Chimeric 6-methylsalicylic acid synthase with domains of acyl carrier protein and methyltransferase from *Pseudallescheria boydii* shows novel biosynthetic activity

Ji-Long Liao,1 Ka-Lai Pang,2 Guang-Huan Sun,3 Tun-Wen Pai,4 Pang-Hung Hsu,1 Jyun-Siou Lin,1 Kuang-Hui Sun,5,6 Chi-Cheng Hsieh7* and Shye-Jye Tang1**

1Department of Bioscience and Biotechnology, Center of Excellence for the Oceans, National Taiwan Ocean University, No. 2 Pei-Ning Road, Keelung, 20224, Taiwan.
2Department of Marine Biology, Center of Excellence for the Oceans, National Taiwan Ocean University, No. 2 Pei-Ning Road, Keelung, 20224, Taiwan.
3Division of Urology, Department of Surgery, National Defense Medical Center, Tri-Service General Hospital, No. 325, Sec. 2, Cheng-gong Rd., Taipei, Taiwan.
4Department of Computer Science and Engineering, National Taiwan Ocean University, No. 2 Pei-Ning Road, Keelung, 20224, Taiwan.
5Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, No. 155, Sec. 2, Linong Street, Taipei, Taiwan.
6Department of Education and Research, Taipei City Hospital, Taipei, Taiwan.
7Section of Urology, Cheng-Hsin General Hospital, Taipei, 11211, Taiwan.

Summary

Polyketides are important secondary metabolites, many of which exhibit potent pharmacological applications. Biosynthesis of polyketides is carried out by a single polyketide synthase (PKS) or multiple PKSs in successive elongations of enzyme-bound intermediates related to fatty acid biosynthesis. The polyketide gene PKS306 from *Pseudallescheria boydii* NTOU2362 containing domains of ketosynthase (KS), acyltransferase (AT), dehydratase (DH), acyl carrier protein (ACP) and methyltransferase (MT) was cloned in an attempt to produce novel chemical compounds, and this PKS harbouring green fluorescent protein (GFP) was expressed in *Saccharomyces cerevisiae*. Although fluorescence of GFP and fusion protein analysed by anti-GFP antibody were observed, no novel compound was detected. 6-methylsalicylic acid synthase (6MSAS) was then used as a template and engineered with PKS306 by combinatorial fusion. The chimeric PKS containing domains of KS, AT, DH and ketoreductase (KR) from 6MSAS with ACP and MT from PKS306 demonstrated biosynthesis of a novel compound. The compound was identified with a deduced chemical formula of C7H10O3, and the chemical structure was named as 2-hydroxy-2-(propan-2-yl)cyclobutane-1,3-dione. The novel compound synthesized by the chimeric PKS in this study demonstrates the feasibility of combinatorial fusion of PKS genes to produce novel polyketides.

Introduction

Secondary metabolites that are obtained from microbes are widely applied in medicine, agriculture, environmental treatment and industrial processes (Cox and Simpson, 2009; Gerke and Braus, 2014). Metabolites of polyketides and non-ribosomal peptides (NRP), such as lovastatin, erythromycin, rapamycin and cytochalasin E, have shown antibiotic, antifungal, antiviral, immune-suppressant, anticholesterol and anticancer biological activities (Udagawa et al., 2000; Cane, 2010; Mulder et al., 2015; Waldner et al., 2016). Devising a feasible strategy for synthesis of secondary metabolites is important to contribute to further biological applications.

