Unprecedented acetoacetyl-coenzyme A synthesizing enzyme of the thiolase superfamily involved in the mevalonate pathway

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Acetoacetyl-CoA is the precursor of 3-hydroxy-3-methylglutaryl (HMG)-CoA in the mevalonate pathway, which is essential for terpenoid backbone biosynthesis. Acetoacetyl-CoA is also the precursor of poly-β-hydroxybutyrate, a polymer belonging to the polyester class produced by microorganisms. The de novo synthesis of acetoacetyl-CoA is usually catalyzed by acetoacetyl-CoA thiolase via a thioester-dependent Claisen condensation reaction between two molecules of acetyl-CoA. Here, we report that \( nphT7 \), found in the mevalonate pathway gene cluster from a soil-isolated Streptomyces sp. strain, encodes an unusual acetoacetyl-CoA synthesizing enzyme. The recombinant enzyme overexpressed in Escherichia coli catalyzes a single condensation of acetyl-CoA and malonyl-CoA to give acetoacetyl-CoA and CoA. Replacement of malonyl-CoA with malonyl-(acyl carrier protein) resulted in loss of the condensation activity. No acetoacetyl-CoA synthesizing activity was detected through the condensation of two molecules of acetyl-CoA. Based on these properties of \( nphT7 \), we propose to name this unusual enzyme of the thiolase superfamily acetoacetyl-CoA synthase. Coexpression of \( nphT7 \) with the HMG-CoA synthase gene and the HMG-CoA reductase gene in a heterologous host allowed 3.5-fold higher production of mevalonate than when only the HMG-CoA synthase and HMG-CoA reductase genes were expressed. This result suggests that \( nphT7 \) can be used to significantly increase the concentration of acetoacetyl-CoA in cells, eventually leading to the production of useful terpenoids and poly-β-hydroxybutyrate.

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Carbon–carbon bond formation is an essential step in natural product biosynthesis. Thiolase superfamily enzymes catalyze the formation of this covalent bond via a thioester-dependent Claisen condensation reaction in various metabolic pathways, such as fatty acid, polyketide, and mevalonate biosyntheses (1, 2). The superfamily enzymes are categorized into two groups according to their mechanism of \( \alpha \)-anion generation in the Claisen condensation: one consists of nondecarboxylative enzymes, including acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase; and the other includes decarboxylative enzymes, such as \( \beta \)-ketoacyl-(acyl carrier protein (ACP)) synthase (KAS) I, KAS II, KAS III, \( \beta \)-ketoacyl-CoA synthase (KCS), and chalcone synthase (CHS) family enzymes (1, 2). The nondecarboxylative enzymes generate an anion by proton elimination from the \( \alpha \)-carbon of acyl-CoA, whereas the decarboxylative enzymes generate an anion by \( \beta \)-carboxy decarbonylation of a di-keto moiety in malonyl-CoA or malonyl-ACP (1–4). An extensive body of biochemical studies concerning decarboxylative KAS III enzymes and their crystal structures has identified a catalytic triad involved in the Claisen condensation that includes Cys, His, and Asn residues (5–9). The Cys residue is involved in acyl loading from a primer substrate acyl-CoA to give an acyl-cysteine intermediate, and the other two residues are involved in decarbonylation of the malonyl moiety of malonyl-ACP to yield a carbanion. The resulting carbanion performs a nucleophilic attack on the carbonyl carbon of the acyl-cysteine intermediate to yield \( \beta \)-ketoacyl-ACP such as acetoacetyl-ACP for fatty acid synthesis.

Recently, we identified a mevalonate pathway gene cluster (\( nphT1 \) to \( nphT6 \)) in Streptomyces sp. strain CL190 (10–12). Mevalonate pathway gene clusters have also been cloned from Actinoplanes sp. strain A40644 (13), Streptomyces sp. strain KO-3984 (14), and Streptomyces anulatus (15). Each gene cluster contains six open reading frames encoding enzymes that catalyze the formation of isopentenyl diphasphate and dimethylallyl diphasphate for terpenoids via mevalonate from acetoacetyl-CoA (EC 2.3.1.9). Therefore, acetoacetyl-CoA thiolase was expected to exist in the previously mentioned mevalonate pathway gene clusters. However, homologs of acetoacetyl-CoA thiolase, involved in the conversion of acetyl-CoA to acetoacetyl-CoA, are missing in the gene clusters. Instead, an open reading frame (for example, \( nphT7 \) of Streptomyces sp. strain CL190) that shares homology with KAS III flanks each gene cluster (Fig. 1).

