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FULL PAPER

# Discovery of the 2,4′-dihydroxy-3′-methoxypropiophenone biosynthesis genes in *Aspergillus oryzae*

Eiichiro Kan,[a,b] Hiroya Tomita,[a] Yohei Katsuyama,[a,c] Jun-ichi Maruyama,[a,c] Yasuji Koyama,[b] and Yasuo Ohnishi* [a,c]

[a] E. Kan, Dr. Hiroya Tomita, Dr. Y. Katsuyama, Dr. J. Maruyama, Dr. Y. Ohnishi
Department of Biotechnology
Graduate School of Agriculture and Life Sciences, The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657 (Japan)
E-mail: ayasuo@mail.ecc.u-tokyo.ac.jp

[b] E. Kan, Dr. Y. Koyama
Noda Institute for Scientific Research
338 Noda, Noda City, Chiba 278-0037

[c] Dr. Y. Katsuyama, Dr. J. Maruyama, Dr. Y. Ohnishi
Collaborative Research Institute for Innovative Microbiology
Graduate School of Agriculture and Life Sciences, The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657 (Japan)

* Corresponding author. E-mail: ayasuo@mail.ecc.u-tokyo.ac.jp

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**Abstract:** The filamentous fungus *Aspergillus oryzae* has 27 putative iterative type I polyketide synthase (PKS) gene clusters, but secondary metabolites produced by them are mostly unknown. Here, we focused on eight clusters which were reported to be expressed at relatively high levels in a transcriptome analysis. By comparison of metabolites between an octuple-deletion mutant of these eight PKS gene clusters and its parent strain, we found that *A. oryzae* produced 2,4′-dihydroxy-3′-methoxypropiophenone (**1**) and its precursor, 4′-hydroxy-3′-methoxypropiophenone (**3**), in a specific liquid medium. Furthermore, an iterative type I PKS (PpsB) encoded by AO090102000166 and an acetyl-CoA ligase (PpsA) encoded downstream from *ppsB* were shown to be essential for their biosynthesis. PpsC, encoded upstream from *ppsB*, was shown to have **3**-binding activity ($K_d$; 26.0 ± 6.2 μM) and is suggested to be involved in the conversion of **3** to **1**. This study deepens our understanding of cryptic secondary metabolism in *A. oryzae*.

## Introduction

The filamentous fungus *Aspergillus oryzae* has long been used for the production of Japanese fermented foods, such as rice wine (*sake*), soy sauce (*shoyu*), and soybean paste (*miso*). Therefore, *A. oryzae* is classified as a GRAS (generally regarded as safe) organism by the United States Food and Drug Administration (FDA). [1] Appropriate for a GRAS organism, *A. oryzae* is known to produce few secondary metabolites, which are potentially toxic. In standard fermentation conditions, most of the endogenous secondary metabolite biosynthetic gene clusters appear to not be expressed in *A. oryzae*. This characteristic has allowed the fungus to serve a suitable host for the heterologous production of fungal secondary metabolites because of its relatively clear background in liquid chromatography (LC) and/or mass spectrometry (MS) analysis of heterologously produced metabolites. For example, atrochrysone, aphidicolin, paxilline, aspyridone, and chrodrimanin B have been heterologously produced in *A. oryzae*, and the functions of their biosynthetic genes have been analyzed. [2-6]

Meanwhile, several *A. oryzae*'s secondary metabolites have been identified. Examples include kojic acid, cyclopiazonic acid, asperfuran, and kojistatin. [7-10] These secondary metabolites are produced on different solid media, such as CYA (Czapek yeast autolysate) agar, YES (yeast extract sucrose) agar, and autoclaved rice. In the analysis of *Aspergillus nidulans*'s metabolites, different solid media were also used; CYA agar for sterigmatocystin, YES agar and CY20 (Czapek yeast salt) agar for monodictyphenone, and RT (Raulin-Thom) agar for emodin. [11] However, liquid media are also sometimes used for the production of fungal secondary metabolites. Lovastatin production in liquid culture by *Aspergillus terreus* is a famous example. [12] It was reported that the expression levels of lovastatin biosynthesis genes were different between solid-state and liquid-submerged cultures. [13] In *A. oryzae*, it was also reported that the expression levels of primary metabolic genes were different between solid and liquid cultures. [14] Therefore, it is very important to consider various culture conditions, including solid and liquid states, for detecting cryptic secondary metabolites of *A. oryzae*.

Polyketides are a major class of secondary metabolites not only in bacteria, but also in fungi. Polyketide synthases (PKSs) synthesize the backbones of various polyketides from acyl-CoA building blocks by repeated cycles of decarboxylative Claisen condensations. After elongation, the β-oxofunctionality is

successively processed by enzymes such as ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and *C*-methyltransferase (cMT). [15] Iterative type I PKSs, which are very common in fungi, have only a single copy of each functional domain necessary for precursor loading, chain assembly, and reduction. [16] Therefore, they use each functional domain multiple times to synthesize polyketides.

In 2005, the whole genome of *A. oryzae* was sequenced, showing that *A. oryzae* contained a number of secondary metabolite biosynthetic gene clusters. [17] Secondary metabolite unknown regions finder (SMURF) analysis indicated that *A. oryzae* has 61 secondary metabolite biosynthesis gene clusters including 27 PKS gene clusters. [18] All 27 PKS gene clusters contain iterative type I PKS genes. However, secondary metabolites produced by these PKS gene clusters remain mostly unknown. Only 2 polyketides, the spore pigment YWA1 [19] and isocoumarin derivatives, [20] have been identified so far. YWA1 is empirically known to be produced only in the course of sporulation on a solid medium. Isocoumarin derivatives are not produced under normal culture conditions, while they were produced and identified when the pathway-specific transcriptional activator for their biosynthetic genes was forcedly expressed. [20]