Polyketides are synthesized by the enzymes of polyketide synthases (PKSs) to form diverse chemical structures from the simplest monocyclic aromatic compounds, such as 6-methylsalicylic acid (6-MSA) (Beck et al., 1990), to complex compound such as 6-deoxyxythrorniolide B (6-dEB) (Katz, 2009). PKSs incorporate a
wide variety of starter unit carboxylates via acyltransferase (AT) or ketosynthase (KS) domain onto acyl carrier protein (ACP) (Jenke-Kodama et al., 2005; Tran et al., 2010). In the extension of the final β-keto-acyl chain, AT domain introduces the extender unit bound ACP, and KS domain containing growing polyketide catalyses decarboxylative Claisen condensation to afford a new intermediate. The elongation product is tailored by dehydratase (DH), ketoreductase (KR), enoyl reductase (ER) or thioesterase (TE) domains to yield the fully processed polyketides (Shen, 2003; Dutta et al., 2014; Weissman, 2014). Leading to increasing various patterns of the polyketides, the most common is programmed methylation by methyltransferase (MT) (Storm et al., 2017). Moreover, phosphopantetheinyl transferase (PPTase) uses a phosphopantetheine to modify a serine residue in the ACP, and the activated ACP may harbour acyl group intermediates in the biosynthesis (Wattana-amorn et al., 2010; Wang et al., 2016). PKSs are characterized as modular or iterative PKSs based on the frequency of usage of their domains. Modular PKSs use their domains only once (Katz, 2009), whereas iterative PKSs may repeatedly use their domains to synthesize polyketides (Chen and Du, 2016).

Numerous PKS genes have been obtained from next-generation sequencing (NGS) and data mining in many fungal species (Helfrich et al., 2014; Amoutzias et al., 2016). Most of the fungal PKS genes have a very long open reading frame (ORF) and are translated to form megaenzymes that have a high molecular weight (Tsunematsu et al., 2013). The first identified PKS gene, 6-methylsalicylic acid synthase (6MSAS), produces 6-methylsalicylic acid (6MSA) in *Penicillium patulum* and *Aspergillus terreus* (Dimroth et al., 1970; Beck et al., 1990). The gene with a 6200 bp ORF and harbouring KS-AT-DH-ACP domains in the protein sequence was successfully and heterologously expressed with PPTase in *Escherichia coli* and *Saccharomyces cerevisiae* (Wattanachaisareekul et al., 2007; Lee et al., 2009; Bond et al., 2016). Moreover, 6MSA, as an intermediate, may play a role in the polyketide biosynthesis of other structurally diverse compounds with comparable differences in biological activities (Holm et al., 2014; Huang et al., 2018; Kong et al., 2018). The enzyme is regarded as a multifunctional catalytic machine and operates sequentially in individual active sites to yield a polyketide product. Therefore, 6MSAS is a model gene that is suitable to develop cloning methodology and expression of PKS for the production of polyketide. Although 6MSAS is the first PKS discovered in the biosynthesis of secondary metabolites, its use in gene engineering to create the novel compound is not well explored.

**Pseudallescheria boydii** NTOU2362 was isolated from mangrove endophytic fungus and identified to produce several polyketides (Chang et al., 2013). The whole-genome sequence of *P. boydii* NTOU2362 was obtained by NGS, and PKS genes were annotated by data mining. PKS306 and PKS64 from *P. boydii* NTOU2362 contain domains of KS-AT-DH-ACP-MT and KS-AT-DH-ER-KR-ACP respectively. These PKSs were transformed into *S. cerevisiae*, but no novel compound was found. In an attempt to produce a novel compound in the transformed *S. cerevisiae*, chimeric 6MSAS was created by fusion of the domains of KS, AT, DH and KR from 6MSAS with ACP and MT from PKS306, and the engineered enzyme produced the novel compound, 2-hydroxy-2-(propan-2-yl) cyclobutane-1,3-dione, which is reported in this study.

**Results**

**Cloning strategy, expression system and detection of 6MSAS**

PKS and PPTase are required for the biosynthesis of polyketides, and a feasible strategy is therefore needed to establish a methodology to construct yeast expression system. The genes of PKS were generated by long-range PCR to clone into a donor vector using an orientation-directed TOPO ligation system, and then, the vectors used DNA recombination to transfer the PKS gene into a suite of Gateway cloning vectors (Fig. 1A) that have various promoters, auxotrophic selection markers, N- or C-terminus GFP tags and replication origins (Fig. 1B). The constitutive promoter glyceraldehyde-3-phosphate dehydrogenase (GPD) and the inducible promoter GAL1 were used in this study. Auxotrophic selection markers *ura3*, *leu2* and *trp1* could combine multiple vectors to transform into *S. cerevisiae* to obtain multiple gene expression in yeast cells. Moreover, GFP may either maintain the efficiency of translation in the N-terminus or detect full-length expression in the C-terminus. To set up the PKS expression system, 6MSAS from *Aspergillus terreus* and PPTase from *A. nidulans* were cloned and constructed.

