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# Synthetic Polyketide Enzymology: Platform for Biosynthesis of Antimicrobial Polyketides

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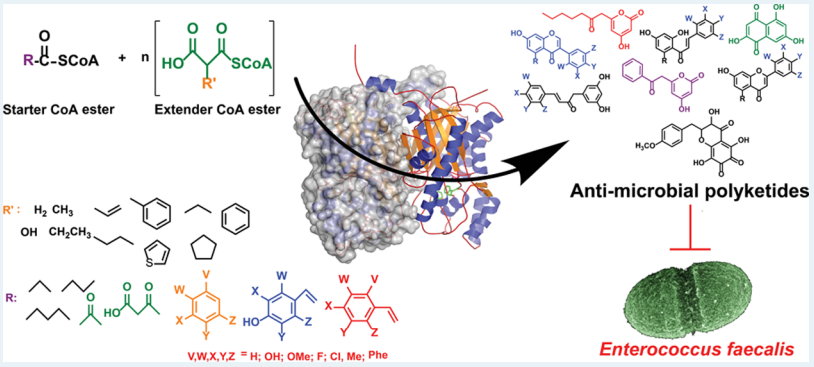
Supporting Information




**ABSTRACT:** Synthetic biology often employs enzymes in the biosynthesis of compounds for purposeful function. Here, we define synthetic enzymology as the application of enzymological principles in synthetic biology and describe its use as an enabling platform in synthetic biology for the purposeful production of compounds of biomedical and commercial importance. In particular, we demonstrated the use of synthetic polyketide enzymology as a means to develop lead polyketide based compounds for antimicrobial therapeutics, as exemplified by the modular coupling of acid:CoA ligases to type III polyketide synthases in the biosynthesis and development of polyketide-based biochemicals. Using wild-type and rationally designed mutants of a type III polyketide synthase isolated from *Oryza sativa* (OsPKS), we produced a chemically diverse library of novel polyketides and identified two bioactive antimicrobials, 4-hydroxy-6-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-2*H*-pyran-2-one (bisnoryangonin) and 3,6,7-trihydroxy-2-(4-methoxybenzyl)-4*H*-1-benzopyran-4,5,8-trione (26OH), respectively, from a screen against a collection of Gram-positive and Gram-negative bacteria. The purification, crystallization, and structural resolution of recombinant OsPKS at 1.93 Å resolution are also reported. Using the described route of synthetic polyketide enzymology, a library of OsPKS mutants was generated as an additional means to increase the diversity of the polyketide product library. We expect the utility of synthetic enzymology to be extended to other classes of biomolecules and translated to various purposeful functions as the field of synthetic biology progresses.

**KEYWORDS:** *synthetic enzymology, platform for synthetic biology, precursor-directed combinatorial biosynthesis, polyketides, anti-microbials, therapeutics development*

## INTRODUCTION

Synthetic biology refers to the design and engineering of biologically based parts and novel devices and systems, as well as the redesign of existing biological systems, for purposeful function. It is enriched by a multitude of foundational disciplines, including synthetic enzymology (defined as the use of enzymological principles such as mechanistic enzymology, rational and directed enzyme engineering, combinatorial biosynthesis, and genomic and functional enzymology, in the intentional design and engineering of biological systems for purposeful function). In this study, we describe the utility of synthetic enzymology for the purposeful production and development of antimicrobial therapeutics.

One of the most commonly required functions of synthetic biology is the production of biochemicals such as polyketides. Polyketides are a large class of biomolecules that are naturally

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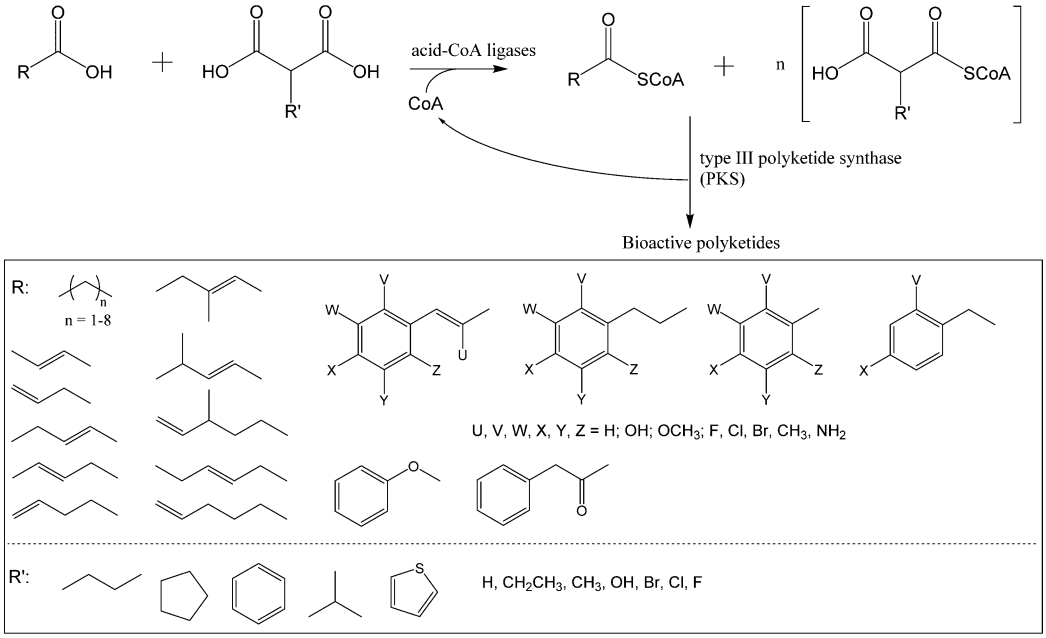
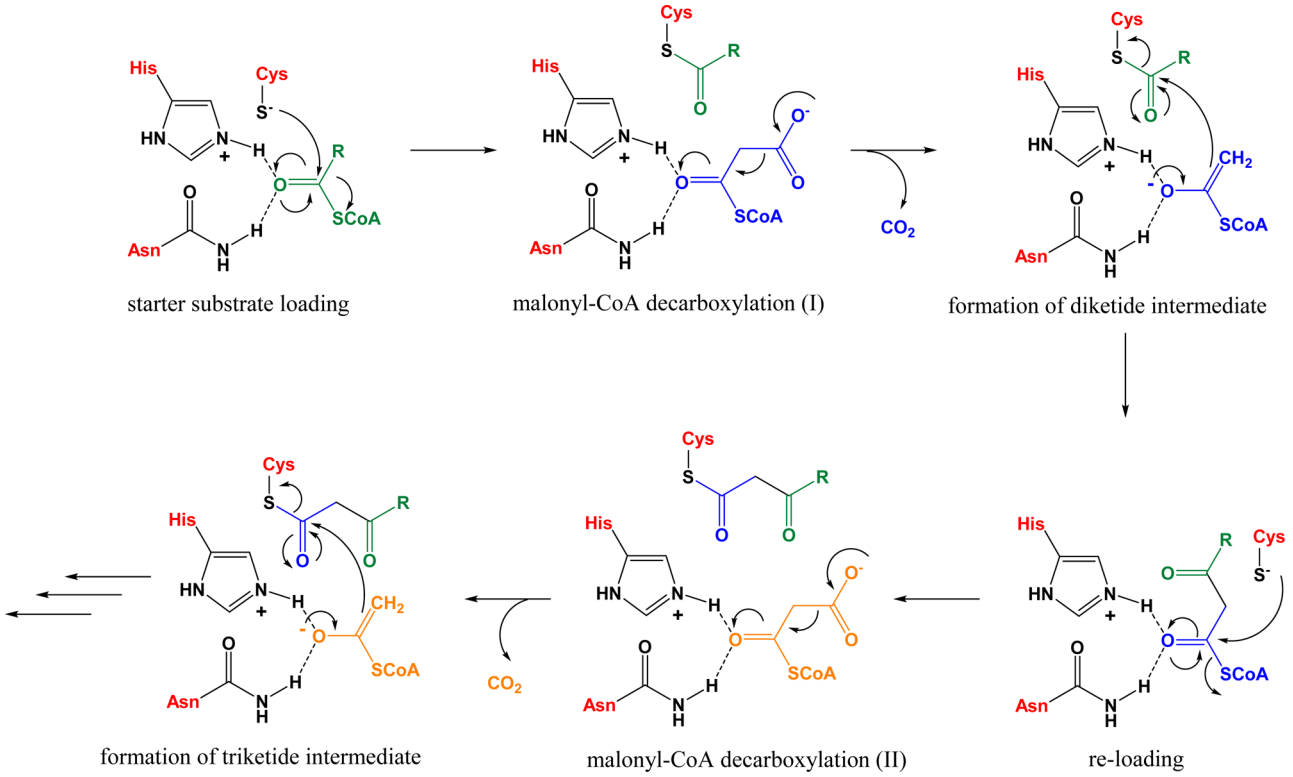
**Scheme 1. Synthetic Polyketide Biosynthesis**