In this study, we aimed to identify cryptic polyketides that could be produced by uncharacterized iterative type I PKS gene clusters in *A. oryzae*. For this purpose, we focused on eight iterative type I PKS gene clusters (named pksc_1 to pksc_8), which had been reported to be expressed at relatively high levels in a minimal medium (CD medium) both in liquid and solid cultures in a comprehensive transcriptome analysis of *A. oryzae*. [21] Although involvement of pksc_4 in the biosynthesis of the spore pigment polyketide YWA1 had been reported, [19] the functions of the remaining seven PKS gene clusters was unknown. We disrupted these eight PKS gene clusters one by one to construct an *A. oryzae* strain that lacked all eight PKS gene clusters. This octuple-deletion mutant (Δpksc_1-8) and its parent strain were cultured under various culture conditions using different culture media, and their metabolites were compared to seek out unidentified polyketides produced by any of these eight PKS gene clusters. As a result, we found that production of three related polyketides was lost in the Δpksc_1-8 strain. A subsequent analysis showed that pksc_1 was responsible for the production of the three polyketides. Structure elucidation of these polyketides and functional analysis of the pksc_1 locus revealed three genes involved in the biosynthesis of 2,4′-dihydroxy-3′-methoxypropiophenone, and indicated a probable biosynthetic pathway for this compound. 2,4′-Dihydroxy-3′-methoxypropiophenone was reported to be a metabolite, evofolin-A, in the heartwood extract of the medicinal plant *Tetradium glabrifolium* and it was also isolated from the herb *Sida acuta* as a compound to induce quinone reductase in cultured Hepa 1c1c7 mouse hepatoma cells. [22, 23] However, no information on its biosynthetic pathway has been provided. Therefore, the isolation of 2,4′-dihydroxy-3′-methoxypropiophenone from microorganisms, as well as the identification of its biosynthesis genes, is unprecedented.

## Results and Discussion

### Generation and metabolic analysis of an *A. oryzae* strain that lacks 8 PKS gene clusters

As described in the introduction, we focused on eight PKS gene clusters in *A. oryzae* (pksc_1 to pksc_8) reported to be expressed at relatively high levels in a minimal medium (Table S1). [21] First, we constructed an *A. oryzae* strain that lacked all eight PKS gene clusters (Δpksc_1-8) by deleting those gene clusters one by one in the order of their numbers. We used *A. oryzae* RIB40 RkuptrP2-1(Δ*ku70*, Δ*pyrG*), which has the advantage of genetic modification, as the original parent strain. Then, the Δpksc_1-8 strain and the parent strain were cultivated under eight different conditions of solid and liquid cultures using four different media (CYA, YES, CY20, and RT). We analyzed the ethyl acetate extract of each culture to compare metabolic profiles between the Δpksc_1-8 and parent strains.

Against our expectations, there were only a few differences in the metabolic profiles between the two strains under the eight culture conditions examined (data not shown). A clear difference was observed when cultured on YES agar. Only the parent strain produced a compound that was predicted to be YWA1 (Figure S1). Because the Δpksc_1-8 strain lacks pksc_4, which is responsible for the production of YWA1, [19] this result is very reasonable. More importantly, another clear difference was observed when the strains were cultured in CYA liquid. Three compounds (**1**, **2**, and **3**) were produced only in the parent strain (Figure 1). Interestingly, a single PKS gene cluster deletion mutant, the Δpksc_1 strain, which was generated at the very beginning of our gene-deletion experiments, also did not produce these three compounds (Figure 1). This result indicates that the pksc_1 gene cluster should be involved in the biosynthesis of these three compounds. Therefore, in this study, we focused on the biosynthesis of these three compounds by the enzymes encoded by the pksc_1 locus. It should be noted that the production of compounds **1**, **2**, and **3** depended on culture conditions; they were observed only when cultivated in liquid CYA.

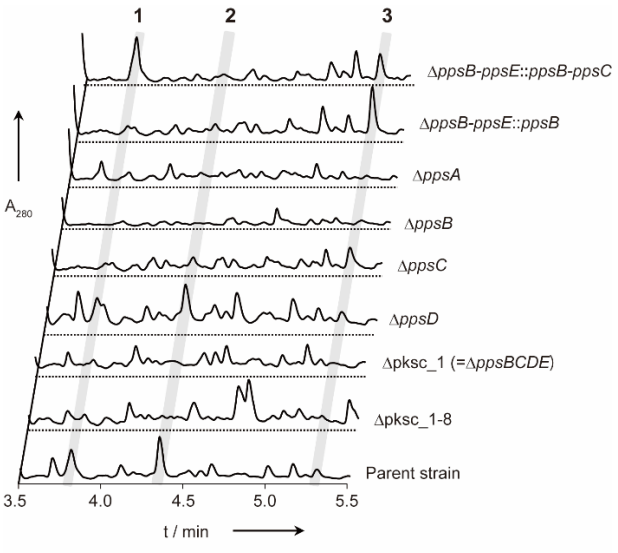
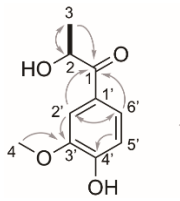



**Figure 1.** UV chromatograms of metabolites produced by *A. oryzae* strains in LC-MS analysis. Peaks including compounds **1**, **2**, and **3** are indicated.

### Purification and structure determination of compounds 1, 2, and 3

We purified compounds **1** and **3** from the ethyl acetate extract of a total of 7 L of culture. By high resolution mass spectroscopy (HR-MS) analysis, the molecular formula of **1** was determined to be $C_{10}H_{12}O_4$ (*m/z* 197.082 $[M+H]^+$; calcd for 197.080). This compound was further analyzed by $^1H$ and $^{13}C$ NMR, and a series of 2D NMR including H-H correlation spectroscopy (COSY), hetero-nuclear multiple quantum coherence (HMQC), and hetero-nuclear multiple-bond connectivity (HMBC). The NMR data suggested that **1** should be 2,4′-dihydroxy-3′-methoxypropiophenone (Figure 2). (2*S*)-2,4′-dihydroxy-3′-methoxypropiophenone was purified from the heartwood extract of the medicinal plant *T. glabrifoliumi* and named evofolin-A. [22, 24-26] We confirmed that our NMR data of **1** corresponded to the reported NMR data of evofolin-A, while the stereochemistry of **1** remained to be elucidated.[22, 24-26] Compound **3** showed a molecular ion of *m/z* 181 $[M+H]^+$ in LC-MS analysis (Figure S2). Assuming that **3** is an analogue of **1** due to their similar UV spectra (Figures S2 and S3), **3** was considered to be 4′-hydroxy-3′-methoxypropiophenone or 2-hydroxy-3′-methoxypropiophenone. By comparing retention time and UV, MS, and $^1H$ NMR spectra with commercially available 4′-hydroxy-3′-methoxypropiophenone, **3** was identified as 4′-hydroxy-3′-methoxypropiophenone (Figure S2). We were not able to obtain sufficient quantities of compound **2** for structure elucidation. However, **2** showed *m/z* 225 $[M+H]^+$ by LC-MS analysis (Figure S3), and was predicted to be a carboxylated form of **3** considering the biosynthetic pathway for **1** (see below).