In this paper, we demonstrate that \( nphT7 \) catalyzes a single condensation of acetyl-CoA and malonyl-CoA to give acetoacetyl-CoA and CoA. Based on the enzymatic properties of \( nphT7 \), we propose that it is an unprecedented acetoacetyl-CoA synthase of the thiolase superfamily. This paper also describes the effect of \( nphT7 \) expression on mevalonate production in the heterologous host Streptomyces albus. Coexpression of \( nphT7 \) with the HMG-CoA synthase gene and the HMG-CoA reductase gene in S. albus resulted in 3.5-fold higher production of mevalonate than when only the HMG-CoA synthase and HMG-CoA reductase genes were expressed. This result suggests that \( nphT7 \) can be used to significantly increase the production of useful terpenoids such as carotenoids, taxol, and artemisinin.

Results

Expression and Purification of Recombinant \( nphT7 \) Gene Product. The \( nphT7 \) gene from Streptomyces sp. strain CL190 was overexpressed in Escherichia coli as an N-terminal His\(_6\)-tagged protein, and the recombinant protein was purified to apparent homogeneity. The molecular mass of the \( nphT7 \) product was estimated to be 37 kDa by SDS-PAGE (Fig. S1) and 63 kDa by gel filtration chromatography, suggesting that \( nphT7 \) is most likely a dimer.

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The authors declare no conflict of interest.

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Assay for KAS III Activity of NphT7. KAS III enzymes possess a catalytic triad of Cys, His, and Asn residues involved in the Claisen condensation of the primer substrate acetyl-CoA and the extender substrate malonyl-ACP. Alignment of NphT7 and the well characterized E. coli KAS III (5, 6) identified the highly conserved catalytic triad at residues Cys115, His256, and Asn286 (Fig. S2). Therefore, we first determined whether NphT7 displays KAS III activity, in which the acetyl group of acetyl-CoA is transferred to malonyl-ACP to yield acetoacetyl-CoA. To detect this acetyl transfer in the KAS III reaction, we used [1-14C]acetyl-CoA as the primer substrate. A high level of radioactivity arising from [3-14C]acetoacetyl-ACP was observed in the KAS III reaction (Fig. S3), whereas the radioactive signal of the ACP derivatives incubated with NphT7 was no greater than the signal in the absence of enzyme (negative control). Interestingly, the NphT7 reaction clearly indicates that NphT7 catalyzes the condensation of acetyl-CoA and malonyl-CoA to yield acetoacetyl-CoA (3; retention time = 9.2 min); and acetyl-CoA (4); retention time = 10.0 min) (Fig. 3A, B, and D). In contrast, no acetoacetyl-CoA synthesizing activity was detected in the absence of malonyl-CoA. These NphT7 in vitro assays unambiguously elucidated the function of NphT7, which catalyzes the condensation of acetyl-CoA and malonyl-CoA to yield CoA and acetoacetyl-CoA. Furthermore, the stoichiometry of the NphT7 reaction clearly indicates that NphT7 catalyzes a single condensation of one molecule of acetyl-CoA and one molecule of malonyl-CoA to yield one molecule of CoA and one molecule of acetoacetyl-CoA (Fig. 3E and Fig. S4A).

The steady-state kinetic constants for NphT7 with acetyl-CoA and malonyl-CoA as substrates (bisubstrate conditions) were determined (Fig. S5). The apparent $K_m$ for malonyl-CoA was estimated to be 68 ± 4 μM at a fixed concentration of malonyl-CoA (100 μM), whereas the apparent $K_m$ for acetyl-CoA was estimated to be 28 ± 2 μM at a fixed concentration of acetyl-CoA (200 μM). The $V_{\text{max}}$ value was 8.9 ± 0.3 μmol · min⁻¹ · mg⁻¹.

As described previously, E. coli KAS III catalyzes the formation of acetyl-ACP from malonyl-ACP by decarboxylating malonyl-ACP in the absence of the primer substrate acetyl-CoA (2, 17). This fact led us to assume that NphT7 catalyzes the formation of acetyl-CoA from malonyl-CoA by decarboxylating malonyl-CoA. To test this assumption, we incubated NphT7 with malonyl-CoA alone (monosubstrate conditions). HPLC of the NphT7 reaction with the addition of only malonyl-CoA revealed the formation of CoA (1) and acetoacetyl-CoA (2) concomitant with consumption of malonyl-CoA (3) (Fig. 3C). The stoichiometry of the NphT7 reaction under monosubstrate conditions indicates that one molecule of CoA and one molecule of acetoacetyl-CoA were formed concomitantly with the consumption of two molecules of malonyl-CoA (Fig. 3F and Fig. S4B). The steady-state kinetic constants for NphT7 under monosubstrate conditions were also determined (Fig. S5C): $K_m$ for malonyl-CoA was 320 ± 20 μM and $V_{\text{max}} = 1.8 ± 0.3$ μmol · min⁻¹ · mg⁻¹.
Prediction of the Reaction Mechanism of NphT7. The cysteine residue, which functions as an anchor for the acetyl moiety of primer substrates, is highly conserved and exists in a catalytic Cys-His-Asn triad in KAS III and other decarboxylative condensing enzymes such as KCS and CHS. Substitution of the cysteine to another amino acid results in the loss of the condensation activity. However, the substituted enzyme retains its decarboxylation activity through the actions of the His and Asn residues to produce acetyl-ACP from malonyl-ACP by decarboxylation of the malonyl moiety (5). To determine the function of Cys115 in NphT7, we constructed the mutant NphT7 (C115A) (Fig. S1) and evaluated its activity. HPLC of the NphT7 (C115A) reaction mixture in the presence of acetyl-CoA and malonyl-CoA (bisubstrate conditions) confirmed the full consumption of malonyl-CoA and an increase in acetyl-CoA (Fig. 4A). Meanwhile, there was no formation of CoA or acetoacetyl-CoA. This result indicates that the C115A mutant enzyme yields acetyl-CoA via its malonyl-CoA decarboxylation activity, presumably by the His256 and Asn286 residues, while it has lost its condensation activity. This loss of function is most likely due to the lack of Cys115, which anchors the acetyl moiety of acetyl-CoA. These results strongly suggest that Cys115 in NphT7 functions as a key catalytic residue for the condensation reaction.