**Scheme 2. Proposed Mechanism for Type III Polyketide Synthases**




produced by bacteria, fungi, and plants and include many clinically important biomolecules with anticancer, antimicrobial, antioxidant, and anti-inflammatory activities.[1,2] They are biosynthesized from acyl-CoA precursors by polyketide synthases (PKSs) and, due to their chemical complexity, are not easily synthesized and structurally manipulated by chemical means. In order to fully realize the potential and use of synthetic biology in the biochemical production of polyketides, tool kits for enzymatic biosynthesis (and the enabling platform technologies that describe these tool kits) must be developed and made available to the synthetic biology community.

There are three types of PKSs:[3] type I PKSs are modular multifunctional enzymes with noniterative catalytic activities for polyketide chain elongation; type II PKSs are multienzyme complexes with iterative catalytic activities for polycyclic polyketide biosynthesis; type III PKSs are single polypeptide homodimers of iteratively acting condensing enzymes. Irrespective of the mechanistic diversity among PKSs, polyketide production relies on the availability of acyl-CoA



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precursors, and a modular coupling of acyl-CoA biosynthesis to polyketide biosynthesis is an amenable system for purposeful design and engineering. We have recently described a tool kit for acyl-CoA biosynthesis that provides new enzymatic routes for the production of a library of acyl-CoA thioesters.[4] Here, we describe a platform for polyketide biosynthesis (a process we term as synthetic polyketide enzymology) that couples the enzymatic systems of acid:CoA ligases to type III PKSs (Scheme 1).

Unlike type I and II PKSs, which require acyl carrier protein (ACP) to activate the acyl-CoA substrates and to channel the growing polyketide intermediates, type III PKSs act directly on the acyl-CoA substrates, allowing precursor-directed biosynthesis to be applied in the biosynthetic route for library diversification.[5] In addition, type III PKSs catalyze (poly)ketide extension and iterative condensation reactions (Scheme 2) within a single active site of the enzyme and are thus ideal and molecularly tractable candidates for rational and directed evolution experiments. In an effort to establish a route for the biosynthesis of a library of polyketides, we chose a type III PKS from *Oryza sativa* (rice, OsPKS, GI: 115485731) isolated from the genome as the enzyme template for synthetic polyketide biosynthesis. Sequence analysis of OsPKS suggested that the enzyme is a functionally orthologous naringenin-chalcone synthase (OsPKS bears 91% sequence identity to a naringenin-chalcone synthase from alfafa[6]). We have previously demonstrated that OsPKS can be used as a downstream enzyme with the availability of a library of acyl-CoA thioesters.[4] This study is a continuation and expansion on the importance of determining substrate promiscuities beyond conventional substrate pools to provide additional tool(s) for establishing precursor-directed combinatorial polyketide biosynthesis.

OsPKS was found to be promiscuous in substrate utilization, consistent with previously characterized type III PKSs.[7] Using precursor-directed biosynthesis (library of 70 starter acyl-CoA thioesters and 12 extender malonyl-CoA derivatives), a catalog of polyketides was obtained from combinations of starter acyl-CoA derivatives and extender malonyl-CoA derivatives. To expand upon the lexicon of polyketides in the catalog, directed mutageneses of OsPKS active site residues were performed (based upon the available X-ray crystallographic structure of the enzyme reported in this study), with the purpose of altering and diversifying product profiles of the OsPKS-derived polyketide catalog.

The utility of this approach was demonstrated by screening the library of biosynthesized polyketides against a panel of Gram-positive and Gram-negative bacteria for antimicrobial activity. Two polyketides, 4-hydroxy-6-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-2*H*-pyran-2-one (bisnoryangonin, a previously described antimicrobial) and 3,6,7-trihydroxy-2-(4-methoxybenzyl)-4*H*-1-benzopyran-4,5,8-trione (26OH, a novel polyketide), were identified from the screen as reasonable lead compounds for antimicrobial therapeutic development. This study also highlights the importance of establishing a catalog of biosynthetic routes, including a detailing (or accurate prediction) of substrate specificities, so that functional orthogonality can be achieved with classes (families and superfamilies) of enzymes for biosynthesis of compounds, such as polyketides, of biomedical and commercial importance.