| position | $\delta_H$ (multiplicity, *J* in Hz) | $\delta_c$ |
|---|---|---|
| 1 | - | 200.8 |
| 2 | 4.95 (br d, 1H) | 68.9 |
| 3 | 1.24 (d, *J*=6.6, 3H) | 23.0 |
| 4 | 3.79 (s) | 56.2 |
| 1′ | - | 126.0 |
| 2′ | 7.46 (s) | 110.5 |
| 3′ | - | 151.2 |
| 4′ | - | 147.0 |
| 5′ | 6.68 (d, *J*=8.4, 1H) | 114.2 |
| 6′ | 7.47 (d, *J*=8.4, 1H) | 124.1 |

**Figure 2.** NMR analysis of **1**. HMBC and COSY signals are indicated.

### Bioinformatic analysis of the *pps* gene cluster

As described above, compounds **1**, **2**, and **3** were revealed to be related polyketides with a propiophenone structure. Therefore, we named the putative gene cluster for the biosynthesis of these compounds the *pps* (propiophenone synthesis) gene cluster (Figure 3). Although four genes (from *ppsB* to *ppsE*) were predicted to comprise pksc_1 by the SMURF analysis, [27] we considered an additional gene (*ppsA*) located downstream from *ppsB* as a member of the pksc_1 gene cluster (Figure 3 and Table 1), because a neighboring gene pair of *ppsA* and *ppsB* homologs were found in several *Aspergillus* species (Figure S4). It should be noted that *ppsC*, *ppsD*, and *ppsE* homologs are not encoded around the *ppsA* and *ppsB* homologs in other *Aspergillus* species, except *A. flavus*. PpsA is an acyl-CoA synthetase homolog and shows the highest sequence similarity to the adenylate-forming enzyme AfeA of *Aspergillus fumigatus*. [28] PpsB was annotated as an iterative type I polyketide synthase, which has a KS-AT-DH-cMT-KR-acyl carrier protein (ACP) domain architecture. PpsC is a small hypothetical protein of 111 amino acids, but shows similarities to lipocalin-like domain-containing proteins. PpsD was annotated as a cytochrome P450 oxygenase. PpsE is a homolog of synaptic vesicle transporters.

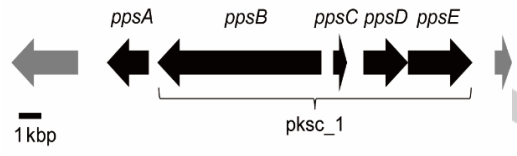



**Figure 3.** Schematic representation of the pksc_1 locus. Arrows represent lengths and directions of open reading frames. Four genes (*ppsB*, *ppsC*, *ppsD*, and *ppsE*) were predicted by the SMURF analysis to comprise a secondary metabolite gene cluster named pksc_1. We assumed that *ppsA* should be functionally related to *ppsB*.

| Accession no. | Gene | Product (amino acids) | Putative function | Homologus protein | Identity |
|---|---|---|---|---|---|
| AO090102000165 | *ppsA* | 561 | Acyl-CoA synthetase | Adenylate-forming enzyme AfeA [*Aspergillus fumigatus Af293*] XP_753401.1 | 273/558 (49%) |
| AO090102000166 | *ppsB* | 2429 | Polyketide synthase | Lovastatin nonaketide synthase [*Aspergillus udagawae*] GFF32295.1 | 1008/2541 (40%) |
| AO090102000167 | *ppsC* | 111 | Hypothetical protein | Lipocalin-like domain-containing protein [*Aspergillus bertholletius*] KAE8382349.1 | 66/93 (71%) |
| AO090102000168 | *ppsD* | 528 | Cytochrome P450 | *O*-Methylsterigmatocystin oxidoreductase [*Aspergillus flavus*] RAQ41204.1 | 365/528 (69%) |
| AO090102000169 | *ppsE* | 490 | Transporter | 1,3-β-Glucanosyltransferase Gel4 [*Aspergillus lentulus*] GFF66718.1 | 216/445 (49%) |

**Table 1.** Predicted functions of proteins encoded by the *pps* gene cluster.

### Analysis of *pps* genes by gene disruption and complementation

To investigate the biosynthetic pathway for compounds **1**, **2**, and **3**, we deleted each of the four *pps* genes to generate the Δ*ppsA*, Δ*ppsB*, Δ*ppsC*, and Δ*ppsD* mutants. These mutant strains were cultured in CYA liquid for 6 days and their metabolites were analyzed by LC-MS. Deletion of *ppsD* did not affect the production of **1**, **2**, and **3** (Figure 1). In contrast, the production of all these 3 compounds was abolished in both the Δ*ppsA* and Δ*ppsB* strains (Figure 1). In the Δ*ppsC* strain, the yields of **1** and **2** decreased, while the yield of **3** increased (Figure 1). When the Δpksc_1 strain, which is called the Δ*ppsBCDE* strain hereafter, was transformed with *ppsB*, a large amount of **3**, as well as small amounts of **1** and **2**, was produced, similar to the Δ*ppsC* strain (Figure 1). Furthermore, when both *ppsB* and *ppsC* were introduced into the Δ*ppsBCDE* strain, the production of **1** was restored (Figure 1). These results revealed that (i) *ppsA* and *ppsB* are essential for the biosynthesis of **1**, **2**, and **3**, (ii) *ppsC* is not necessarily required for the biosynthesis of **1**, but is important for improving its yield and probably related to the hydroxylation of **3** at C-2, and (iii) *ppsD* and *ppsE* are not involved in the biosynthesis of **1**, **2**, and **3**.