Green fluorescent protein fused in frame with 6MSAS and the fusion protein 6MSAS-GFP exhibited green fluorescence, as observed by fluorescence microscopy (Fig. 2A). Remarkably, spots were observed in the yeast cells instead of a dispersed pattern in the cytosol, implying that 6MSAS is localized to a specific organelle in *S. cerevisiae*. To examine the expression of 6MSAS, galactose was added to activate the GAL1 promoter, and the protein was detected by anti-GFP antibody. The expression of 6MSAS with a molecular weight of 230 kDa was represented by the 194 kDa (6MSAS) and 28 kDa (GFP) band, as demonstrated in Fig. 2B after an induction of 96 h, showing that the vector is translated in...
frame and does not contain a frameshift or a stop codon before GFP. The *S. cerevisiae* BJ2168 harbouring either pGAL426-6MSAS-GFP (6MSAS-GFP) only or pGAL426-6MSAS-GFP with or without pGAL425-PPTase (PPTase) were cultured for 48 h and were induced expression by galactose for 96 h. The medium was collected and extracted with the same volume of ethyl acetate. The extract was concentrated by evaporation and was analysed by HPLC. In Fig. 2C, a distinct peak is shown in the yeast harbouring pGAL426-6MSAS-GFP and pGAL425-PPTase, but the peak was not observed in the yeast harbouring pGAL426-6MSAS-GFP or pGAL425-PPTase.

Heterologous expression of PKS from *P. boydii* NTOU2362

The genomic sequence of *P. boydii* NTOU2362 was obtained by NGS. PKS64 (MK134561) and PKS306 (MK134562) contain the functional domains, as shown in Fig. 3A. Compared with the functional domains of 6MSAS, PKS306 contained the MT domain in the carboxyl terminus, and PKS64 contained the ER domain. Because PKS64 harbours intron, cDNA was prepared, whereas the intronless PKS306 was obtained by PCR using genomic DNA as the template. By fluorescence microscopy, PKS64 and PKS306 showed a dispersed pattern of green fluorescence in the cytosol (Fig. 3B). Fusion of GFP with PKS306 and PKS64 in the amino (pGPD424-GFP-PKS306 and pGPD424-GFP-PKS64) or carboxyl terminus (pGAL426-PKS306-GFP and pGAL426-PKS64-GFP) shows a molecular weight of 250 and 280 kDa respectively. Despite the fluorescence signal, reduced protein expression was demonstrated by Western blotting using an anti-GFP antibody (Fig. 3C). Moreover, no compound was identified by HPLC from the ethyl acetate extract (data not shown).

Chimeric PKS constructed by the fusion of 6MSAS with PKSs from *P. boydii* NTOU2362

To explore the diversity of 6MSAS, chimeric 6MSAS was constructed by replacing its ACP with the C-termini of PKS64 and PKS306 to construct the expression vector of R6MSAS\(^\Delta\)-64-ER-KR-ACP and R6MSAS\(^\Delta\)-306-ACP-MT, respectively, as shown in Fig. 4A. Remarkably, the pattern of green fluorescence of R6MSAS\(^\Delta\)-306-ACP-MT and R6MSAS\(^\Delta\)-64-ER-KR-ACP was similar to that of 6MSAS analysed by fluorescence microscopy (Fig. 4B). By Western blotting, the fusion of GFP with R6MSAS\(^\Delta\)-64-ER-KR-ACP and R6MSAS\(^\Delta\)-306-ACP-MT in the carboxyl terminus shows molecular weight of 250 and 280 kDa respectively (Fig. 4C). Regarding HPLC analysis, the extract from R6MSAS\(^\Delta\)-306-ACP-MT revealed a new peak (Fig. 4D), whereas R6MSAS\(^\Delta\)-64-ER-KR-ACP was not observed a new peak. Fortunately, chimeric PKS showed the novel biosynthetic activity to differ from that of 6MSAS. To rule out variation during HPLC, the distinct peak was demonstrated by the mixture containing the extract from R6MSAS\(^\Delta\)-306-ACP-MT and R6MSAS\(^\Delta\) (Fig. 4D). Using the GAL1 promoter, the peak was observed after galactose induction but not in the extract from the culture containing glucose (Fig. S1). Our findings showed that the novel PKS was constructed by the fusion of the domains of KS, AT, DH and KR from 6MSAS with those of ACP and MT from PKS306.
The MT domain is involved in the biosynthesis of the compound