## MATERIALS AND METHODS

The carboxylic acid substrates for acid-CoA ligases were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), Tokyo Chemical Industry (TCI) Co. (Tokyo, Japan), Extrasynthese Co. (Genay Cedex, France), and Lier Chemical Co. (Sichuan, People's Republic of China). A total of 81 carboxylic acids were used as precursors for acyl-CoA ester synthesis catalyzed by five acyl-CoA ligases previously characterized.[4] They are as follows: (*cinnamate type*) cinnamic acid, 2-fluorocinnamic acid, 3-fluorocinnamic acid, 4-fluorocinnamic acid, $\alpha$-fluorocinnamic acid, 3-chlorocinnamic acid, 4-chlorocinnamic acid, 4-methylcinnamic acid, $\alpha$-methylcinnamic acid, 4-hydroxycinnamic acid, 4-methoxycinnamic acid, 3-chloro-4-methoxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid, 2-hydroxycinnamic acid; (*phenylpropanoate type*) 3-phenylpropanoic acid, 3-(3′-chloro-4′-methoxy)-phenylpropanoic acid, 3-(3′-methoxy)phenylpropanoic acid, 3-(4′-fluoro)phenylpropanoic acid, 3-(4′-methoxy)-phenylpropanoic acid, 3-(3,4-dihydroxy)phenylpropanoic acid, 3-(3′-chloro)phenylpropanoic acid, (*benzoate type*) benzoic acid, 2-fluorobenzoic acid, 3-fluorobenzoic acid, 4-fluorobenzoic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, 2-bromobenzoic acid, 3-bromobenzoic acid, 4-bromobenzoic acid, 2-iodobenzoic acid, 3-aminobenzoic acid, 4-aminobenzoic acid, 2-hydroxybenzoic acid, 2-methoxybenzoic acid, 2-methylbenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,6-difluorobenzoic acid, 2.5-dihydroxybenzoic acid; (*phenylacetate type*) phenylacetic acid, phenoxyacetic acid, 4-hydroxyphenylacetic acid, phenylpyruvic acid, 4-methoxyphenylacetate, 2-hydroxyphenylacetic acid, (*naphthalene, pyridine, and quinoline type*) 1-naphthalenecarboxylic acid, 2-naphthalenecarboxylic acid, 2-quinolinecarboxylic acid, 3-quinolinecarboxylic acid, (*saturated aliphatic type*) propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid; (*unsaturated aliphatic type*) 2-methyl-2-butenoic acid, 2-pentenoic acid, 3-butenoic acid, 3-hexenoic acid, 3-methyl-2-butenoic acid, 3-methyl-4-pentenoic acid, 3-pentenoic acid, 4-pentenoic acid, 5-hexenoic acid, 2-butenoic acid; (*malonic type*) malonic acid, methylmalonic acid, phenylmalonic acid, 3-thiophenemalonic acid, allylmalonic acid, butylmalonic acid, ethylmalonic acid, hydroxymalonic acid, isopropylmalonic acid, 2-chloromalonic acid, 2-fluoromalonic acid, 2-bromomalonic acid.

The chemical structures of all substrates tested are represented in Figure S1 in the Supporting Information. All reagents were the highest quality grade commercially available.

The cloning, expression, and protein purification of the four acyl-CoA ligases and OsPKS were previously reported.[4] The five acyl-CoA ligases are 4-coumarate-CoA ligase from *Nicotiana tabacum* (4CL),[8] benzoate-CoA ligase from *Rhodopseudomonas palustri* (BZL),[9] phenylacetate-CoA ligase from *Streptomyces coelicolor* (PCL),[4] and malonyl-CoA synthetase from *Rhizobium trifolii* (MCS).[4]

**In Vitro and in Vivo Biosynthesis of Naringenin using 4CL, MCS, and OsPKS.** Naringenin was biosynthesized using two methods: purified enzymes (in vitro) and *E. coli* cell hosting the genes of the enzymes (in vivo). Both methods produced naringenin; this was confirmed by mass analysis (theoretical mass of 232.0614, experimental mass of 232.0612 ± 0.0002). This validates the ability of the in vivo system to produce polyketides.

**Profiling the Substrate Specificities of OsPKS using in Vivo Combinatorial Biosynthesis.** The biosynthesis of polyketides was adopted from the method by Katsuyama et al.[10] The *E. coli* strain Rosetta II(DE3) (Novagen) was used as


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the host cell for the biosynthesis of polyketides. Figure 1 shows the plasmid constructs transformed into the *E. coli* cell. The

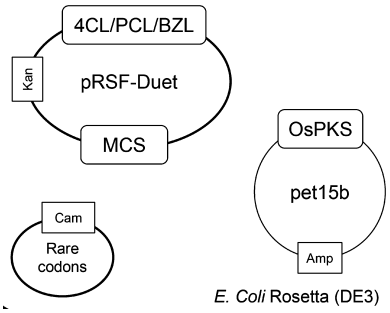


**Figure 1.** Construct of *E. coli* Rosetta (DE3) strain containing the acyl-CoA genes (4CL, PCL, or BZL with MCS) and the OsPKS gene.

exogenous plasmid contains three genes, an acyl-CoA ligase (4CL, PCL, or BZL), MCS, and OsPKS. The acyl-CoA ligase gene and MCS gene were inserted into a pRSFDuet-1 vector (Novagen). This plasmid (acyl-CoA ligase/MCS/pRSF) will produce the corresponding proteins and in turn catalyze the synthesis of a starter acyl-CoA and an extender malonyl-CoA upon addition of exogenous carboxylic acid and malonic acid. The OsPKS gene was inserted into a pET15b-derived vector. As the starter acyl-CoA and extender malonyl-CoA are produced, OsPKS will catalyze the production of polyketide(s). An *E. coli* cell without the OsPKS gene was also prepared; this served as the control for the detection of novel polyketides. The engineered *E. coli* host cells were separately fed with a matrix of 70 starter acids and 12 extender acids, giving a possible combination of 840 substrate profiles.

The engineered *E. coli* harboring the biosynthetic genes was grown in 10 mL of LB medium containing 100 $\mu$g/mL of ampicillin, 30 $\mu$g/mL of kanamycin, and 34 $\mu$g/mL of chloramphenicol at 25 °C. When an OD of 0.6 was reached, 0.5 mM isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) was added for protein induction for 16 h. The cells were harvested, resuspended in minimal medium containing 0.5 mM IPTG, 1 mM starter carboxylic acid, and 3 mM extender malonic acid, and incubated at 25 °C for an additional 48 h. The supernatant was acidified to pH 3.0 with 6 M hydrochloric acid (HCl) and extracted twice with ethyl acetate. Ethyl acetate was removed with a vacuum concentrator, and the sample was dissolved in 100 $\mu$L of dimethyl sulfoxide (DMSO). The sample was considered as the "organic extract" and contained polyketides produced by the host cell. The organic extract was used for the detection of polyketides and tested for inhibitory effect against various Gram-positive and Gram-negative bacteria.

The organic extract (10 $\mu$L) was loaded onto an Atlantis Analytical C18 (Waters) reverse-phase high-performance liquid chromatography (HPLC) column. The column was equilibrated using a 10% acetonitrile/90% water mixture containing 0.1% trifluoroacetic acid (TFA), at a flow rate of 1 mL/min for 10 min. The acetonitrile concentration was increased to 50% acetonitrile over a period of 40 min. For the next 5 min, the concentration was further increased to 100% acetonitrile. Potential polyketides were detected by comparing the chromatograms between control (without OsPKS) and wild type (with OsPKS). New peaks that have a minimum height of 20 mAU at 259 nm were considered as potential polyketide products.