### Bioconversion of compound 3 into compound 1

As mentioned above, PpsC appears to be related to the hydroxylation of **3** to produce **1**. To obtain a further insight into PpsC function, bioconversion of **3** into **1** by the Δ*ppsB* and Δ*ppsC* strains was examined. These two strains were inoculated

into CYA liquid, and **3** was added into the culture medium after 24 h. After an additional 5 days of cultivation, we analyzed their metabolites and found that **3** was efficiently converted to **1** in the Δ*ppsB* strain (Figure 4). In the Δ*ppsC* strain, **1** was also detected in large amounts, but a considerable amount of **3** was also detected. We assumed that exogenously added **3** was able to be converted into **1**, but that endogenously synthesized **3** was not converted in the absence of PpsC. These results further indicate that PpsC does not catalyze the hydroxylation of **3**, but facilitates the conversion of **3** into **1**. In addition, **2** was not observed in the Δ*ppsB* strain, while it was observed in the Δ*ppsC* strain. This result indicates that **2** is produced by the function of the PKS PpsB and not produced by the conversion of **3**. We postulate that **2** should be the precursor of **3** in the biosynthetic pathway for **1** (see Scheme 1).

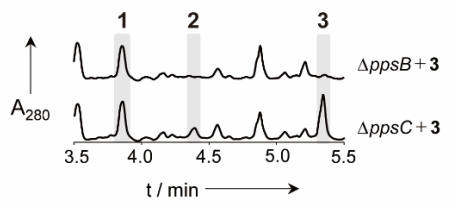



**Figure 4.** LC-MS analysis for the bioconversion of exogenously added **3** into **1**. UV chromatograms of the metabolites produced by the Δ*ppsB* and Δ*ppsC* strains cultivated in the presence of **3** are shown. Peaks including compounds **1**, **2**, and **3** are indicated.

### *In vitro* analysis of PpsC

According to the feeding study mentioned above, PpsC was not proposed to be a hydroxylase of **3**. In fact, our repeated attempts to detect any hydroxylation activity of the recombinant PpsC protein failed, even though various co-factors and metal ions were examined (data not shown). To elucidate the function of PpsC, we next used the Phyer2 software [29] to predict the folding of PpsC. PpsC was predicted to contain a fold similar to the fold of fatty acid-binding proteins (FABPs, e.g., PDB ID: 2n93). [30] FABPs are proposed to function as a lipid chaperone in the transport of lipids to specific cellular compartments, such as lipid droplets for storage, the endoplasmic reticulum for signaling and membrane synthesis, and mitochondria and peroxisomes for oxidation. [31] They share the same overall tertiary structure composed of a ten-stranded anti-parallel β-barrel structure, which is formed by two five-stranded β-sheets. The binding pocket is located inside the β-barrel, and a fatty acid molecule is bound to the interior cavity. [32] Therefore, we expected that PpsC functioned as a carrier protein to transport the biosynthetic intermediate of **1**, presumably **3**, to a specific cell compartment in which **3** is hydroxylated to **1**.

To verify our hypothesis, we prepared recombinant PpsC protein (Figure S7A) and analyzed *in vitro* binding of PpsC to **3** by a competition assay using 8-anilinonaphthalene-1-sulfonic acid (ANS) as an indicator according to previous reports. [33, 34] First, we determined the $K_d$ value between ANS and PpsC to be 26.6 ± 9.3 μM (Figure S7B). Although the value was higher than the reported $K_d$ value (1.58 ± 0.29 μM) between ANS and the epidermal fatty acid-binding protein E-FABP, [35] the affinity of ANS to PpsC was high enough to carry out displacement experiments. Then, we calculated the $K_d$ value between **3** and PpsC to be 26.0 ± 6.2 μM (Figure S7C). This value is much larger than the $K_d$ (154.6 ± 35.3 nM) between E-FABP and oleic acid, [35] but it clearly showed that PpsC can function as a carrier protein of **3**. In addition, PpsC hardly bound to octanoic acid ($K_d$ = 355.5 ± 54.5 μM), but showed a weak binding activity to lauric acid ($K_d$ = 61.3 ± 19.0 μM) (Figure S7C). This result indicates that PpsC prefers **3** to fatty acids as its ligand.

### CoA-ligase activity of PpsA

PpsA was annotated as a CoA-ligase, and was therefore expected to provide a starter substrate for a PKS, PpsB. To elucidate its function, we analyzed the CoA-ligase activity of PpsA *in vitro*. Recombinant PpsA was produced in *Escherichia coli* using the pCold system and purified by $Co^{2+}$ affinity chromatography (Figure 5A). The putative substrates of PpsA were assumed to be acetate, propionate, pyruvate, α-ketobutyric acid, acetoacetate, and crotonate. When PpsA was incubated with these substrate candidates in the presence of CoA, only acetate and propionate resulted in the production of acyl-CoAs (Figure 5BC). The products were identified as acetyl-CoA and propionyl-CoA, respectively, by comparing their retention time, UV, MS, and MS/MS spectra with those of authentic standards (Figures 5BC, S5, and S6). These results showed that PpsA has a CoA-ligase activity, and recognizes acetate and propionate as substrates. In addition, PpsA seems to prefer acetate to propionate as a substrate, because the yield of acetyl-CoA was much higher than that of propionyl-CoA (Figure 5BC). This result is not surprising, since most acetyl-CoA synthases of bacterial, eukaryotic, and archaeal origins, show a strong preference for acetate as the acyl substrate with propionate being a less efficient acyl substrate. [36]

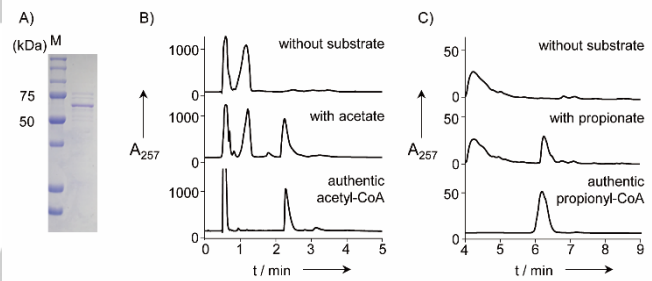
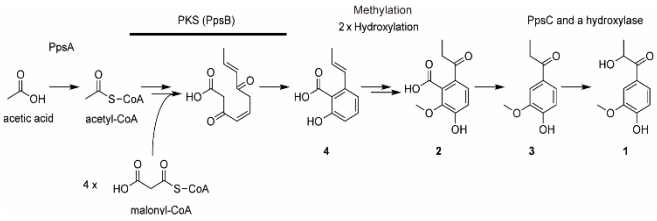



**Figure 5.** *In vitro* analysis of PpsA. A) SDS-PAGE of recombinant PpsA. Lane M, molecular weight marker; right lane, purified PpsA. B) and C) LC-MS analysis for the PpsA-catalyzed conversion of carboxylic acids to acyl-CoAs. UV chromatograms are shown. Conversion of acetate to acetyl-CoA (B) and that of propionate to propionyl-CoA (C) were experimentally confirmed. Commercially available acyl-CoAs were used as authentic standards. Note that the scales of Y axis are different between B and C.