To determine the function of the His256 and Asn286 residues in NphT7, we attempted to construct the mutants NphT7 (H256A) and NphT7 (N286A) (Fig. S1). However, we could not obtain NphT7 (N286A) as a soluble protein. Therefore, we investigated the activity of only NphT7 (H256A). HPLC of the NphT7 (H256A) reaction mixture revealed that this mutant enzyme exhibited detectable acetoacetyl-CoA synthesizing activity, but its specific activity (0.23 μmol·min⁻¹·mg⁻¹) was approximately 40-fold lower than that of wild-type NphT7 (8.9 μmol·min⁻¹·mg⁻¹) (Fig. 4B). This low activity of the NphT7 (H256A) suggests that His256 functions as a catalytic residue for the decarboxylation of the extender substrate malonyl-CoA.

Taken together, the results suggest that the NphT7-catalyzed reaction presumably proceeds through an almost identical reaction mechanism to that of KAS III.

in Vivo Acetoacetyl-CoA Synthesizing Activity of NphT7. To verify the in vivo acetoacetyl-CoA synthesizing activity of NphT7, we evaluated the effect of nphT7 expression on mevalonate production by constructing two plasmids (pSEMV25 and pSEMV40) and then transforming each into S. albus. pSEMV25 contains nphT5 and nphT6 (18), whereas pSEMV40 contains nphT5, nphT6, and

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**Fig. 3.** Assay for acetoacetyl-CoA synthesizing activity. All reactions were performed as described in Materials and Methods. HPLC analysis was performed for the NphT7-catalyzed reactions (A) with acetyl-CoA and malonyl-CoA and (C) with malonyl-CoA alone. Chromatograms monitored at 259 nm. Peaks: 1, CoA; 2, acetoacetyl-CoA; 3, malonyl-CoA; and 4, acetyl-CoA. A: solid line, after a 1-min incubation with NphT7; dotted line, after a 2-min incubation with heat denatured NphT7. C: solid line, after a 2-min incubation with NphT7; dotted line, after a 2-min incubation with heat denatured NphT7. B and D: high-resolution mass spectra (negative mode) of peaks 1 and 2, respectively. E and F: stoichiometries of the acetoacetyl-CoA synthesizing activity of NphT7 under bisubstrate and monosubstrate conditions, respectively. Filled triangles, acetoacetyl-CoA; circles, CoA; triangles, acetyl-CoA; and filled circles, malonyl-CoA. The standard errors of each point were generated by repeating the experiment three times. The NphT7-catalyzed reaction formulas are described inside. Also see chromatograms in Fig. S4.

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**Fig. 4.** Assays for the acetoacetyl-CoA synthesizing activity of NphT7 (C115A) and NphT7 (H256A). Peaks: 1, CoA; 2, acetoacetyl-CoA; 3, malonyl-CoA; and 4, acetyl-CoA. A: HPLC analysis of the NphT7 (C115A)-catalyzed reaction monitored at 259 nm. Solid line, after a 10-min incubation of the reaction mixture with heat denatured NphT7 (C115A); dotted line, after a 10-min incubation of the reaction mixture with heat denatured NphT7 (C115A). B: HPLC analysis of the NphT7 (H256A)-catalyzed reaction monitored at 259 nm. Solid line, after a 5-min incubation of the reaction mixture with heat denatured NphT7 (H256A); dotted line, after a 5-min incubation of the reaction mixture with heat denatured NphT7 (H256A).
We demonstrated in the present study that NphT7, a KAS III homolog flanking the mevalonate pathway gene cluster of *Streptomyces* sp. strain CL190, displays an unusual acetoacetyl-CoA synthesizing activity. Synthesis of acetoacetyl-CoA is usually catalyzed by acetoacetyl-CoA thiolase via the condensation of two molecules of acetyl-CoA, yet NphT7 synthesizes acetoacetyl-CoA via the condensation of acetyl-CoA and malonyl-CoA. Importantly, NphT7 cannot use malonyl-ACP as its extender substrate, whereas KAS III enzymes can. We therefore propose to name NphT7 “acetoacetyl-CoA synthase,” an unprecedented enzyme of the thiolase superfamily.