**Site-Directed Mutagenesis of OsPKS and in Vitro Biosynthesis of Polyketides using Mutant Libraries.** The libraries of site-specific random mutants of OsPKS (S136X, T197X, T200X, G259X, F218X, F268X, and S341X) were created using the QuikChange kit (Stratagene). The primers for each construct were modified to contain a "NNS" codon targeted toward the site of mutagenesis. Expression and purification of each mutant library were carried out analogously to those described for wild-type OsPKS.[4]

Deconvolution of the site-specific random library of OsPKS was carried out by picking 16 random clones and grouping them into four sets, with each set containing four clones. The recombinant enzymes from each set were purified and assayed for activity. Dividing the set into four individual clones further deconvoluted the set of mutants possessing the required activity, and each enzyme was purified and assayed for activity.

**Determination of Minimal Inhibition Concentration (MIC) against Various Bacteria.** The MIC assay was performed on the basis of the standard guidelines from the National Committee for Clinical Laboratory Standards (NCCLS), sixth edition. The library of organic extracts was tested for inhibitory properties against five different bacteria: *Acinetobacter baumannii*, *Burkholderia thailandensis*, *Enterococcus faecalis* V583, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* subsp. aureus. A log-phase culture of the bacterium was diluted to a concentration of $1 \times 10^6$ CFU/mL. The diluted culture (50 $\mu$L) was mixed with 48 $\mu$L of LB and 2 $\mu$L of organic extract. The culture was then incubated at 37 °C for 18 h. The MIC value is the lowest concentration of a compound that completely inhibits the growth of the bacteria. After incubation, the cell density at $OD_{600}$ was measured for each culture. If the cell density of the organic extract containing culture was less than 50% in comparison to the cultures that did not have any organic extract, the organic extract was deemed to have polyketides with antimicrobial properties. These organic extracts were analyzed using HPLC as mentioned above.

**Identification of Antimicrobial Polyketides.** Organic extracts with antimicrobial properties were further analyzed. The engineered *E. coli* harboring the biosynthetic genes that produced the organic extract with antimicrobial properties was grown on a larger scale (100 mL). The organic extract was analyzed in the same manner using an Atlantis Prep C18 (Waters) HPLC column. Fractions containing novel peaks were pooled and concentrated to dryness and redissolved in DMSO, analogous to the treatment of organic extracts. The fractions were then tested using the MIC assay mentioned above. The pool that showed antimicrobial properties was then submitted for mass analysis and NMR analysis.

**Mass and NMR Analysis of Bisnoryangonin and 26OH.** $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AVANCE 500 MHz NMR spectrometer. All NMR spectra were recorded in $D_2O$. The mass of the polyketide was determined by electrospray ionization (ESI) high-resolution mass spectrometry (HR-MS) performed at the Mass Spectrometry Laboratory at the Department of Chemistry, National University of Singapore.

*Bisnoryangonin (Figure 2).* HR-MS (ESI, negative mode): $m/z$ 229.0378 (experimental); $m/z$ 229.0506 (theoretical). $^1$H NMR: $H_a$ (2H, d) 7.49 ppm, $J_{H_a-H_b}$ = 8.5 Hz; $H_b$ (2H, d) 6.79 ppm, $J_{H_a-H_b}$ = 8.5 Hz; $H_c$ (1H, d) 6.78 ppm, $J_{H_c-H_d}$ = 16 Hz; $H_d$ (1H, d) 7.22 ppm, $J_{H_c-H_d}$ = 16 Hz; $H_e$ (1H, s) 6.13 ppm; $H_f$ (s) 6.49 ppm.



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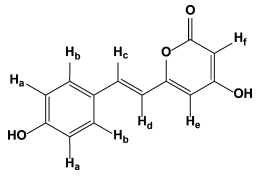

**Figure 2.** Chemical structure of bisnoryangonin.

*3,6,7-Trihydroxy-2-(4-methoxybenzyl)-4H-1-benzopyran-4,5,8-trione (Figure 3)*. HR-MS (ESI, negative mode): $m/z$ =

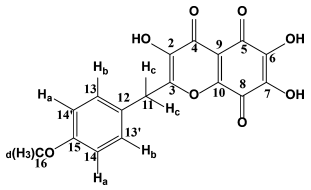

**Figure 3.** Chemical structure of 3,6,7-trihydroxy-2-(4-methoxybenzyl)-4*H*-1-benzopyran-4,5,8-trione.

343.2671 (experimental); $m/z$ 343.0453 (theoretical). $^1$H NMR: $H_a$ (2H, d) 7.2 ppm, $J_{H_a-Hb}$ = 8.5 Hz; $H_b$ (2H, d) 6.9 ppm, $J_{H_a-H_b}$ = 8.5 Hz; $H_c$ (2H, s) 5.2 ppm; $H_d$ (3H, s) 3.8 ppm. $^{13}$C NMR: $C_2$ 88 ppm; $C_3$ 100 ppm; $C_4$ 170 ppm; $C_5$ and $C_8$ 164 ppm; $C_6$ and $C_7$ 166 ppm; $C_9$ 130 ppm; $C_{10}$ 128 ppm; $C_{11}$ 38 ppm; $C_{12}$ 130 ppm; $C_{13,13'}$ 128 ppm; $C_{14,14'}$ 114 ppm; $C_{15}$ 158 ppm; $C_{16}$ 55 ppm.

**Crystallization and Structure Determination.** OsPKS at 50 mg/mL was crystallized by the hanging drop vapor diffusion method in 1:1 ratio of protein and precipitant (0.1 M Tris-HCl, pH 8.5, 2 M ammonium sulfate) at 25 °C. Crystals were observed within 1−3 days. X-ray diffraction data were collected at the National Synchrotron Radiation Research Center (NSRRC, Taiwan), beamline BL13B1. Data were processed and scaled using the HKL2000 program package (HKL Research). Molecular replacement was performed using chalcone synthase from alfalfa (PDB: 1BI5) as a search model in PHASER.[11] Restrained refinement was carried out using REFMAC,[12] and model building was performed in COOT.[13] The data collection and refinement statistics are shown in Table 7 in the Supporting Information. All molecular structure figures were generated using PyMOL (Delano Scientific LLC). The crystal structure coordinates were deposited in the Protein Data Bank with accession code 4YJY).

## RESULTS AND DISCUSSION

We have previously reported on the assembly of a tool kit that cataloged a list of acyl-CoA thioesters that can be biosynthesized by acid-CoA ligases, including the definition of substrate specificities of these enzymes for sequential use in polyketide production. The previously characterized four acid-CoA ligases, MCS, 4CL, BZL, and PCL, were assembled for the biosynthesis of a library of chemically diverse acid-CoA thioesters.[4] We sought to demonstrate the functional orthogonality of acid-CoA ligases with polyketide synthases by defining the substrate specificities of a previously uncharacterized OsPKS. The modularity of synthetic polyketide