To analyse the role of the MT domain, R6MSASΔ-306-ACP was constructed by removal of the MT domain from R6MSASΔ-306-ACP-MT (Fig. 5A). However, the pattern of green fluorescence of R6MSASΔ-306-ACP was similar to that of 6MSAS analysed by fluorescence microscopy (Fig. 5B). The product of this construction was not found in the ethyl acetate extract, showing that MT domain is

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required for the biosynthesis of the novel compound (Fig. 5D). To explore whether MT plays a role in the biosynthesis of 6MSA, R6MSAS-306-MT was constructed by the fusion of MT from PKS306 (Fig. 5A). Regarding the HPLC results, no difference was observed in the production of 6MSA (Fig. 5D). Our finding shows that MT is unable to change 6MSAS biosynthetic activity.

**Chemical structure of the novel compound**

To identify the molecular weight of the novel compound, a mass spectrum was generated [M]+ and [M-H]+ at 142.0579 and 141.0546 respectively (Fig. 6A). Moreover, the FT-IR spectrum revealed the presence of carbonyl (1746 cm⁻¹) and hydroxyl (3372 cm⁻¹) groups.
(Fig. 6B), but not benzyl and unsaturated acyl groups (2260–2000 cm\(^{-1}\)), in the novel compound. Based on the elemental analysis result, carbon and hydrogen were confirmed to be the elements in this compound without nitrogen (data not shown). The results of NMR spectra for the product of R6MSAS\(\Delta\)-306-ACP-MT including \(^1\)H, \(^{13}\)C (Fig. S2), \(^1\)H-\(^{13}\)C HSQC (Fig. S3), \(^1\)H-\(^{13}\)C HMBC (Fig. S4) and \(^1\)H-\(^1\)H COSY (Fig. S5) are summarized in Table 1. From the \(^1\)H-\(^{13}\)C HSQC spectrum, we conclude that two methyl groups (\(\delta_\text{H} 0.945, \delta_\text{C} 15.263, \text{C-7}; \delta_\text{H} 0.992, \delta_\text{C} 16.135, \text{C-6}\)), one methylene group (\(\delta_\text{H} 2.791, \delta_\text{C} 40.832, \text{C-4}\)) and one methanetriyl group (\(\delta_\text{H} 2.046, \delta_\text{C} 35.288, \text{C-5}\)), are involved in this novel compound. Additionally, three carbons were determined to have no proton attached (\(\delta_\text{C} 77.087, \text{C-2}; \delta_\text{C} 173.326, \text{C-1}; \delta_\text{C} 176.849, \text{C-3}\)). The \(^1\)H-\(^{13}\)C HMBC (Fig. S3) and \(^1\)H-\(^1\)H

\(\text{Fig. 5. ACP-MT contributes to the biosynthetic activity of R6MSAS}\(\Delta\)-306-ACP-MT.}\)

A. The diagram shows the functional domains of chimeric PKS as indicated.
B. The transformants as indicated were observed by fluorescence microscopy.
C. The expression of recombinant proteins was detected by Western blotting using an anti-GFP antibody. “--” indicates the molecular weight of PKS.
D. The chemical product was analysed by HPLC.

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COSY spectra (Fig. S5) showed two methyl groups correlated with C-2, C-5 and each other (C-6 or C-7); the methylene group correlated with C-1, C-2, C-3 and C-5; and methanetriyl group correlated with C-2, C-3, C-6 and C-7. The 1H-1H COSY indicated two methyl groups interacted with the methanetriyl group that constituting the CH(CH₃)₂ group, and two protons showed interacting on the methylene group. According to the above data, the chemical structure of the product of R6MSAS-306-ACP-MT included a cyclobutane-like backbone, C-1 and C-3 were keto groups, and the CH(CH₃)₂ group was at the diagonal position of the methylene group. C-2 contains a hydroxyl group and links with two carbonyl groups as well as the CH(CH₃)₂ group. The molecular formula is C₇H₁₀O₃ termed 2-hydroxy-2-(propan-2-yl) cyclobutane-1,3-dione, and the chemical structure is shown in Fig. 7.