**Scheme 1.** Putative biosynthetic pathway for 2,4′-dihydroxy-3′-methoxypropiophenone (1). See text for details.

## Discussion

In this study, we sought out previously unidentified polyketides produced by *A. oryzae*, and found a monocyclic polyketide, 2,4′-dihydroxy-3′-methoxypropiophenone (**1**). We also successfully identified its biosynthetic genes. An iterative type I PKS (PpsB) encoded by AO090102000166 was indicated to be responsible for its biosynthesis. Although (2*S*)-**1** (evofolin-A) was previously isolated from medicinal plants, [22-26] the isolation of **1** from microorganisms has not been reported thus far. Therefore, identification of **1** as one of the rare secondary metabolites of *A. oryzae* is intriguing. Elucidation of the function of one of the 25 uncharacterized iterative type I PKS gene clusters of *A. oryzae* is of significance as a step to understand potential secondary metabolism in *A. oryzae*.

Regarding secondary metabolite production in *A. oryzae*, we think that this study supports an idea that many *A. oryzae*'s secondary metabolite gene clusters are not sufficiently expressed to produce any compounds at a detectable level. [20] Even though eight different culture conditions were examined, only two of the eight selected gene clusters, pksc_1 and pksc_4, were clearly activated from the viewpoint of the metabolic profile. The spore pigment YWA1, which is produced by the PKS encoded by pksc_4, was detected only when cultured on YES agar. Compound **1** was also detected in only one condition (CYA liquid) among the eight culture conditions examined. Thus, judging from metabolic profile, six of the eight PKS gene clusters were not functioning under the conditions examined, and two of them were functioning only under specific conditions, although we cannot exclude the possibility that we just failed to detect the metabolites produced by these six PKS gene clusters in our extraction and analysis methods. It is most likely that insufficient transcription of these six PKS gene clusters is the cause of no detection of the resulting secondary metabolites, though we cannot exclude the possibility that some of these six PKS gene clusters are sufficiently transcribed but not functioning because a key biosynthetic enzyme gene is mutated to lost its function. In any cases, our results show the difficulty in identification of cryptic secondary metabolites of *A. oryzae* by gene disruption, although we examined only 8 gene clusters of the 61 predicted secondary metabolite gene clusters.

However, our strategy to examine secondary metabolite production by the eight PKS gene clusters was very efficient from the viewpoint of screening of culture conditions in which a cryptic secondary metabolite biosynthetic gene cluster may be activated. We constructed the octuple-deletion mutant (Δpksc_1-8), and its metabolites were compared to those of the parent strain in eight different culture conditions. In this experiment, we performed a functional analysis of the eight PKS gene clusters at once. If we tried to compare metabolic profiles between each of the eight single gene-deletion mutants (Δpksc_1 to Δpksc_8) and the parent strain, we would have needed more than a 7-fold increase both in cultivation and metabolic analysis. In this strategy using the octuple-deletion mutant, however, we also needed eight single gene cluster-deletion mutants to figure out which PKS gene cluster is responsible for the production of target compounds in a specific culture condition. In addition, generation of the octuple-deletion mutant was much more time-consuming than parallel generation of eight single gene cluster-deletion mutants. In spite of these disadvantages, we believe that our strategy using the octuple-deletion mutant was appropriate, because screening of many culture conditions should be the most important and laborious task to find cryptic secondary metabolites.

Involvement of three genes, *ppsA*, *ppsB*, and *ppsC*, in the biosynthesis of 2,4'-dihydroxy-3'-methoxypropiophenone (**1**) was shown. According to the results obtained by the gene inactivation experiments and *in vitro* analysis of PpsA and PpsC, we propose the biosynthetic pathway of **1** as follows (Scheme 1). First, acetate is converted to acetyl-CoA by PpsA, and this acetyl-CoA is used as a starter substrate of the PKS PpsB. Then, this acetyl-CoA is condensed with 4 molecules of malonyl-CoA, and the pentaketide **4** is probably synthesized and released from the PKS. During the polyketide extension, the polyketide chain should be reduced and dehydrated by the KR and DH domains, respectively. *O*-Methylation seems to be catalyzed by an unknown methyltransferase rather than the MT domain of PpsB, because this MT domain was predicted as a cMT. In addition to this *O*-methylation, two hydroxylation reactions catalyzed by an unknown enzyme(s) should be required for the conversion of **4** into **2**. Then, the decarboxylation of **2** forms **3**. Finally, **3** is transported to a different cell organelle by means of PpsC and converted to **1** by hydroxylation catalyzed by an unknown enzyme.

By our *in vitro* analysis, we showed that PpsA has a CoA-ligase activity and prefers acetate as a substrate. Therefore, we expect that PpsB uses acetyl-CoA that is produced by PpsA as a starter substrate. However, there is a doubt in this hypothesis. Generally, a significant amount of acetyl-CoA should exist in the cytosol, because acetyl-CoA is a very common compound in primary metabolism. Therefore, it is a mystery that the acetyl-CoA ligase PpsA is essential for the biosynthesis of **1**. One possibility is that PpsB requires a high concentration of acetyl-CoA, and PpsA provides enough acetyl-CoA to drastically enhance the yield of **1**. Another possibility is that PpsA is not a CoA-ligase but an ACP-ligase, like a fatty acyl-AMP ligase, which activates a fatty acid as an acyladenylate, and subsequently catalyzes its transfer onto the ACP of PKS. [37, 38] If PpsA catalyzes the acylation of the ACP domain of PpsB to start polyketide biosynthesis, it is very reasonable that PpsA is essential for the biosynthesis of **1**. Wide distribution of a neighboring gene pair of *ppsA* and *ppsB* homologs among *Aspergillus* species suggests a close functional relationship between PpsA and PpsB. The detailed functional analysis of the iterative type I PKS PpsB, including the possible involvement of PpsA in the PpsB reaction, is our future subject.