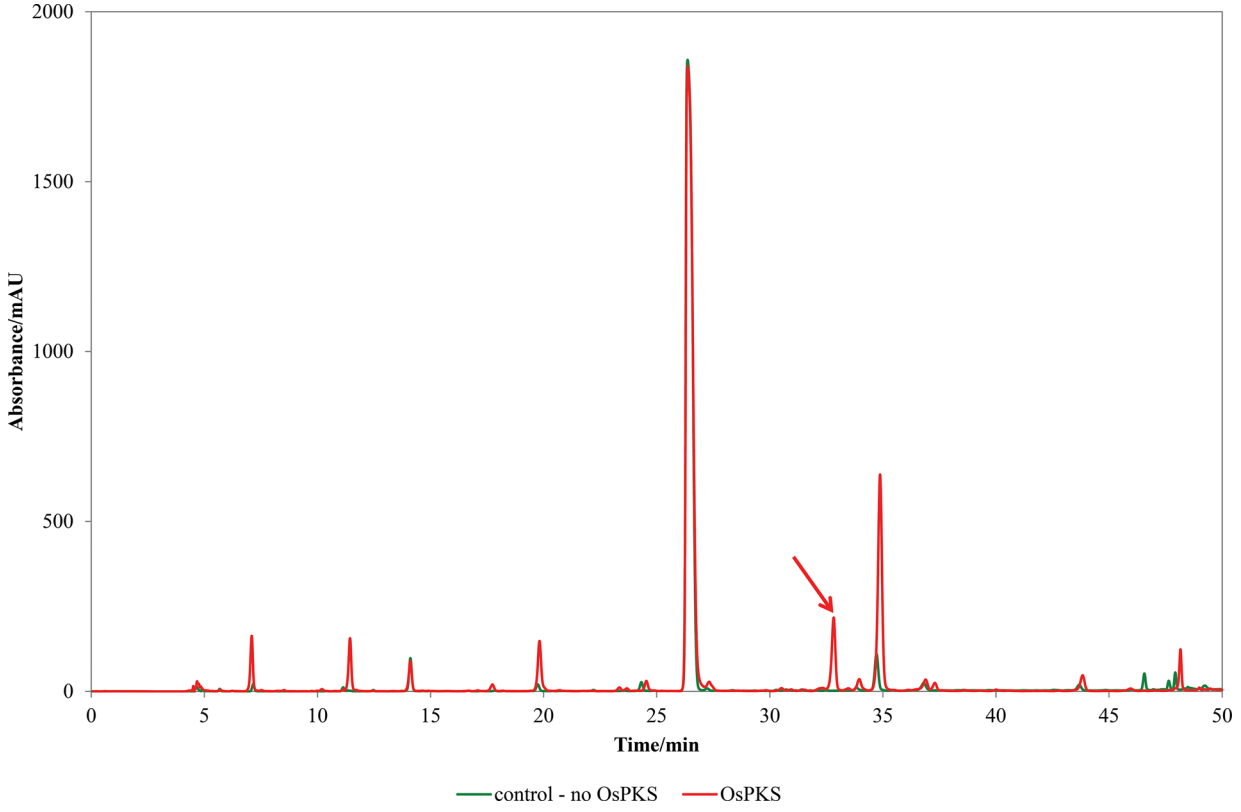


**Figure 4.** HPLC chromatogram of the reaction mixture from the in vivo biosynthesis of polyketides using 4-methoxyphenylacetyl-CoA as the starter molecule and hydroxymalonyl-CoA as the extender molecule. The reaction contains two acylCoA-ligase genes and OsPKS gene (red line). The control does not contain the OsPKS gene (green line). A novel peak at 32.8 min was observed (indicated by a red arrow).



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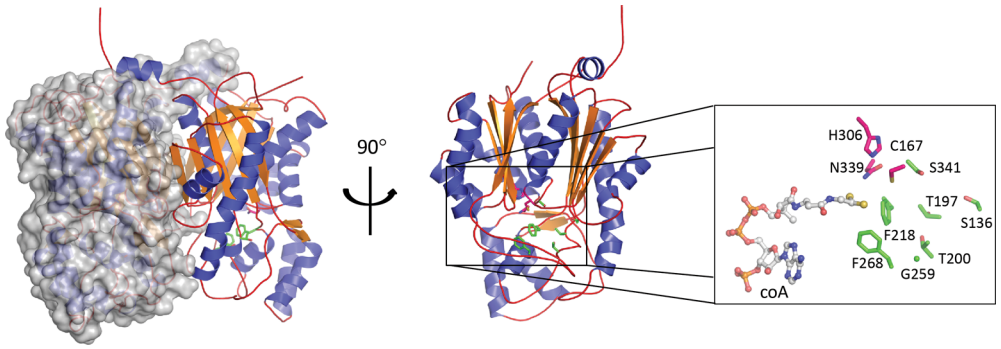


**Figure 5.** Crystal structure of dimeric OsPKS, with highlighted active site residues. Catalytic triad residues and residues in OsPKS that were targeted for mutagenesis are highlighted in magenta and green, respectively. CoA from the alfalfa CHS/CoA complex (PDB 1BQ6) was modeled into the active site through superimposition of the proteins and is shown as a ball and stick diagram.

enzymology (coupling of acid-CoA ligases to PKSs) should be universal among PKSs; despite the mechanistic differences between the three paradigmatic classes of PKSs, polyketide biosynthesis is still dependent on the availability of novel (or chemically diverse) acyl-CoA precursors and extenders.

**Profiling the Substrate Specificities of OsPKS.** The *E. coli* host system was tested for its biosynthesis capability. Naringenin was biosynthesized in vitro from precursor acid substrates using the purified acid-CoA ligases 4CL and MCS, and OsPKS. We observed the formation of naringenin using 4-hydroxycinnamate and malonate as the precursor acid substrates (generating 4-hydroxycinnamyl-CoA as starter CoA and malonyl-CoA as extender CoA, catalyzed by 4CL and MCS, respectively). With the expectation that OsPKS is promiscuous,[4] we wanted to explore the extent of substrate promiscuity exhibited by the type III PKS.

The range of substrate promiscuity exhibited by OsPKS was explored by coupling the biosynthesis of acid-CoA thioesters to the biosynthesis of polyketides (Scheme 1): a starter-CoA ligase (CCL, 4CL, BZL, or PCL, respectively), an extender-CoA ligase (MCS or PCL, respectively), and OsPKS were simultaneously expressed within the synthetic *E. coli* host, and a panel of acid precursors (the chemical structures of the 80 carboxylic acids used are illustrated in Figure S1 in the Supporting Information) were separately fed to the synthetic host for in vivo polyketide production. The modular coupling of in vivo acyl-CoA thioester biosynthesis with polyketide biosynthesis circumvented the need to use purified enzymes and acyl-CoA thioesters; in addition, only stoichiometric amounts of carboxylic acid precursors and catalytic amounts (present endogenously in the synthetic hosts) of CoA and other cofactors such as $Mg^{2+}$ ion and adenosine 3′-triphosphate were required for polyketide production.

Synthetic host cells were separately fed with a matrix of 70 starter acids and 12 extender acids (giving a possible combination of 840 substrate profiles), and successful polyketide production (positive substrate identification) was detected by HPLC analyses of organic extracts of the cell culture media. We compared the HPLC chromatograms of the construct containing OsPKS with the construct without the OsPKS, to find the possible production of new polyketides. A novel peak height of at least 100 milli-absorbance units or 10% of the highest peak in the chromatogram of the construct containing OsPKS was considered as a new polyketide. Figure 4 is an HPLC chromatogram on the in vivo biosynthesis of polyketides using 4-methoxyphenylacetyl-CoA as the starter molecule and hydroxymalonyl-CoA as the extender molecule. A new peak with a retention time of 32.8 min was detected at 280 nm. All HPLC chromatograms were analyzed in the same manner (Figures S2−S7 in the Supporting Information).

