The differences of C-8 and C1′–C6′ in the γ-pyridol form were particularly large. Because the optical rotation of **1** was almost zero, − 1.8 ($c = 0.33$), **1** may be a racemate.

Revision of structure description of penicidone D (3)

The structure of **3** was previously reported as a γ-pyridone form (**3b**), like **2**, without any detailed analyses [11]. We found that the ^{13}C signals at C-3′ (δ 153.7) and C-5′ (δ 147.9) (Table 3; our data) of **3** were shifted downfield, which

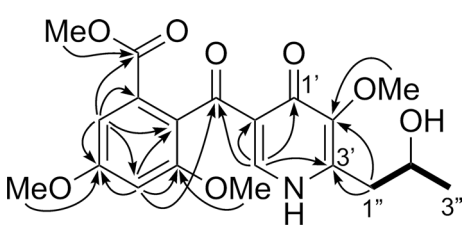

Fig. 3 COSY and HMBC correlations of **1**

Table 2 Comparison of ^{13}C NMR chemical shifts between observed and calculated data for γ-pyridone and γ-pyridol forms of **1**

No.	δ_{obs}	γ-pyridone form		γ-pyridol form	
		δ_{cald}	$\delta_{cald} - \delta_{obs}$	δ_{cald}	$\delta_{cald} - \delta_{obs}$
1	165.9	169.3	3.4	167.0	1.1
2	129.2	131.9	2.7	132.0	2.8
3	104.9	105.7	0.8	105.1	0.2
4	159.6	158.3	− 1.3	160.1	0.5
5	102.7	105.1	2.4	103.9	1.2
6	157.2	159.4	2.2	157.1	− 0.1
7	127.5	131.3	3.8	123.5	− 4.0
8	192.0	194.9	2.9	204.9	12.9
9	52.0	51.8	− 0.2	52.4	0.4
10	55.7	54.2	− 1.5	54.7	− 1.0
11	56.1	57.1	1.0	54.6	− 1.5
12	58.8	57.3	− 1.5	58.3	− 0.5
1′	170.9	173.8	2.9	163.0	− 7.9
2′	148.9	150.6	1.7	142.2	− 6.7
3′	139.6	136.3	− 3.3	163.8	24.2
5′	138.9	139.2	0.3	149.6	10.7
6′	124.6	127.7	3.1	119.7	− 4.9
1″	36.9	31.5	− 5.4	36.9	0.0
2″	65.0	69.7	4.7	66.2	1.2
3″	23.6	23.4	− 0.2	23.2	− 0.4
SD			2.5		7.0

SD standard deviation

is characteristic of the γ-pyridol form. We confirmed this by DFT calculation (Table 3). SDs of the differences between the observed and calculated shifts for the γ-pyridone and γ-pyridol forms clearly showed that **3** should be represented as a γ-pyridol form; notably, the differences of C-7, C-8, C2′–C6′, and C-2″ in the γ-pyridone form were large.

Conclusions

Co-culture of *T. pinophilus* (17F4103) and *Paraphaeosphaeria* sp. (17F4110) afforded a new metabolite, **1**, along with five known compounds, **2**–**6**. Among these compounds, the productions of **2** and **3** were enhanced 27- and sixfold, respectively, by the co-culture (Fig. 4), while those of **4**–**6** were not changed. It is noteworthy that the γ-pyridone/γ-pyridol forms in **1**–**3** in an aprotic solvent are converted by the substituted groups. Among them, only **3** has a γ-pyridol form. Compared to **1** and **3**, the substituents at C-3′ are different, i.e., a 2-hydroxypropyl group in **1** and a 1-propenyl group in **3**, and thus **3** corresponds to a 2′-methoxy derivative of **2**. The compounds isolated in this study did not show cytotoxicity or antimicrobial activity.

Table 3 Comparison of ^{13}C NMR chemical shifts between observed (150 MHz, acetone-d_6) and calculated data for γ-pyridone and γ-pyridol forms of **3**

No.	δ_{obs}	γ-pyridone form		γ-pyridol form	
		δ_{cald}	$\delta_{cald} - \delta_{obs}$	δ_{cald}	$\delta_{cald} - \delta_{obs}$
1	166.4	169.1	2.7	167.2	0.8
2	131.7	132.2	0.5	132.3	0.6
3	107.8	105.0	− 2.8	105.4	− 2.4
4	163.0	158.4	− 4.6	159.8	− 3.2
5	103.9	105.1	1.2	103.5	− 0.4
6	159.2	159.4	0.2	156.9	− 2.3
7	122.6	131.5	8.9	123.5	0.9
8	201.0	194.6	− 6.4	204.6	3.6
9	52.9	52.0	− 0.9	52.1	− 0.8
10	56.4	54.2	− 2.2	54.7	− 1.7
11	57.0	57.0	0.0	54.4	− 2.6
12	61.0	56.9	− 4.1	58.6	− 2.4
1′	162.9	174.1	11.2	163.2	0.3
2′	142.4	150.8	8.4	140.4	− 2.0
3′	153.7	133.4	− 20.3	155.7	2.0
5′	147.9	139.3	− 8.6	151.1	3.2
6′	119.9	127.8	7.9	119.1	− 0.8
1″	124.8	123.6	− 1.2	124.7	− 0.1
2″	136.4	124.5	− 11.9	135.6	− 0.8
3″	18.9	18.9	0.0	18.9	0.0
SD			7.2		1.9