We demonstrated in this study that NphT7 can synthesize acetoacetyl-CoA in assay mixtures in which only malonyl-CoA is added as the substrate (monosubstrate conditions). Furthermore, a C115A mutant enzyme loses its condensation activity while retaining its malonyl-CoA decarboxylation activity to yield acetyl-CoA. These results indicate that NphT7 converts malonyl-CoA into acetyl-CoA via decarboxylation of malonyl-CoA. This finding explains why NphT7 displays acetoacetyl-CoA synthesizing activity even with no addition of acetyl-CoA into the reaction mixture, as it alternatively generates acetyl-CoA primer substrate by decarboxylating malonyl-CoA in an irreversible reaction. A similar reaction has been reported for the plant type III polyketide synthase 2-pyrene synthase (2-PS) (22). 2-PS catalyzes two Claisen condensations of one molecule of acetyl-CoA with two molecules of malonyl-CoA to yield 6-methyl-4-hydroxy-2-pyrene. When acetyl-CoA is not present in this reaction, 2-PS synthesizes the primer substrate acetyl-CoA via decarboxylation of malonyl-CoA and further synthesizes 6-methyl-4-hydroxy-2-pyrene. In contrast, a bacterial type III polyketide synthase, 1,3,6,8-tetrahydroxynaphthalene (THN) synthase, synthesizes THN by four Claisen condensations of a single malonyl-CoA extender substrate with no incorporation of an acetyl-CoA primer substrate, although THN synthase can also convert malonyl-CoA into acetyl-CoA (23). It is known that THN synthase does not load acetyl-CoA as a primer substrate despite possessing the ability to decarboxylate malonyl-CoA. In view of the substrate structures and the condensation number of NphT7, the NphT7-catalyzed reaction is simplest among the decarboxylative condensing enzymes possessing the catalytic triad (Fig. S6). Therefore, a crystal structure of NphT7 will provide new insights into the substrate recognition mechanism and the reaction mechanism of not only KAS III, 2-PS, and THN synthase, but also other decarboxylative enzymes possessing the Cys-His-Asn catalytic triad.

To gain insight into the substrate recognition of NphT7, we compared amino acid sequences of the NphT7 homologs with that of well-characterized *E. coli* KAS III (5, 6, 16). N-terminal architectures and recognition residues for acetyl-CoA are relatively conserved among the NphT7 homologs and *E. coli* KAS III (ecoKASIII), whereas partial C-terminal architectures and ACP recognition residues are not conserved (Fig. 6 and Fig. S2). Recognition residues Thr32 and Arg151 for the adenine ring of acetyl-CoA (5, 6) are completely conserved, but Thr28 is replaced with Val or Ile in the NphT7 homologs. Residues Thr37, Arg151, Met207, Gly209, Ala246, and Asn247, which are responsible for the recognition of phosphopantetheine of ACP or CoA (5, 6), are completely conserved, but a variety of residues equivalent to Arg36, Asn210, Arg249, and Ile250 in ecoKASIII are found in the NphT7 homologs. The Gly305–307 residues that form an oxaymion hole (5, 6) are highly conserved. Residues Phe87, Leu142, Phe157, Leu188, and Leu205 form a hydrophobic pocket for recognition of the acetyl methyl group of acetyl-CoA (5, 6). Of the five residues, Phe157 and Leu188 are highly conserved, but both Leu142 and Leu205 are replaced with the bulky residue Tyr in the NphT7 homologs. In addition, the Phe87 residue that dictates primer substrate specificity in ecoKASIII (5, 6) is replaced with Gin in the NphT7 homologs. Since the NphT7 homologs share a STPDXPQ sequence motif in the predicted L3 loop region containing the important Gin residue, the motif may be a determinant site of the primer substrate specificity of the NphT7 homologs. The Ccr1 and Ccr2 regions of ecoKASIII interact with the helix II region of ACP to recognize the ACP molecule (16). The ACP recognition residues Lys214, Arg249, Lys256, and Lys257 in the Ccr1 and Ccr2 regions (16) are replaced with various amino acids in the NphT7 homologs. Most importantly, the predicted L9 loops in the NphT7 homologs are inserted by extra sequences and share an (A/G)GGSR sequence motif (Fig. 6). This (A/G)GGSR motif, characteristic of the NphT7 homologs, may serve as one of recognition sites for the CoA moiety of the extender substrate malonyl-CoA. Further insights into the structural basis for the substrate recognition mechanism require crystal structures of NphT7 (acetoacetyl-CoA synthase) complexed with the substrate or the product.