Assuming that each novel peak corresponds to a single polyketide, a total of 315 substrate combinations (38% of the matrix) were identified through the observation of polyketide (peak) formation in HPLC chromatograms. OsPKS was able to use a number of cinnamyl-CoA (Table S2 in the Supporting Information), phenylpropanoyl-CoA (Table S2), phenylacetyl-CoA (Table S3 in the Supporting Information), benzoyl-CoA (Table S4 in the Supporting Information), saturated and unsaturated aliphatic CoA (Table S5 in the Supporting Information), and bicyclic aromatic CoA thioester derivatives (Table S5) as the starter acyl-CoA, in combination with various malonyl-CoA derivatives as the extender acyl-CoA (Table S6 in the Supporting Information). The list of acids and corresponding acid-CoA ligases used for in vivo biosynthesis is given in Table S1 in the Supporting Information.

**Providing a Tractable Route for Future Predictions of Substrate Specificities of PKSs.** We sought a molecular explanation for the observed promiscuity of OsPKS, so that a tractable route could be formulated for future predictions (through computational means) of substrate specificities of PKSs to enhance the utility and universality of synthetic polyketide enzymology. With regard to each class of starter molecules, we tallied the total number of possible products each class of starter acyl-CoAs can produce in the following way: starting with a library of 14 cinnamyl-CoA starter molecules, an assumption was made to have one product for each combination of a starter cinnamyl acyl-CoA and extender malonyl acyl-CoA, with the corollary that a combination of 14 cinnamyl-CoA starters with 12 malonyl-CoA extenders will form a total of 168 new products. From our HPLC analyses, we detected 78 new products from this combination of starters and extenders, representing 46% of the total possible 168 new products. Using this route, we observed the following preference for the choice of starter acyl-CoA by OsPKS: phenylpropanoyl-CoA (57%) > unsaturated aliphatic acyl-CoA (48%) > cinnamyl-CoA (46%) > bicyclic aromatic acyl-CoA (42%) = phenylacetyl-CoA > saturated aliphatic acyl-CoA (40%) > benzoyl-CoA (18%). We propose that the starter acyl-CoA preference was associated with (1) the degree of resemblance to the "natural" or cognate substrate of OsPKS, 4-hydroxycinnamyl-CoA, (2) the polarity of the substrates, (3) the position and size of the substituent groups in thioesters with an aromatic ring, and (4) the length of the acyl-CoA thioester substrate.



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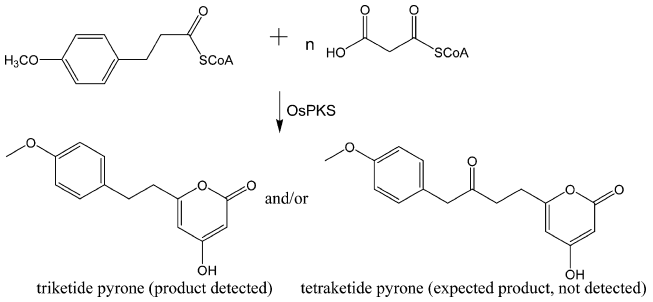
In addition to the choice of starter acyl-CoAs, there was an observed preference for extender acyl-CoA (malonyl-CoA derivatives) by OsPKS: allylmalonyl-CoA (39%) > fluoromalonyl-CoA (37%) > hydroxymalonyl-CoA (31%) > malonyl-CoA (30%) > butylmalonyl-CoA (27%) > isopropylmalonyl-CoA (26%) > methylmalonyl-CoA (25%) = ethylmalonyl-CoA (25%) > chloromalonyl-CoA (24%) > bromomalonyl-CoA (23%) > 3-thiophenemalonyl-CoA (20%) > phenylmalonyl-CoA (11%). We propose that the cosubstrate (extender acyl-CoA) preference was associated with (1) the stability of the carbanion intermediate due to inductive or resonance effects and (2) steric effects of the ensuing reactive intermediates during polyketide extension.

**Structure of OsPKS, a Primer for Synthetic Polyketide Enzymology.** As an extension to the development of a tool kit for polyketide biosynthesis, we recognized that a rational and tractable route for synthetic enzymology must involve the structural elucidation of polyketide synthases: structure-based mutagenesis can be used as a reliable strategy to control and direct enzymatic catalysis and further increase the diversity of polyketides formed by OsPKS. The crystal structure of apo OsPKS was determined and refined at 1.93 Å resolution. Two subunits of OsPKS comprise the asymmetric unit, which are related by a noncrystallographic 2-fold axis forming a dimer (Figure 5). This is in agreement with type III PKS characteristics as a homodimeric enzyme carrying independent active sites. Within each subunit, the four conserved residues for all CHS-related enzymes, Cys-167, Phe-218, His-306, and Asn-339, are present together with other common residues that shape the active site of CHS-like proteins (Pro-141, Gly-166, Gly-170, Leu-217, Asp-220, Gly-265, Pro-307, Gly-308, Gly-309, Gly-338, Gly-377, Pro-378, and Gly-379).[6] Structural superposition to previously characterized alfalfa PKS (PDB ID: 1BI5) demonstrates an RMSD of 0.296 Å including 2122 atoms. The active site topology clearly suggests that OsPKS is functionally orthologous to type III naringenin-chalcone synthases.

**Extending the Biosynthetic Repertoire of PKSs by Directed Mutagenesis.** Structure-based mutagenesis was used as an additional route to increase the diversity of the polyketide product library: by incorporating strategic mutations into the enzymes (often at the catalytically relevant active sites), variations in substrate specificities and product profiles may be induced. Selected residues (S136, T197, T200, G259, F218, F268 and S341) were mutated to alter product profiles biosynthesized by OsPKS (Figure 4). These residues were previously reported in other type III PKSs to affect product formation.[7] A possible explanation for observed changes in product formation may involve changes in volume (sterics) and three-dimensional shape (geometry) of the enzyme active site cavity that led to differing rounds of polyketide iterations (extension and subsequent cyclization).