SD standard deviation

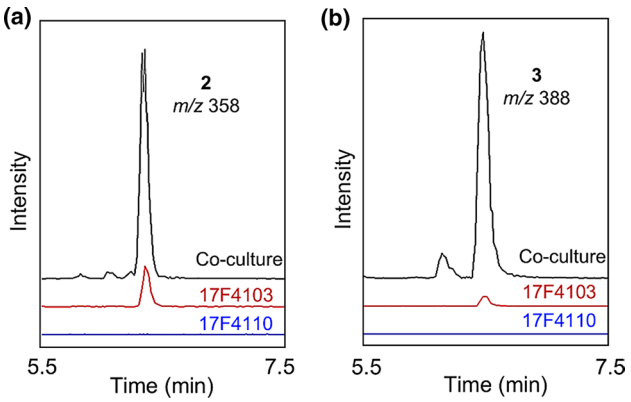

Fig. 4 LC–MS profiles of the extracts of the co-culture and monocultures for **2** and **3** extracted with *m/z* 358 (**a**) and 388 (**b**), respectively

Experimental

General experimental procedures

Optical rotations were measured on a JASCO DIP-1000 polarimeter in 50% MeOH–H_2O. UV spectra were measured on a JASCO V-550 spectrophotometer in 50% MeOH-H_2O. IR spectra were recorded on a Perkin Elmer Frontier FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance III HD 500 NMR spectrometer in DMSO-d_6 or acetone-d_6. Chemical shifts were referenced to the residual solvent peaks (δ_H 2.49 and δ_C 39.5 for DMSO-d_6, δ_H 2.04 and δ_C 29.8 for acetone-d_6). ESIMS spectra were measured on a Bruker amaZon speed ETD or Waters Xevo G2-XS Qtof mass spectrometer. The preparative HPLC system comprised a Waters 515 HPLC pump, Waters 2489 UV/visible detector, and Pantos Unicorder U-228.

Fungal material

The fungal strains 17F4103 and 17F4110 used in this study were isolated from soil collected in Miyazaki Prefecture, Japan, in March 2017, and deposited at the Graduate School of Pharmaceutical Sciences, Kumamoto University. The strains were identified according to their ITS sequences. A 600 base pair ITS sequence of 17F4103 had 100% sequence identity to that of *Talaromyces pinophilus* (CP017345), and a 624 base pair ITS sequence of 17F4110 had 99.42% sequence identity to that of *Paraphaeosphaeria* sp. (KM103318). The sequence data of these strains had been deposited to GenBank with accession numbers, MT093464 (17F4103), and MT093465 (17F4110).

Fermentation, extraction, and isolation

The fungi *T. pinophilus* (17F4103) and *Paraphaeosphaeria* sp. (17F4110) were co-cultured on a malt extract agar medium (2.0% malt extract, 0.5% peptone, and 1.5% agar; 40 mL) in 200 plastic square plates (140 × 100 × 14.5 mm) at 25 °C for 35 days. The culture was extracted with MeOH. The MeOH extract was concentrated and partitioned between EtOAc and H_2O. The EtOAc fraction was then partitioned between *n*-hexane and 90% MeOH–H_2O, and the H_2O fraction was partitioned between *n*-BuOH and H_2O. The *n*-BuOH fraction (2.5 g) was subjected to ODS chromatography with a stepwise gradient of MeOH–H_2O. The fraction eluted with 40% MeOH–H_2O was purified by HPLC [Asahipak GS-310P (21.5 × 500 mm), Asahi Chemical Industry Co., Ltd., 40% MeCN–H_2O; COSMOSIL 5C_{18}-MS-II column (20 × 250 mm), Nacalai Tesque Inc., 20% MeOH–H_2O (0.1% acetic acid)] to afford talarodone A (**1**, 0.80 mg). The 90% MeOH–H_2O fraction (1.1 g) was subjected to SiO_2 chromatography with 5, 10, and 20% MeOH–CH_2Cl_2. The fraction eluted with 5% MeOH–CH_2Cl_2 was purified by SiO_2 HPLC [Inertsil SIL-100A (20 × 250 mm), GL Sciences Inc., CH_2Cl_2–MeOH (319:1)] to afford **2** (6.7 mg), **3** (14.0 mg), **4** (3.0 mg), and **5** (4.5 mg). The fraction eluted

with 10% MeOH–CH_2Cl_2 was subjected to ODS chromatography with 75% MeOH–H_2O and MeOH. The fraction eluted with MeOH was purified by HPLC [Inertsil Diol (20 × 250 mm), GL Sciences Inc., CH_2Cl_2–MeOH (24:1)] to afford **6** (3.4 mg).

Talarodone A (**1**): white amorphous powder. $[\alpha]_D^{20}$ − 1.8 (c = 0.33, 50% MeOH–H_2O). UV λ_{max} (50% MeOH–H_2O) (log ε): 318 (3.82), 260 (3.97), 236 (4.39), 206 (4.63), 196 (4.59) nm. IR (film) υ_{max}: 3244, 2922, 2849, 1664, 1599, 1330, 1063, 843, 791 cm^{-1}. ^1H and ^{13}C NMR data (DMSO-d_6), see Table 1. HRTOFMS *m/z* 406.1528 $[M+H]^+$ (calcd for $C_{20}H_{24}NO_8$, 406.1502).

Conformational analyses and chemical shift calculations for the γ-pyridone and γ-pyridol forms of 1 and 3

These experiments were performed as previously described [15] using spartan'18 instead of spartan'16. Chemical shift calculations were performed at the ωB97X-D/6-31G* level.

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