We determined the steady-state kinetic constants for NphT7 under both monosubstrate and bisubstrate conditions to determine which condition the NphT7 enzyme prefers. The *Km* value...
for malonyl-CoA under monosubstrate conditions was approximately 10-fold higher than that for malonyl-CoA under bisubstrate conditions. In addition, the \( V_{\text{max}} \) value under monosubstrate conditions was approximately 5-fold lower than that under bisubstrate conditions. These notable differences of the kinetic constants imply that NphT7 synthesizes acetoacetyl-CoA via the predominant bisubstrate reaction in vivo.

We demonstrated in this study that NphT7 functions as an acetoacetyl-CoA synthase involved in the mevalonate pathway. Although acetoacetyl-CoA thiolase (EC 2.3.1.9) also produces acetoacetyl-CoA by reversible noncarboxylative condensation of two molecules of acetyl-CoA, this enzyme prefers acetoacetyl-CoA thiolysis to acetoacetyl-CoA synthesis (2). Because NphT7 exhibits no thiolysis activity against acetoacetyl-CoA, and since acetoacetyl-CoA synthase involved in the mevalonate pathway.

BLAST searches using the NphT7 sequence as a query sequence found 55 sequences from protein databases of the National Center for Biotechnology Information annotated as putative KAS III sequences displaying E-values less than 3 \( e^{-99} \). The 55 sequences include 14 actinobacterial sequences (Table S1). Of the 14 sequences annotated as putative KAS III sequences, six NphT7 homologs flank the mevalonate pathway gene clusters for terpenoid biosynthesis in an engineered strain of \( E. coli \) (24–26). Therefore, nphT7 could be used for heterologous production of biofuel (butteroil), biodegradable plastic (poly-\( \beta \)-hydroxybutyrate), and useful terpenoids, such as carotenoids, taxol, and artemisinin (27), all of which use acetoacetyl-CoA as common precursors.

BLAST searches using the NphT7 sequence as a query sequence found 55 sequences from protein databases of the National Center for Biotechnology Information. These results are consistent with the hypothesis that NphT7-like enzymes are widely involved in the biosynthesis of terpenoid biosynthesis in a variety of organisms.

Materials and Methods
Preparation of NphT7, NphT7 (C115A), and NphT7 (H156A). Using previously prepared pCLC (accession numbers AB807666 and AB800131) (11) including nphT7 as a template, a PCR amplification for ligation into the E. coli expression vector pHis8 (28) was carried out with the following primers S-GGGGATCCACCGACGTCCGATTCCGC-3′ (BamHI site underlined) and the reverse primer S-GGGGAATTCCTACCTCGATCGGCGG-3′ (EcoRI site underlined) to generate pHis_nphT7. Using pHis_nphT7 as a template, site-directed mutagenesis for NphT7 (C115A) and NphT7 (H256A) was performed to construct pHis_nphT7_C115A and pHis_nphT7_H256A, respectively, following the QuickChange XL (Stratagene) protocol. The primers for mutagenesis were: forward for NphT7 (C115A), S-GAATTCCTACCACTCGATCAGGGCG-3′; reverse for NphT7 (C115A), S-GAACACCGTGCCCGGACGCGCGAGCG GTTGCATG-3′; forward for NphT7 (C115A), S-GAACACCCGTTTGCGGACGCGGAC CGTTGACGTC-3′; and reverse for NphT7 (H256A), S-GACACCTGTTG CCTGCGCCGGGAGAAGTCCT-3′. For full experimental details of protein expression and purification of NphT7, (C115A), and NphT7 (H256A), see SI Text.

Assay for KAS III Activity. The assay contains 4 \( \mu \)g NphT7, 84.7 \( \mu \)M [2-\( ^{14} \)]acetate-CoA, and 13.5 \( \mu \)M malonyl-ACP in 10 \( \mu \)L reaction buffer (100 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol). As a positive control, 0.3 \( \mu \)g FabH (S. coelicolor KAS III) was added instead of NphT7. As a negative control, the reaction buffer was added instead of NphT7. The reaction was carried out at 25 °C for 2 h and terminated by boiling with 1× loading dye for 5 min. Proteins were separated by SDS-18% PAGE. After gel drying, the gel was exposed to an imaging plate (Fujiﬁlm) for 18 h and the radiolabeled ACP derivatives were visualized on an FLA-3000 imaging system (Fujiﬁlm). For full experimental details of the preparation of acetoacetyl-CoA (29), see SI Text.