The natural substrates of wild-type OsPKS are 4-hydroxycinnamyl-CoA and malonyl-CoA. OsPKS catalyzes the formation of the tetraketide naringenin using one unit of 4-hydroxycinnamyl-CoA and three units of malonyl-CoA. We found that when an "unnatural" substrate such as 3-(4-methoxyphenyl)-propanoyl-CoA is used, this resulted in premature polyketide chain termination, resulting in the formation of a triketide product instead of the analogous tetraketide product (Scheme 3). This supports the prevailing hypothesis that the sterics of the active site cavity of a polyketide synthase may be responsible for the extent of

**Scheme 3. Triketide Product Formation by OsPKS**




polyketide chain extension, and that premature chain termination was likely a consequence of the larger volume occupied by the unnatural substrate, 3-(4-methoxyphenyl)-propanoyl-CoA.[14]

In an attempt to reverse "premature" polyketide chain termination, seven mutant libraries of OsPKS (S136X, T197X, T200X, G259X, F218X, F268X, and S341X, respectively) were constructed. The enzyme libraries were purified and used for in vitro polyketide biosynthesis. Analyses of the product profiles obtained from the respective mutant libraries of OsPKS corroborated the expectation of altered product profiles. Using site-directed mutagenesis, we were able to compensate for the steric effects of using 3-(4-methoxyphenyl)-propanoyl-CoA and malonyl-CoA as substrates for polyketide biosynthesis. Although wild-type OsPKS produced (almost exclusively) a triketide pyrone, 4-hydroxy-6-[2-(4-methoxyphenyl)-ethyl]-2*H*-pyran-2-one (Figure 6, product Y), a second polyketide, a tetraketide pyrone, 4-hydroxy-6-[2-(4-methoxyphenyl)2-oxobutyl]-2*H*-pyran-2-one (Figure 6, product Z), was obtained in various stoichiometric ratios (to the triketide pyrone) for the mutant libraries of OsPKS. The identities of products Y and Z identities were confirmed by mass spectrometry (positive mode): product Y (theoretical mass 245.0819; experimental mass 245.011); product Z (theoretical mass 287.0925; experimental mass 287.0915).

The mutant library of OsPKS S341X was further deconvoluted to determine which specific mutant catalyzed the biosynthesis of the tetraketide pyrone. We found that S341N OsPKS mutant biosynthesized equivalent amounts of triketide and tetraketide pyrone products. The identification of the tetraketide pyrone (likely produced from a lactone cyclization (C5-O → C1) of the tetraketide chain) produced from the mutant OsPKS libraries suggested an alteration in the geometry of the enzyme active site that allowed the incorporation of 3 equiv of malonyl-CoA extenders, in a biosynthetic route similarly observed with wild-type OsPKS using 4-hydroxycinnamyl-CoA as starter acyl-CoA substrate. The identities of the triketide and tetraketide pyrones were confirmed by mass spectrometry.

**Characterization of Bisnoryangonin.** Bisnoryangonin (0.5 mg/L of culture) is generated from the condensation of 4-hydroxycinnamyl-CoA with two units of malonyl-CoA. The linear triketide formed then undergoes a C5−O → C1 lactone cyclization of the triketide precursor (Scheme 4).

Bisnoryangonin was found to inhibit the growth of *E. faecalis* and *S. aureus* with MIC values of 60 and 120 μg/mL, respectively. This is consistent with an earlier study by Benedict and Brady. It was identified as a metabolite from mushrooms and was shown to display broad-spectrum antibacterial activity against several Gram-positive bacteria (*Bacillus subtilis*, *S.*



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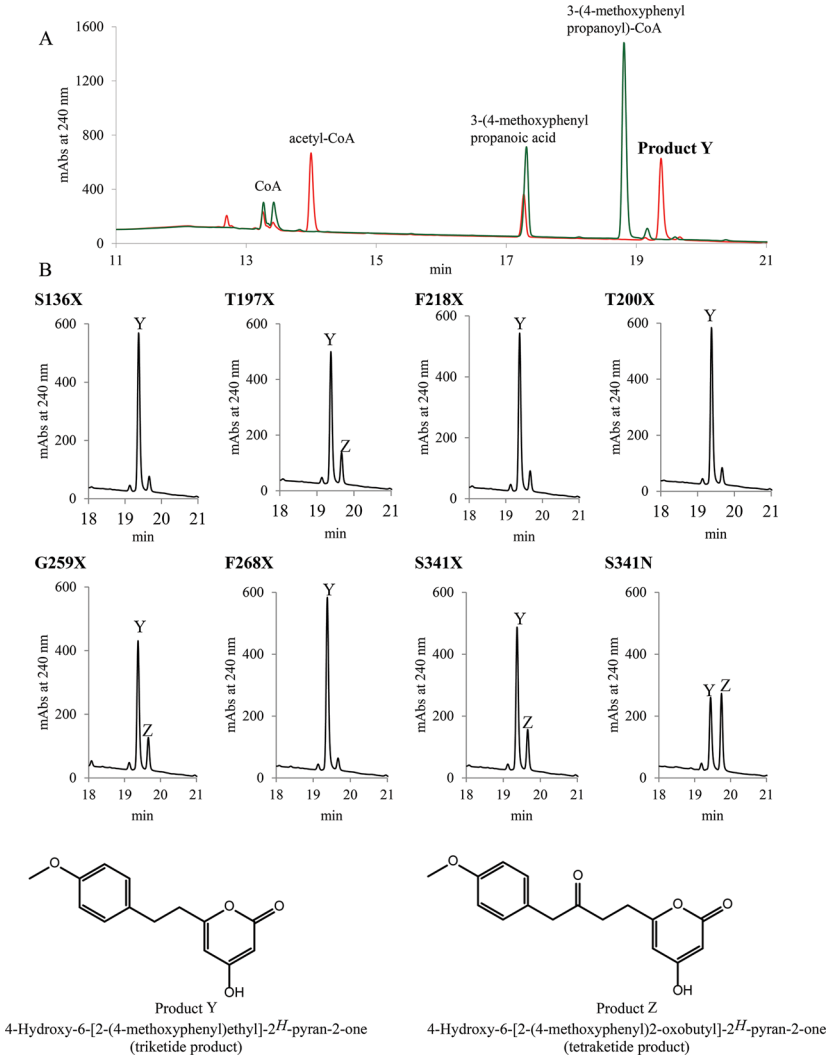
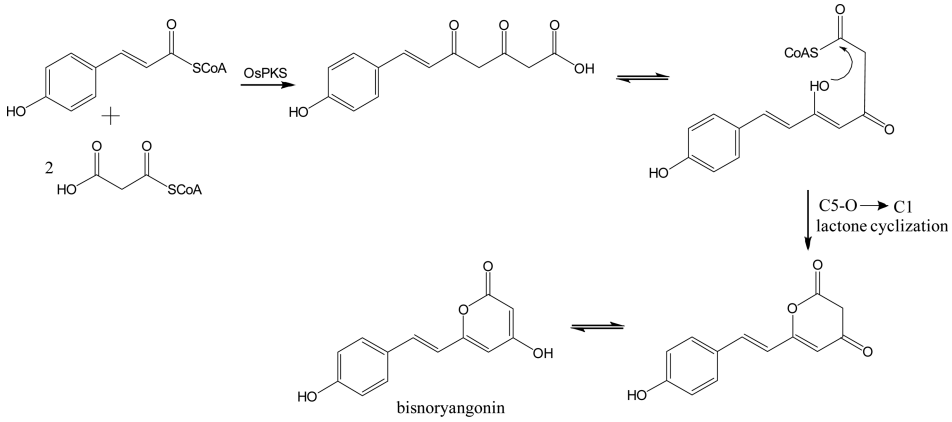



**Figure 6.** Partial HPLC chromatograms of polyketide synthesis catalyzed by wild-type and mutant OsPKS using 3-(4-methoxyphenyl)propanoyl-CoA and malonyl-CoA: (A) nonenzymatic control without OsPKS (green line) and with wild-type OsPKS (red line); (B) various OsPKS mutants. The new product that is produced only in the OsPKS mutants is product Z. The identities of products Y and Z are shown below the partial chromatograms.