Discussion

Although a reliable and feasible strategy for the heterologous expression of PKSs in S. cerevisiae is developed in this study, functional enzymatic activity and novel compounds were not demonstrated except by 6MSAS. The failure of recombinant PKS from P. boydii NTOU3362 to generate biosynthetic activity may be due to the following reasons. FAS, biosynthetic machinery for fungal melanin and mycotoxins biosynthesis localize in the endoplasmic reticulum, endosome and peroxisome, respectively, implying that the localization signal harbouring in PKSs may have important roles in the biosynthesis of polyketides (Tehlivets et al., 2007; Kistler and Broz, 2015; Upadhyay et al., 2016). However, localization signal of heterologous expression of recombinant PKS genes was not recognized in the machinery of protein transport in S. cerevisiae (Hesketh and Chater, 2003). Moreover, an oligomer is needed various PKSs to form a functional enzyme, whereas recombinant PKS expressed from one gene or inadequate PKS genes cannot form functional oligomers (Studt et al., 2015; Herbst et al., 2018).

Chimeric PKS comprising KS, AT, DH and KR from 6MSAS with ACP and MT from PKS306 can biosynthesize 2-hydroxy-2-(propan-2-yl) cyclobutane-1,3-dione. ACPs consist of 70-100 amino acids typically and participate in the biosynthesis of polyketides to relay growing acyl chains through the assembly line of PKS (Chen et al., 2011; Crosby and Crump, 2012). Remarkably, ACP collaborates with the functional domains of PKS to add a specific chemical moiety such as a chain length of acyl groups (Moore and Hertweck, 2002; Crawford et al., 2006; Ray and Moore, 2016; Keatinge-Clay, 2017). Compared with the protein sequence of ACP from 6MSAS, a low similarity of ACP from PKS306 is shown except at the phosphopantetheine attachment site.

Table 1. ¹H and ¹³C chemical shifts of 2-hydroxy-2-(propan-2-yl) cyclobutane-1,3-dione in methanol-d₄.

| Position | δC (ppm) | δH (ppm) |
|----------|----------|----------|
| 1        | 173.326 (C) | 4.932 (–OH) |
| 2        | 77.087 (C) |          |
| 3        | 178.849 (C) |          |
| 4        | 40.832 (CH₂) | 2.791 |
| 5        | 35.288 (CH)  | 2.046 |
| 6        | 16.135 (CH₃) | 0.992 |
| 7        | 15.263 (CH₃) | 0.945 |

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which is recognized by phosphopantetheinylltransferase (Fig. S6). ACP and MT from PKS306 showed cooperativity to biosynthesize the compound. Comparison of ACP and MT from PKS306 with \textit{Scedosporium apiospermum} (XP_016643174.1), \textit{Oidiodendron maius} Zn (KIM92947.1), \textit{Rutstroemia sp} NJR-2017a BNV2 (PQE24163.1) and \textit{Madurella mycetomatis} (KXX79217.1) reveals identities of 89\%, 53\%, 51\% and 47\% respectively. However, these PKSs are not characterized. The role of KS, AT, DH, KR, ACP and MT in the synthesis of the novel compound remains to be explored.