Recently, the mycophenolic acid biosynthetic enzymes in a filamentous fungus (*Penicillium brevicompactum*) were reported to be elegantly compartmentalized in the cytosol, Golgi apparatus, endoplasmic reticulum membrane, and peroxisomes, enabling the unique cooperation between the secondary

metabolite biosynthetic machinery and the β-oxidation catabolism machinery. [39] In the last step, a biosynthetic intermediate of mycophenolic acid is transported into peroxisomes to undergo multiple oxidation reactions. [39] This remarkable study highlights the importance of organelle-associated catalytic mechanism for secondary metabolite biosynthesis in fungi and other higher organisms. According to this notion, as well as the results of *in vivo* and *in vitro* analysis of PpsC, we propose that PpsC having **3**-binding activity should play a role in the transport of **3** to some cell compartment in which hydroxylation of **3** occurs. At present, the cell compartment responsible for the hydroxylation of **3** is unknown, but it should be emphasized that exogenously added **3**, but not endogenously synthesized **3**, was efficiently converted into **1** without PpsC. This result supports compartmentalized biosynthesis of **1**, and PpsC's function in the transport of **3**. The detailed function of PpsC is worthy to elucidate, because no study on such a biosynthetic intermediate-binding protein for the transport between cell compartments has been reported in the secondary metabolism of filamentous fungi.

## Conclusion

We identified a monocyclic polyketide, 2,4′-dihydroxy-3′-methoxypropiophenone (**1**), as a secondary metabolite of *A. oryzae*, and proposed its biosynthetic pathway. The involvement of a biosynthetic intermediate-binding protein in the transport between cell compartments was suggested in its biosynthesis. In addition, this study supports the idea that *A. oryzae* scarcely produces endogenous secondary metabolites. Thus, this study deepened our knowledge on cryptic secondary metabolism in *A. oryzae*, and will be important for the food industry using *A. oryzae* as a safe microorganism, as well as future basic research in this field.

## Experimental Section

#### Fungal strains and culture media

*A. oryzae* RIB40 RkuptrP2-1(Δ*ku70*, Δ*pyrG*), which lacks aflatoxin biosynthesis gene cluster, was used as the parent strain for gene deletion. [40] *A. oryzae* RIB40 RkuptrP2-1::*pyrG* (*pyrG*-complemented strain) [41] was used for the purification of **1**, **2**, and **3**. Polypeptone dextrin (PD) medium [polypeptone (1%), dextrin (2%), $KH_2PO_4$ (0.5%), $NaNO_3$ (0.1%), $MgSO_4 \cdot 7H_2O$ (0.05% ), and casamino acids (0.1%); pH 6.0] was used for liquid cultivation of fungal strains. Czapek-Dox (CD) minimal medium agar (Difco Laboratories, Detroit, MI, USA) supplemented with 20 mM uridine and 1.2 M sorbitol was used as a selective medium for transformation. CD minimal medium containing 20 mM uridine and 5-fluoroorotic acid (5-FOA) (2 mg/ml; Sigma, St. Louis, MO, USA) was used for positive selection of *pyrG*-deficient strains. CYA medium [sucrose (3%), yeast extract (0.5%), $NaNO_3$ (0.3%), $K_2HPO_4$ (0.1%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.05%), and $FeSO_4 \cdot 7H_2O$ (0.001%); pH 6.0] was used for polyketide production. YES medium [sucrose (15%), yeast extract (2%), $MgSO_4 \cdot 7H_2O$ (0.05%), $CuSO_4 \cdot 5H_2O$ (0.0005%), and $ZnSO_4 \cdot 7H_2O$ (0.001%); pH 6.0], CY20 medium (a modified CYA medium; sucrose concentration is increased to 20%), and RT medium [sucrose (3%), $C_4H_4CaO_6 \cdot 4H_2O$, $NaH_2PO_4$ (0.04%), $(NH_4)_2SO_4$ (0.016%), $MgSO_4 \cdot 7H_2O$ (0.028%), $K_2CO_3$ (0.04%), $FeSO_4 \cdot 7H_2O$ (0.006%), $CuSO_4 \cdot 5H_2O$ (0.0005%), and $ZnSO_4 \cdot 7H_2O$ (0.007%); pH 6.0] were also used for the analysis of metabolites.

#### Analysis of metabolites produced by *A. oryzae* strains

For liquid culture, a portion of the *A. oryzae* spore solution ($5.0 \times 10^7$ spores in water containing 0.02% Tween 20) was added into a liquid medium (50 mL) in an Erlenmeyer flask, and the culture was incubated with shaking (150 rpm) at 30 °C for 6 d. After removal of mycelia by filtration, the culture medium was acidified to pH 3 with 6 M HCl and extracted with ethyl acetate. For solid culture, a portion of the *A. oryzae* spore solution ($1.0 \times 10^5$ spores/mL in water containing 0.02% Tween 20) was spotted on an agar medium plate (8.2 cm in diameter), and incubated for 7 d at 30 °C. The agar disk whose surface was covered with mycelium was detached from the plastic case and broken into small pieces. Then, they were soaked in a solvent (methanol:dichloromethane:ethyl acetate = 1:2:3, 40 mL) containing 0.5% formic acid and sonicated for 30 min. After the sample was kept at room temperature for 30 min, the agar pieces and insoluble materials were removed by filtration. The organic layer of the ethyl acetate extraction (liquid culture) and the filtrated solvent (solid culture) were evaporated to dryness under reduced pressure, and the residual materials were dissolved in methanol (300 μL and 1 mL, respectively) for liquid chromatography (LC)-electrospray ionization (ESI) mass spectrometry (MS) analysis using the LC-2040C 3D plus (Shimadzu, Kyoto, Japan) coupled to a LCMS-8040 (Shimadzu). LC-ESIMS was equipped with a COSMOCORE 2.6C18 column (2.1×150 mm; Nacalai Tesque, Kyoto, Japan), and eluted with a linear gradient of water-acetonitrile containing 0.1% formic acid (5–100% acetonitrile) at a flow rate of 0.6 mL/min.