Assay for Acetoacetyl-CoA Synthesizing Activity. Under bisubstrate conditions, acetoacetyl-CoA synthesizing activity was assayed in 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM acetyl-CoA, and 1 mM malonyl-CoA in a total volume of 50 \( \mu \)L. After the reaction mixture devoid of enzyme was incubated at 30 °C for 1 min, the reaction was started by adding 15 \( \mu \)g NphT7, 50 \( \mu \)g NphT7 (C115A), or 70 \( \mu \)g NphT7 (H256A). The reactions, in the case of NphT7, were carried out at 30 °C for 10, 20, 30, 60, and 120 sec. In the case of NphT7 (C115A), the reaction was carried out for 10 min. For NphT7 (H256A), the reactions were carried out for 30, 60, 120, and 300 sec. Under monosubstrate conditions, acetoacetyl-CoA synthase activity was assayed in 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM acetyl-CoA, and 1 mM malonyl-CoA in a total volume of 50 \( \mu \)L. The reactions under monosubstrate conditions were carried out at 30 °C for 30, 60, 90, and 120 sec. The reactions were terminated by boiling with 1× loading dye for 5 min. Proteins were separated by SDS-18% PAGE. After gel drying, the gel was exposed to an imaging plate (Fujiﬁlm) for 18 h and the radiolabeled ACP derivatives were visualized on an FLA-3000 imaging system (Fujiﬁlm). For full experimental details of the preparation of acetoacetyl-CoA (29), see SI Text and Table S2.
and NphT7 (H256A) were calculated from respective consumption of malonyl-CoA and acetyl-CoA were detected at 9.2 min, 9.6 min, 9.8 min, and 10.0 min, respectively.

**Steady-State Kinetic Parameters.** A spectrophotometric NphT7 assay based on the formation of a Mg\(^{2+}\)–acetoacyl-CoA enolate complex was employed for steady-state kinetic studies of NphT7 (31). Under bisubstrate conditions, acetoacyl-CoA and acetyl-CoA were detected at 9.2 min, 9.6 min, 9.8 min, and 10.0 min, respectively.


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Supporting Information

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SI Text

SI Material and Methods. Reagents. [1-14C]Acetyl-CoA was purchased from GE Healthcare. The other CoA derivatives, cerulenein, and thiocolaminoc were from Sigma-Aldrich. Di-n-butyl-ammonium acetate and ammonium acetate used for ion pair chromatography were purchased from Tokyo Chemical and Wako Pure Chemical, respectively. The Ligation High used for ligation was from Toyobo. Restriction enzymes and the DNA Blunting kit were from Takara Bio. The Expand High Fidelity PCR system used for amplification of genes was from Roche. The Ni-NTA Superflow used for purification of recombinant proteins was from Qiagen. The Bradford method protein assay kit was from BioRad.

Bacterial strains and vectors. Escherichia coli DH5α was used for plasmid cloning. E. coli BL21(DE3) and JM109 were used for expressing recombinant proteins. Streptomyces albus was used for the heterologous production of mevalonate. The pT7Blue T-Vector (Novagen) was used for cloning PCR products. pET15b (Novagen) and pHis8 (1) were used for protein expression. A Streptomyces-E. coli shuttle vector, pSE101 (2), was used for heterogeneous production of mevalonate.

Primer design, gene cloning, and construction of expression plasmids. N-terminal and C-terminal primers of ACP (acp) involved in the fatty acidity biosynthesis (FAS) system, holo-ACP synthase (acps), and malonyl-ACP transferase (fabD) were designed and referred to the genome database sequence of the Genome Information Broker (http://gib.genes.nig.ac.jp/) of Streptomyces coelicolor A3 (2). The gene numbers of these genes are as follows: acp, SCO2387; acpC, SCO2388; acps, SCO4744; and fabD, SCO2378. The acp gene was amplified by PCR using the chromosomal DNA of Streptomyces sp. strain CL190 as a template. The acps and fabD genes were also amplified by PCR using the chromosomal DNA of S. coelicolor A3(2) as a template. The PCR conditions were set by the instruction manual (Roche). These PCR products were inserted into pT7Blue T-Vector by TA-cloning. The plasmids were introduced into E. coli DH5α competent cells. The resulting transformants were grown on LB containing 50 μg/mL of ampicillin for 12 h at 37 °C and colonies were selected by colony-directed PCR using the M13, M4, and M13 RV universal primers. The positive transformants were grown in LB containing 50 μg/mL of ampicillin for 12 h at 37 °C. The plasmids were then recovered from the transformants and the inserted DNA sequences were verified by sequencing. Note that the sequence of FAS acp amplified using the genomic DNA of Streptomyces sp. strain CL190 as a template DNA was completely identical to that of S. coelicolor A3(2). Plasmids were digested with the appropriate restriction enzymes and then separated by agarose gel electrophoresis. Recovered DNA was subcloned into the appropriate restriction sites of pHis8 or pET15b by ligation to construct pHis_fabD, pHis_acps, and pET_acp.