The MT domain is necessary for the biosynthesis of the compound because removal of the MT domain may deprive the biological activity of the chimeric PKS. Moreover, 6MSAS fused with the MT domain did not affect the biosynthesis of 6MSA and is not found in the novel compound, demonstrating that biosynthesis of the compound is not exclusively attributed to the cooperation of ACP and MT domain. Methyltransferase in PKS functional domains may perform iterative C-methylation of fungal non-reduced polyketides using S-adenosylmethionine as the donor of methyl groups one or more times to a tetra- or pentaketide (Kroken et al., 2003; Ahuja et al., 2012). The novel compound containing an isopropyl group demonstrates activity of the MT domain that is essential for biosynthesis because removal of the domain abolishes the synthesis of the compound. Although 6MSAS fused with the MT domain, 6MSA was observed suggesting that the domain did not alter the biosynthetic pathway of the iterative PKS. ACP involved in the biosynthesis of apratoxin A, contains two MT domains, MT1 and MT2 (Skiba et al., 2017). MT1 performs two methylations to produce Me2Mal-ACP using Mal-ACP as the substrate. MT2 is a bifunctional enzyme, carrying out both decarboxylation and methylation, and is active on multiple substrates to produce pivaloyl-, isobutyryl-, propionyl- or acetoacetyl-ACP. MT from PKS306, compared with MT2, shows 27\% identity and 44\% similarity, whereas MT1 shows \(<\)15\% and 29\% identity and similarity (Fig. S7 and S8) respectively. Interestingly, ACP and MT from PKS306 provide unpredictable biological activity to rebuild the 6MSAS biosynthetic logic.

The first discovered PKS is 6MSAS, which has demonstrated remarkable biosynthesis to produce the secondary metabolite, 6MSA. Moreover, the PKS may provide an intermediate that is involved in the polyketide biosynthesis of other structurally diverse compounds with comparable differences in biological activities. The enzyme is regarded as a multifunctional catalytic machine and operates sequentially in individual active sites to yield a polyketide product. Our findings imply that biosynthesis of the novel compound involves the functional domain of KS, AT, DH and KR as well as ACP and MT. By replacing ACP in 6MSAS with ACP-MT from PKS306, the chimeric PKS produces the novel compound 2-hydroxy-2-(propan-2-yl) cyclobutane-1,3-dione leading to a remarkable possibility to general secondary metabolites using combinatorial functional domains from various PKSs.

**Experimental procedures**

**Bacterial strains and culture conditions**

The strains and plasmids used in this study are described in Table S1. \textit{E. coli}, \textit{S. cerevisiae}, \textit{A. nidulans} and \textit{A. terreus} cultures were grown at 37, 28 and 25°C respectively. \textit{E. coli} Mach1TMT1 was used for cloning, and vectors containing ccdB used \textit{E. coli} DB3.1 as the host. \textit{E. coli} strains were cultured in Luria–Bertani medium. \textit{S. cerevisiae} BJ2168, \textit{A. nidulans} and \textit{A. terreus} were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose).

**Preparation of genomic DNA and RNA**

\textit{Aspergillus nidulans} or \textit{P. boydii} NTOU2362 was cultured in 3 ml YPD overnight and pelleted by centrifugation. Genomic DNA was prepared using the Puregene Yeast/Bac kit (Qiagen Hilden, Germany) according to the manufacturer's protocol. The amount of DNA was estimated by OD\textsubscript{260} and gel electrophoresis by compared the loaded DNA markers. \textit{A. terreus} or \textit{P. boydii} NTOU2362 was cultured in 3 ml YPD for 48 h and harvested. To the pellet, 2 grams of acid-washed glass beads in lysis buffer (50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 0.5% (v/v) Nonident\textsuperscript{P}-40, 1000 U ml\textsuperscript{-1} of RNase inhibitor and 1 mM DTT) was added and vortexed vigorously for 3 min. After the beads
settled, 1 ml of the supernatant was transferred to a new 15 ml centrifuge tube, and then, 1 ml of lysis buffer was added to the sample to replace the aspirated supernatant. The sample was shaken, and the beads were allowed to settle again, and then, 1 ml of the supernatant was transferred the same 15 ml centrifuge tube. The lysate was centrifuged for 5 min at 3000 g. Next, 2 ml of 70% ethanol was added to the supernatant, mixed thoroughly and subjected to RNeasy midi column purification following the manufacturer’s instructions (Qiagen, Hilden, Germany). The amount and quality of RNA were determined by OD260 and RNA electrophoresis respectively.