#### Isolation and structural identification of compounds 1 and 3

A total of $5.0 \times 10^7$ *A. oryzae* spores were inoculated into CYA liquid (50 mL) in an Erlenmeyer flask and incubated with shaking (150 rpm) at 30 °C for 6 d. After removal of mycelia by filtration, the culture medium was acidified to pH 3 with 6 M HCl and extracted with ethyl acetate. The organic layers obtained from a total of 7 L of culture were collected together and evaporated to dryness. The residual materials were dissolved in methanol (300 μL), applied to a Cosmosil 75C18-OPN column (Nacalai Tesque, Kyoto, Japan), and eluted by a linear gradient of water/methanol using the Purif-Compact A medium pressure liquid chromatography (MPLC) system (Shoko Scientific, Kanagawa, Japan). The fractions containing **1** or **3** were collected and evaporated to dryness. Then, **1** and **3** were purified by high performance liquid chromatography (HPLC, Prominence series, Shimadzu, Kyoto, Japan) equipped with a reversed-phase COSMOSIL Cholester column (20 × 250 mm; Nacalai Tesque). Both compounds were eluted in 30% methanol containing formic acid (0.1% in water) at a flow rate of 10 mL/min. Compounds **1** and **3** were further purified by HPLC with a reversed-phase COSMOSIL πNAP column (4.6 × 150 mm; Nacalai Tesque). These compounds were eluted at a flow rate of 1 mL/min with a linear gradient of water containing 0.1% formic acid (A) and methanol

containing 0.1% formic acid (B) as follows: 2 min, 15% B; 22 min, 40% B; 22.5 min, 100% B, to obtain pure **1** (1.1 mg) and **3** (1.0 mg). The $^{1}$H NMR, $^{13}$C NMR, COSY, HMQC and HMBC spectra of **1** were recorded using the JNM-A600 NMR system (JEOL, Tokyo, Japan).

**Construction of gene-deleted strains**

Primers used for PCR are listed in Table S2. The whole gene cluster was deleted by loop-out recombination. [40] The details of the gene deletion procedure are described below using the pksc_1 deletion (Δ*ppsBCDE*) as a representative (Figure S8). First, four DNA fragments for pksc_1 deletion by 5-FOA selection were prepared by PCR. The genomic DNA of *A. oryzae* was used as a template for PCR unless otherwise noted. KOD-Plus-Neo DNA polymerase (Toyobo CO., LDT., Osaka, Japan) was used for PCR. DNA fragments 1 and 4 are for the homologous recombination in the first step, fragment 2 carries an auxotrophic marker, and fragment 3 is for the homologous recombination in the second step (Figure S8). DNA fragments 1–4 were amplified by PCR with primers U and L. For the amplification of fragment 3, a DNA fragment containing *pyrG* was used for the template. Four amplified DNA fragments were connected by fusion PCR using primers U1_nest and L4_nest. [42] Then, *A. oryzae* RIB40 RkuptrP2-1 was transformed with the fused DNA fragment to generate the first recombination strain, in which a DNA fragment containing *pyrG* and a downstream region from *ppsB* was inserted between *ppsB* and *ppsA* (Figure S8). Transformation was performed using the protoplast-polyethylene glycol method described previously. [43, 44] Finally, to remove the DNA region containing *pyrG* and *ppsBCDE* by loop-out recombination (Figure S8), the transformants were inoculated on CD agar containing 11.5 mM 5-FOA. Strains that grew well on CD agar were further inoculated to uridine-containing CD agar. The deletion of *ppsBCDE* was confirmed by amplification of the deleted region using primers U3_nest and L4. Other pksc gene clusters were deleted similarly.

**Disruption of *pps* genes**

*ppsA*, *ppsB*, *ppsC*, and *ppsD* were disrupted by substituting each of them with *pyrG* (Figure S9). The *pyrG*-containing DNA fragment was amplified using primers pyrG_U and pyrG_L. Two 1.2 kb DNA fragments (upstream and downstream regions from each target gene) were amplified using the corresponding LU/LL and RU/RL primer pairs, respectively. These three DNA fragments were connected and amplified by fusion PCR. *A. oryzae* RIB40 RkuptrP2-1(Δ*ku70*, Δ*pyrG*) was transformed with the amplified DNA fragment. Each *pps* gene-deleted strain resulting from double crossover homologous recombination was confirmed by amplifying the deleted region using the corresponding LU/RL primer pair (Figure S10).

**Introduction of *ppsB* and *ppsB-ppsC* into the Δ*ppsBCDE* strain**

*ppsB* was introduced into the *A. oryzae* Δ*ppsBCDE* strain by substituting the amylase B gene (*amyB*) with *ppsB* accompanied with an auxotrophic marker, *pyrG*. Previously, we constructed the pPamyB-CUS plasmid, which includes (i) an upstream region (1.2 kb) from the *amyB* promoter (*amyB*-P), (ii) a *pyrG* cassette, (iii) the *amyB*-P region (625 bp), (iv) the curcumin synthase gene (*CUS*), and (v) a downstream region from *amyB* (1.2 kb), in this order (Figure S11). [44] Here, *CUS* on pPamyB-CUS was substituted with *ppsB*. For this purpose, *ppsB* was amplified by PCR with primers ppsB_amyB_U and ppsB_amyB_ L. The insert DNA fragment of pPamyB-CUS except *CUS* was also amplified by PamyB_L and amyB_RU. These two amplified fragments were connected using the In-Fusion HD cloning Kit (Clontech Laboratories, Mountain View, CA, USA), resulting in pPamyB-ppsB (Figure S11). pPamyB-ppsB was linearized by HindIII digestion and introduced into the Δ*ppsBCDE* strain. The introduction of *ppsB* was confirmed by amplifying using primers amyB_u and amyB_d.

For the construction of an expression plasmid for *ppsB* and *ppsC*, the region containing *CUS* and *amyB*-P on pPamyB-CUS was substituted with *ppsB-ppsC*. The *ppsB-ppsC* region including both the *ppsB* and *ppsC* promoters and the *ppsC* terminator was amplified by PCR with primers ppsC_amyB_U and ppsB_amyB_L. The region other than *CUS* and *amyB*-P on pPamyB-CUS was amplified with primers *pyrG*_L and amyB_RU. These two amplified DNA fragments were connected by the In-Fusion HD cloning Kit, resulting in pPamyB-ppsB-ppsC (Figure S12). pPamyB-ppsB-ppsC was linearized by AclI digestion and introduced into the Δ*ppsBCDE* strain. The introduction of *ppsB-ppsC* was confirmed by amplifying using primers amyB_u and amyB_d.