Protein expression and purification. E. coli BL21(DE3) was used for the expression of pHis_nphT7, pHis_fabD, pHis_acps, pET_acp, pHis_nphT7_C115A, pHis_nphT7_H256A, and pHis_nphT7_N286A. E. coli JM109 was used for the expression of pQE-fabH. These transformants were grown in Terrific Broth medium containing 100 μg/mL of ampicillin (pET_acp and pQE_fabH) or 50 μg/mL of kanamycin (pHis_nphT7, pHis_nphT7_C115A, pHis_nphT7_H256A, pHis_nphT7_N286A, pHis_fabD, and pHis_acps) at 37 °C. IPTG was added at a final concentration of 1 mM to induce expression when the absorbance at 600 nm reached about 1.0. After an additional 12–14 h of cultivation at 18 °C, cells were harvested by centrifugation at 10,000 x g for 10 min. The harvested cells were then suspended in lysis buffer containing 100 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1% (v/v) Tween 20, and 20% (v/v) glycerol. Then cell suspensions were sonicated with a Branson Sonifier 250 (Emerson Japan) on ice. To separate the cellular debris from the soluble protein, the lysates were centrifuged at 30,000 x g at 4 °C for 20 min. The resulting supernatants were loaded onto Ni-NTA Superflow resin. After washing with the wash buffer containing 100 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 20% (v/v) glycerol, the desired proteins were eluted using the same buffer containing 250 mM imidazole. All purification steps for NphT7, NphT7 (C115A), NphT7 residue (H256A), and FabH were performed under reductive conditions by adding 1 mM dithiothreitol. The purified His-tagged recombinant FabH protein was dialyzed against dialysis Buffer A containing 100 mM Hepes (pH 7.5), 200 mM NaCl, 1 mM dithiothreitol, and 20% (v/v) glycerol for 12 h at 4 °C. The purified His-tagged recombinant NphT7, NphT7 (C115A), and NphT7 (H256A) proteins were dialyzed against dialysis Buffer B containing 100 mM Hepes (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, and 20% (v/v) glycerol. The other His-tagged recombinant ACP (apo form), holo-ACP synthase (ACPs), and FabD proteins were dialyzed against dialysis Buffer C containing 100 mM Hepes (pH 7.5), 50 mM NaCl, and 20% (v/v) glycerol. All recombinant proteins were concentrated by centrifugation using Viva Spin (Sartorius) at 4 °C. The concentrations of the recombinant proteins were determined by the Bradford method using bovine serum albumin as a standard.

Molecular weight analysis. NphT7 was analyzed by SDS-10% PAGE and gel filtration chromatography to estimate the protein’s multimeric form and native molecular weight. The purified NphT7 was applied to a Superdex 200 20/60 prep grade gel filtration column (GE Healthcare) equilibrated with the Buffer C. The column was calibrated with three molecular weight markers (GE Healthcare), Catalase (232 kDa), Albumin (67 kDa), and Chymotrypsinogen A (25 kDa). Recombinant proteins other than ACP were analyzed by SDS-10% PAGE, whereas ACP was analyzed by SDS-18% PAGE.

Preparation of malonyl-ACP. Malonyl-ACP was enzymatically modified by a modified method (3). Apo-ACP (73 μM) was converted to holo-ACP by incubation with 1 nM ACPs in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 1 mM CoA, 5 mM MgCl2, and 1 mM dithiothreitol for 12 h at 25 °C. After further incubation with 1 mM malonyl-CoA and 1 mM FabD for 12 h at 30 °C, the reaction mixture was analyzed to confirm the generation of malonyl-ACP by LC-MS (Bruker) with PEGASIL 300 C4 (2μ × 150 mm, 5 μm, Senshu Scientific) under the following conditions: mobile phase (A = 0.1% TFA in acetonitrile, B = 0.1% TFA in water), 5% A for 5 min, 5%–30% A over 5 min, 30%–70% A over 30 min, 70%–100% A over 5 min, 100%–5% A over 1 min, 5% A for 10 min; flow rate, 1.0 mL/min. Mass spectrometry analysis was performed by electrospray ionization in positive ion mode. After the analysis, deconvolution was automatically performed using the equipped software (Bruker). Products P1 and P2 displayed [M + 5H]5+ = 2276.5 and [M + 5H]5+ = 2258.9, respectively. Both values were recalculated by deconvolution to the observed molecular weights of 11,377.4 Da and 11,289.7 Da, which are consistent with the monoisotopic masses of 11,375.6 Da for...
malonyl-ACP and of 11,289.3 Da for holo-ACP. Having confirmed the formation of malonyl-ACP, we collected the fractions that included malonyl-ACP, although we could not completely isolate them from the remaining holo-ACP. Concentrating the collected fractions by centrifugal evaporation at 30 °C followed by ultrafiltration with Amicon Ultrafree-MC (5,000 NMWL; Millipore) yielded malonyl-ACP.