Reverse transcription and polymerase chain reaction

One microgram of the total RNA was used as the template for reverse transcription (RT) using random primers, and the mixture was incubated at 42°C for 30 min in a total volume of 20 μl. Following RT, 2 μl of the RT reaction mixture was utilized for PCR. Primer sequences are shown in Table S2. For directional cloning, 5’ end of the forward primer included the 4-base pair sequence (CACC). KOD HOT Start DNA polymerase (TOYOBO) was used in this study due to the high fidelity and processivity of the enzyme. PCR was performed following GC-rich and genomic DNA template protocol described by the manufacturer (TOYOBO). The polymerase was activated by heating at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and extension at 68°C for 1 kb min⁻¹. To analyse the PCR products, a 10 μl reaction mixture was utilized for PCR. Primer sequences

Construction of donor vectors

The vector pENTR/D-TOPO was used to construct donor vectors for the Gateway cloning technology. The PCR products were purified from agarose gel using the Gel Elution kit (Qiagen Hilden, Germany). To directionally clone the purified blunt-end DNA harbouring CACC in the 5’- end, the pENTR Directional TOPO Cloning kit (Invitrogen, Waltham, MA, USA) was used. The DNA was transformed into Mach1™T1® competent E. coli. The vectors were confirmed by DNA sequencing. The constructed plasmids are shown in Table S1.

Yeast expression vectors were constructed by LR recombination or Multisite Gateway technology

A suite of Gateway cloning vectors (pAGs) was used to establish PKS expression in S. cerevisiae (Alberti et al., 2007). The yeast expression vectors were constructed using the Gateway® LR Clonase™ II enzyme mix (Invitrogen) with the attL-containing donor vector pENTR and attR-containing destination vector. Briefly, the LR reaction was performed by mixing 150 ng of pENTR-containing vectors with 150 ng of pAG-containing vectors, 4 μl of TE buffer and 2 μl of LR Clonase enzyme to form a 10-μl reaction mixture that was incubated at 25°C for 1 h. The reaction was stopped by adding 2 μl of Proteinase K solution at 37°C for 10 min. Chimeric PKSs were obtained to perform a BP recombination reaction between the attB-flanked DNA fragment and the appropriate attP-containing donor vector by 2-fragment recombination in Multisite Gateway (Invitrogen). The generating donor might undergo LR recombination to construct the expression vector (Fig. S9). The expression vectors were obtained after DNA sequencing and are shown in Table S1. To perform a positive control of 6MSAS in a 2-fragment Multisite Gateway reaction, pAG242GPD-ccdB-EGFP, pDONR-6MSAS KS-KR P1-P5r and pDONR-6MSAS ACP P5-P2 were perform to construct the pGPD424-RMSAS-GFP and to generate R6MSAS (Fig. S10). The chimeric PKSs of R6MSAS-6-ER-KR-ACP, R6MSAS-306-ACP-MT, R6MSAS-306-ACP and 6MSAS-306MT were obtained as described in Table S1.

Saccharomyces cerevisiae transformation

Circular forms (1 μg) of expression vectors were transformed into S. cerevisiae using the lithium chloride method. The transformants were incubated without shaking at 30°C for 2 h, and then, 1 ml of medium was added before incubation at the same temperature for 2 h. The cells were centrifuged at 1500 g for 5 min and were spread onto an SDC (0.67% [w/v] yeast nitrogen base medium supplement without His, Leu, Try and uracil, and 0.192% [w/v] yeast synthetic dropout medium supplement without His, Leu, Try and uracil, and 2% [w/v] dextrose) agar plate. The colonies were collected, and PCR was performed to detect the plasmids harboured in these clones. Fluorescence of GFP fusion protein in transformants was examined by Olympus fluorescence microscopy (Olympus, Tokyo, Japan).

Western blot

The yeast cells were collected by centrifugation at 1500 g for 5 min at 4°C. The pellet was resuspended with 300 μl of lysis buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 1% glycerol, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride), and 1 gram of glass beads was added to break the cells under vigorous vortexing for 2 min. The supernatant was collected by centrifugation at 14 000 g for 10 min at 4°C. The protein concentration was determined using the Bio-Rad protein kit. The lysate (20 μg) was resolved by SDS-PAGE and electrotransferred onto nitrocellulose paper.
The blot was hybridized with anti-GFP antibody (MAB1083, Merck Millipore), followed by detection using ECL reagent (Merck Millipore). The signals were examined using the Fujifilm LAS-4000 BioSpectrum system and were quantified using Fujifilm software (Akasaka, Tokyo, Japan).