**Scheme 4. Proposed Bisnoryangonin Biosynthesis by OsPKS**




*aureus*, and *Mycobacterium smegmatis*), with MIC values between 12 and 100 μg/mL.[15] Our findings corroborated their earlier observations that bisnoryangonin is an inhibitor specific for Gram-positive bacteria. In addition, we were able to extend the inhibitory effect to *E. faecalis*, a Gram-positive bacterium that had not been tested earlier by the authors.

**Characterization of 26OH.** 26OH (1.2 mg/L of culture) is biosynthesized from one unit of 4-methoxyphenlyacetyl-CoA and four units of hydroxymalonyl-CoA. The linear pentaketide



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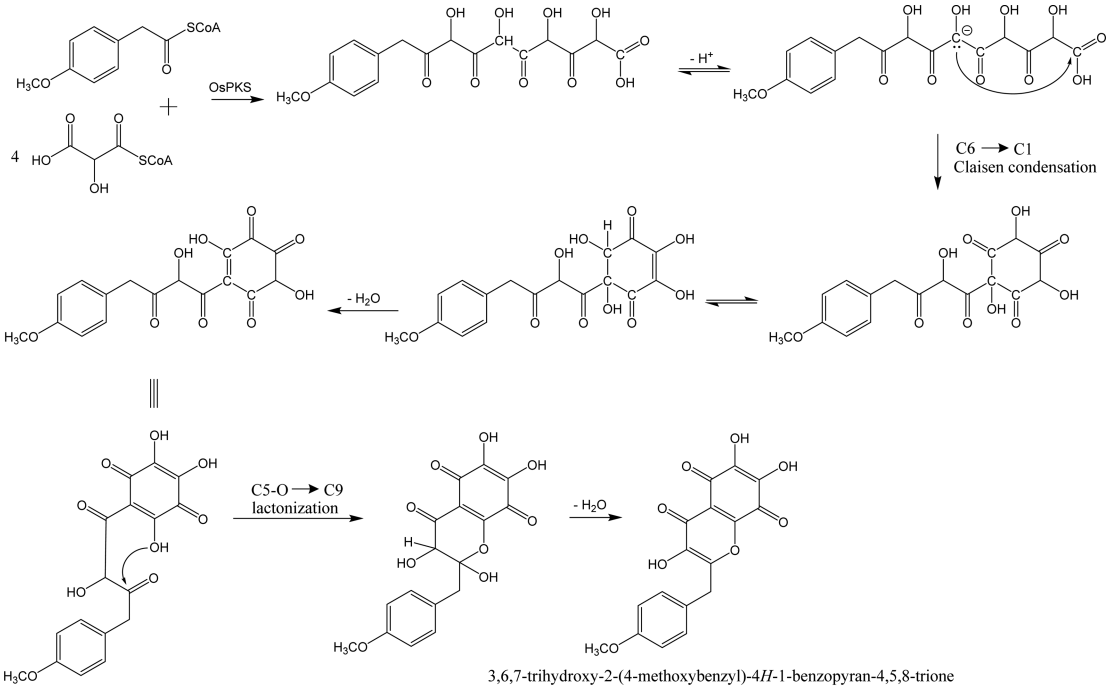
**Scheme 5. Proposed 26OH Biosynthesis by OsPKS**




undergoes a series of cyclizations to generate the final polyketide (Scheme 5). The first cyclization is the C6 → C1 intramolecular Claisen condensation with the subsequent release of the pentaketide from the catalytic cysteine residue. The second cyclization is the C5−O → C9 lactone cyclization, forming the final product.

The biosynthesized benzopyrantrione was used for the MIC assay and yielded MIC values of 256 and 128 μg/mL for *E. faecalis* and *B. thailandensis*, respectively. We propose that the presence of multiple hydroxy groups on the compounds could have contributed to its inhibition effect. Phenolic compounds are known as membrane-damaging microbiocides, as they affect the bacterium membrane permeability, which could cause an adverse effect leading to cell death.[16,17] Another possible antimicrobial mechanism of 26OH against Gram-positive bacteria could be due to the lack of an outer membrane which would facilitate the diffusion of phenolic compounds, resulting in hyperacidification at the plasma membrane interphase from the dissociation of phenolic acids.[18] The hyperacidification would affect the permeability of the cell membrane, in turn affecting the sodium−potassium ATPase pump which would lead to cell death.[19]

## CONCLUDING REMARKS

We have successfully demonstrated the use of synthetic polyketide enzymology as a means to develop lead polyketide based compounds for antimicrobial therapeutics. The modular coupling of acid:CoA ligases to type III polyketide synthases in the biosynthesis and development of polyketide-based biochemical was established. The product profile of OsPKS, a type III polyketide synthase from *Oryza sativa*, was characterized. Using OsPKS, we were able to produce a chemically diverse library of novel polyketides and identified two bioactive antimicrobials, 4-hydroxy-6-[(1*E*)-2-(4-hydroxyphenyl)-ethenyl]-2*H*-pyran-2-one (bisnoryangonin) and 3,6,7-trihydroxy-2-(4-methoxybenzyl)-4*H*-1-benzopyran-4,5,8-trione (26OH). The structure of OsPKS (PDB ID: 4YJY) was determined and used as a rational means to diversify the product profiles of polyketide biosynthesis. Using the described route of synthetic polyketide enzymology, a library of OsPKS mutants was generated as an additional means to increase the diversity of the polyketide product library. We expect the utility of synthetic enzymology to be extended to other classes of biomolecules and translated to various purposeful functions as the field of synthetic biology progresses.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b00477.

Figure S1, showing the structure and names of the 80 carboxylic acids used, Tables S1−S6, giving the product profiles of the various carboxylic acids utilized by the modular coupling of an acid:CoA-ligase and OsPKS, and Table S7, giving the data collection and refinement statistics for OsPKS (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

26OH, 3,6,7-trihydroxy-2-(4-methoxybenzyl)-4*H*-1-benzopyran-4,5,8-trione; 4CL, 4-coumarate:CoA ligase; BZL, benzoate:-CoA ligase; CoA, coenzyme A; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; HCl, hydrochloric acid; HPLC, high performance liquid chromatography; HR-MS, high resolution mass spectrometry; IPTG, isopropyl $\beta$-D-1-thiogalactopyranoside; MCS, malonate:CoA synthetase; MIC, minimum inhibition concentration; NMR, nuclear magnetic resonance; $OD_{600}$, optical density at 600 nm; OsPKS, polyketide synthase from *Oryza sativa*; PCL, phenylacetate:CoA ligase; PKS, polyketide synthase; TFA, trifluoroacetic acid; Tris, tris-(hydroxymethyl)aminomethane

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