**Bioconversion of compound 3 into compound 1**

A total of 5.0 × 10$^{7}$ *A. oryzae* spores were inoculated into CYA medium (50 mL) and incubated with shaking (150 rpm) at 30 °C. After 24 h, compound **3** (60 μg; in 60-μL methanol) was added into the culture medium. After an additional 5-d cultivation, a portion (20 mL) of the culture medium was extracted with ethyl acetate. The organic layer was evaporated to dryness under reduced pressure. The residual material was dissolved in methanol (500 μL) and applied to the LC-MS analysis as described above.

**Preparation of recombinant PpsC and PpsA proteins**

A 550 bp DNA fragment containing *ppsC* was amplified from cDNA of *A. oryzae* RIB40 by PCR with primers pColdII_ppsC_U and pColdII_ppsC_L. The amplified fragment and the pColdII vector digested with NdeI and EcoI were connected using the In-Fusion HD cloning Kit to obtain pColdII-ppsC.

A 1.8 kb DNA fragment containing *ppsA* was amplified from cDNA of *A. oryzae* RIB40 by PCR with primers pET_ppsA_U and pET_ppsA_L. pET16b was amplified with the primer pair pET_U and pET_L. These two fragments were connected using the In-Fusion HD cloning Kit to obtain pET16b-ppsA. Then, the *ppsA* gene was obtained from pET16b-ppsA by SacI and HindIII digestion, and this fragment was inserted in the SacI and HindIII sites of pColdII to obtain pColdII-ppsA.

For the production of N-terminally His-tagged PpsC and PpsA, *E. coli* BL21(DE3) strains harboring pColdII-ppsC and pColdII-ppsA, respectively, were grown at 37 °C in TB medium containing ampicillin (100 mg $L^{-1}$) until $OD_{600}$ reached 0.4. Then, the cultures were supplemented with isopropyl β-D-1-thiogalactopyranoside (100 and 20 μM, respectively), cooled to 15 °C, and further incubated at this temperature for 24 h. Then, the cells were harvested by centrifugation and suspended in a buffer containing Tris-HCl (20 mM, pH 7.5), NaCl (200 and 300 mM, respectively), and glycerol (10 and 20%, respectively). After sonication, cellular debris was removed by centrifugation and filtration. The clear lysate was subjected to purification by $Ni^{2+}$ affinity chromatography using His60 Ni Superflow Resin (Takara) for PpsC and $Co^{2+}$ affinity chromatography using TALON® Metal Affinity Resin (Takara) for PpsA according to the manufacture's manuals. Imidazole was removed from the elution buffer using a PD-10 column (GE Healthcare, Chicago, IL, USA), and the purified protein was stored in a buffer containing Tris-HCl (20 mM, pH 7.5) and glycerol (10% for PpsC and 20% for PpsA).

**PpsC binding assay**

To investigate the binding of PpsC to ANS, the reaction mixture (100 μL) containing Tris-HCl (20 mM, pH 7.5), NaCl (100 mM), recombinant PpsC (10 μM), and ANS (0, 1, 5, 10, 20, 30, 40, 50, 60, 80, and 100 μM) were incubated at 30 °C for 10 min. Fluorescence (excitation at 350 nm and emission at 450 nm) was measured by Spectramax M2 (Molecular Devices, San Jose, CA, USA). To investigate the binding of PpsC to **3**, octanoic acid, or lauric acid, the reaction mixture including PpsC (10 μM), and ANS (50 μM) was incubated with **3** (0, 4, 20, 40, 60, 80, 120, 160, and 200 μM), octanoic acid (0, 4, 40, 100 and 200 μM), or lauric acid (0, 4, 40, 100 and 200 μM) at 30 °C for 10 min. Fluorescence was similarly measured. The binding affinity or the apparent dissociation constant ($K_d$) of PpsC to ANS was calculated using the following equation.

$$F = F_{max} \cdot L_T/(K_d + L_T)$$

The $K_d$ values of PpsC to putative ligands were calculated using the following equation. [45]

$$F = F_0\{[1+(P_T+L_T)K_a - [(P_T - L_T)^2K_a^2+2(P_T+L_T)K_a]^{1/2}]/[2P_TK_a]\}(F_0 - F_{max})$$

$K_d = 1/K_a$, F: the measured fluorescence, $F_0$: the fluorescence in the absence of ligand, $P_T$: the total protein concentration, $L_T$: the ligand concentration, $K_a$: the apparent association constant for the titrant ligand, and $F_{max}$: the fluorescence emission after complete saturation of PpsC with a ligand.

***In vitro* analysis of PpsA**

The reaction mixture (100 μL) containing Tris-HCl (20 mM, pH 7.5), $MgCl_2$ (10 mM), substrate (5 mM; potassium acetate, sodium propionate, sodium pyruvate, α-ketobutyric acid, crotonic acid, or lithium acetoacetate), coenzyme A (0.8 mM), adenosine triphosphate (1 mM), and PpsA (1.9 μM) was incubated at 30 °C for 1 h. Then, methanol (100 μL) was added to precipitate proteins. After centrifugation, the supernatant was collected and evaporated to dryness under reduced pressure. The residual material was dissolved in water (20 μL) to apply to LC-ESI-MS equipped with a COSMOCORE 2.6C18 column (2.1 × 100 mm; Nacalai Tesque). Samples were eluted at a flow rate of 0.4 mL/min with a gradient of 25 mM ammonium acetate (pH 7.0) (A) and acetonitrile (B) as follows: 3 min, 2% B; 16 min, 15% B.

**Keywords:** *Aspergillus oryzae* • Biosynthesis • Gene disruption • Polyketides • Secondary metabolism

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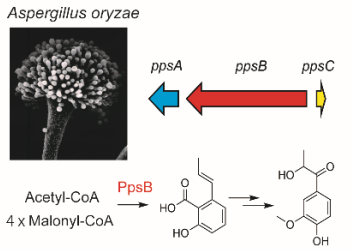
## Entry for the Table of Contents




**Fungal natural product**: A monocyclic polyketide, 2,4′-dihydroxy-3′-methoxypropiophenone, was identified as a rare secondary metabolites of the filamentous fungus *Aspergillus oryzae*. A gene cluster containing an iterative type I PKS gene (AO090102000166; *ppsB*) was shown to be responsible for the production of this compound and its putative biosynthetic pathway was proposed.