Extraction of 6MSA

Saccharomyces cerevisiae transformants containing the expression vector of pGAL426-6MSA-GFP and pGAL425-PPTase or pGAL426-6MSA-GFP only as the control were seeded into a 250 ml flask with 50 ml of SDC medium and were incubated at 25°C on a rotary shaker (200 rpm) for 48 h. The culture was transferred into 500 ml of SDC medium in a 2.5 litre shaker (200 rpm) for 48 h, and then, the cells were collected by centrifugation and refreshed with 500 ml of SDC medium containing galactose (2%) to activate the GAL1 promoter for 96 h. The medium was collected by centrifugation at 500 g for 10 min. The supernatant was extracted with 500 ml of ethyl acetate (EA), and the sample was concentrated by an evaporator to 20 ml.

Detection of 6MSA by HPLC

The dried extract was redissolved with 200 µl of buffer containing 5% methanol and 0.05% TFA in water for HPLC analysis. The chromatographic separation was performed using a Waters e2695 HPLC system equipped with a reversed phase C18 column (Agilent ZORBAX Eclipse XDB-C18, 9.4 mm I.D. × 250 mm, 5µm) and a Waters 2998 photodiode array detector. The column was equilibrated with 95% solvent A (0.05% TFA in water) and 5% solvent B (0.05% TFA in methanol) at a flow rate of 1 ml min⁻¹. The gradient elution steps were as follows: 0–1 min, 5% solvent B; 1–30 min, a linear gradient to 50% solvent B; 30–40 min, to 99% solvent B, and the UV absorption was 254 nm.

Determination of the chemical compound

Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AV600 NMR spectrometer. All NMR spectra were resolved by iNMR software. The molecular weight was determined by Synapt G2 mass spectrometer (Waters). IR spectra were obtained using a Fourier transform infrared spectrometer (Bruker TENSOR II). Element analysis was performed using the Elementar Vario EL III system.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The product of the chemical compound was analyzed by HPLC after the induction using galactose or glucose as indicated.
Fig. S2. $^1$H NMR (600MHz) and $^{13}$C NMR (150MHz) spectrum of novel compound in Methanol-d$_4$.

Fig. S3. $^1$H-$^{13}$C HSQC (600MHz) spectrum of novel compound in Methanol-d$_4$.

Fig. S4. $^1$H-$^{13}$C HMBC (600MHz) spectrum of novel compound in Methanol-d$_4$.

Fig. S5. $^1$H-$^1$H COSY (600MHz) spectrum of novel compound in Methanol-d$_4$.

Fig. S6. Sequence alignment of the ACP from of 6MSAS and PKS306. The phosphopantetheine attachment site is labeled by "*".

Fig. S7. Amino acid sequence alignment of the methyltransferase (MT) domain of PKS306 and MT1 domain of AprA (WP_075900460).

Fig. S8. Amino acid sequence alignment of the MT domain of PKS306 and MT2 domain of AprA.

Fig. S9. Strategy for the construction of the chimeric PKSs. Chimeric PKSs were generated by performing a BP recombination reaction between the attB-flanked DNA fragment and appropriate attP-containing donor vector by 2-fragment recombination in Multisite Gateway system. The indicated peptide sequence from attB5 would replace the similar sequence in the target gene.

Fig. S10. Detection of R6MSAS after 2-fragment recombination of Multisite Gateway. (A) The diagram shows the functional domains of R6MSAS, and the indicated peptide sequence from attB5 would replace the sequence of 6MSAS to generate R6MSAS. (B) The yeast harboring R6MSAS-GFP was examined by fluorescence microscopy. (C) The expression of R6MSAS-GFP was detected by western blotting using an anti-GFP antibody. (D) The product of 6MSAS or R6MSAS was analyzed by HPLC.

Table S1. Strains and plasmids used in this study.
Table S2. Primer sequences.