**Plasmid construction for mevalonate production.** pSEMV40, which contains three open reading frames encoding HMG-CoA synthase (nphT6), HMG-CoA reductase (nphT5), and NphT7 (nphT7), was constructed as follows. Previously prepared pCLC7 (accession numbers AB037666 and AB540131) (4), containing the mevalonate pathway gene cluster from *Streptomyces* sp. strain CL190, was digested with AccIII, which is located downstream of nphT7. The site was then blunted with a DNA blunting kit and the DNA fragment was further digested with Sse8387I, located in nphT6, to yield a 2.1 kb DNA fragment containing the C terminus of nphT6 and the full nphT7. Next, a heterologous expression plasmid pSEMV25 (5) containing nphT5 and nphT6 from *Streptomyces* sp. strain CL190 was digested with HindIII located in the multicloning site of pSE101 (2), followed by blunting of the digestion site. Then the DNA fragment was further digested with Sse8387I, located in nphT6, to yield a 8.7 kb DNA fragment containing the full nphT5 and the N terminus of nphT6 in pSE101. Finally, the resulting 2.1 kb and 8.7 kb DNA fragments were ligated to give pSEMV40 (Fig. 6). All nphT genes in pSEMV25 and pSEMV40 are most likely transcribed as a single operon by read-through from the promoter of the replication protein in the vector pSE101 (6, 7).

**Heterologous production of mevalonate.** Transformation of *S. albus* was performed by the protoplast method (6). The resulting transformants were grown in 10 mL of Typtone Soya Broth liquid medium containing 30 μg/mL thiostrepton for three days at 30 °C. The precultures (200 μL) were inoculated into 10 mL KG medium (5) containing 30 μg/mL thiostrepton, and the transformants were grown for mevalonate production for nine days at 30 °C.


**Fig. S1.** Expression and purification of the recombinant proteins. Lanes: 1, molecular mass markers; 2, the extract from the recombinant *E. coli* culture after induction with IPTG; 3, the supernatant of the same extract; 4, protein passed through a Ni-NTA column; 5, the fraction eluted with the wash buffer; and 6, purified enzyme eluted with the same buffer containing 250 mM imidazole. NphT7 (N286A) could not be obtained as a soluble protein.
Fig. S2. Multiple alignment of the amino acid sequences of NphT7 homologs and E. coli KAS III. This alignment was constructed using ClustalW ver. 1.82. Dots represent gaps introduced for optimization of the alignment. ecoKASIII, E. coli KAS III (PDB ID code 1EBL); S. sp KO3988-1 and S. sp KO3988-2, NphT7 homologs from Streptomyces sp. strain KO-3988 (Protein IDs, BAD86806 and BAE78983, respectively); S. anulatus, NphT7 homolog from S. anulatus strain 9663 (CAX48662); A. sp A40644, NphT7 homolog from Actinoplanes sp. strain A40644 (BAD07381); M. ulcerans, NphT7 homolog from Mycobacterium ulcerans Ag99 (YP_907152); M. marinum, NphT7 homolog from M. marinum M (YP_001851502). The (A/G)GGSR motif found in the present study is underlined. Secondary structures of ecoKAS III are indicated above the alignment (1, 2). The symbols represent the following functional amino acid residues in ecoKASIII: filled circles, catalytic triad; filled squares, hydrophobic residues responsible for recognition of the acetyl moiety; filled triangles, residues responsible for recognition of ACP; bold letters “P”, residues responsible for recognition of phosphopantetheine. filled pentagon, residues responsible for recognition of adenine ring of CoA; bold letters “O”, residues that form an oxyanion hole.

Fig. S3. HPLC/MS analysis of enzymatically prepared malonyl-ACP. A: total ion count (upper) and UV (lower) chromatograms. Peaks: 1, malonyl-ACP, Rt = 21.2 min; and 2, holo-ACP, 21.5 min. B: the corresponding mass spectra of peaks 1 and 2.
Fig. S4. Time course of the NphT7-catalyzed reaction. A: under bisubstrate conditions. B: under monosubstrate conditions. Peaks: 1, CoA; 2, acetoacetyl-CoA; 3, malonyl-CoA; and 4, acetyl-CoA. Fig. 3 E and F were created based on these time courses.
Fig. S5. Michaelis-Menten kinetics of the NphT7 reaction. Lineweaver-Burk plots are inserted. A, Michaelis-Menten kinetics (for acetyl-CoA) under bisubstrate conditions. B, Michaelis-Menten kinetics (for malonyl-CoA) under bisubstrate conditions. C, Michaelis-Menten kinetics under mono substrate conditions.
**Fig. S6.** Claisen condensations catalyzed by the decarboxylative condensing enzymes.
### Table S1. NphT7 homologs retrieved by BLAST search

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### Table S2. Primer sequences used in this study

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