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Substrate Profile Analysis and ACP-Mediated Acyl Transfer in *Streptomyces coelicolor* Type III Polyketide Synthases

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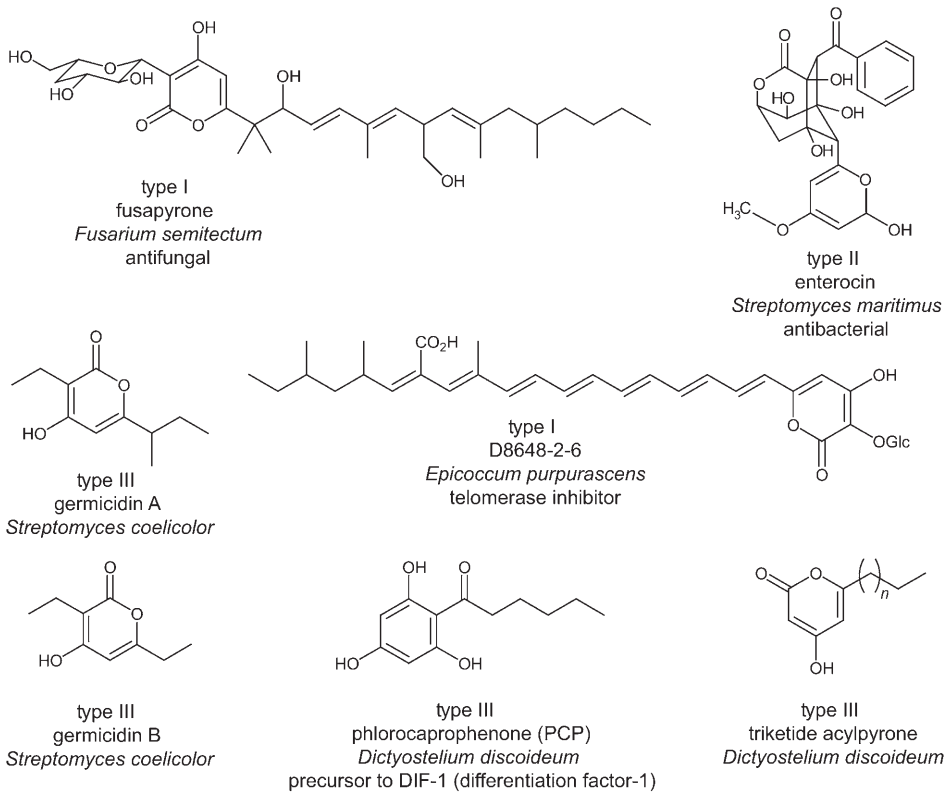
Polyketides represent a diverse family of natural products synthesized by bacteria, fungi, plants, and animals, and have found extensive use as pharmaceuticals (e.g., antimicrobials, antivirals, and antineoplastics) and other commercially important products (e.g., food ingredients, nutraceuticals, and pigments).[1–4] Although naturally occurring polyketides have diverse structures, the majority are produced by three broad classes of polyketide synthases (PKSs) that share a common mechanism involving sequential decarboxylative condensation reactions to form C–C bonds between simple carboxylic acid extender units and the nascent acyl chain.[4] Whereas the vast majority of studies have focused on the modular and iterative type I and iterative type II PKSs, recent work has led to a new appreciation for the homodimeric type III PKSs[5–7] that give rise to a range of aromatic compounds, including flavonoids and chalcones as well as some α-pyrone acylketides. Here we report the potential for removing type III PKSs from their natural biosynthetic context and adapting them to accept unnatural substrates in order to access a more structurally diverse chemical space. In addition, we show the remarkable ability of one *Streptomyces coelicolor* type III PKS to accept efficiently an acyl-ACP as its starter unit.

Pyrone-containing natural products exhibit a wide range of biological activities; this is a consequence of their structural diversity (Scheme 1). Pyrones are synthesized by PKSs through successive condensations of malonyl-CoA derived C_2 units followed by lactonization. The fungal metabolites fusapyrone[8] and D8646-2-6[9] are most likely derived from iterative type I PKSs, and enterocin[10] is produced by a type II PKS. On the other hand, germicidins A, B, and C,[11] as well as the signaling molecule precursor, phlorocaprophenone,[12] have been shown to be produced by PKS proteins from the type III family.



Scheme 1. Structures of α-pyrone metabolites and closely related molecules from type I, type II, and type III polyketide synthases.

Type III PKSs differ from type I and type II PKSs in several key aspects. First, they function as homodimers in which each subunit consists of a single domain of ~40 kDa. Additionally, rather than utilizing substrates that are covalently linked to acyl-carrier proteins (ACP), they are thought to mainly use acyl-coenzyme A (acyl-CoA) thioesters as substrates. Two notable exceptions have recently been reported and will be discussed below. Furthermore, the same active site that catalyzes the Claisen condensations is also responsible for loading of the starter unit and cyclizing the polyketide intermediate. Finally, type III PKSs have demonstrated a relaxed substrate specificity, and have been shown to convert non-native acyl-CoA substrates to pyrones in vitro.[13–15] Taken together, these characteristics make type III PKSs ideal candidates for engineering of ar-

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tificial, enzyme-based systems for the creation of diverse pyrone-containing small molecules.

Streptomyces coelicolor hosts a wide array of genes that are homologous to various types of natural-product biosynthetic enzymes, including type I modular PKSs, type II PKSs, type III PKSs, nonribosomal peptide synthases, sesquiterpene synthases, siderophore synthetases, among others.[16] Based upon proteins predicted to be encoded by its genome, *S. coelicolor* is thought to have the genetic capacity to produce more than 20 natural products.[17] Though some of the physiological roles for these gene clusters remain unclear, we sought to harness the rich potential of the type III PKSs to develop a method to create novel small molecules. Of the three type III PKS clusters found in *S. coelicolor* (see the Supporting Information), tetrahydroxynaphthalene synthase (THNS) has been extensively studied; its homologue from *Streptomyces griseus* was the first bacterial type III PKS to be identified and biochemically characterized.[18,19] THNS is known to catalyze the sequential decarboxylative condensation, intramolecular cyclization, and aromatization of an oligoketide derived from five units of malonyl-CoA to give 1,3,6,8-tetrahydroxynaphthalene (THN), which spontaneously oxidizes to flaviolin. In addition, the kinetics of THN formation and the crystal structure of this enzyme have been reported.[20,21] Based on previous studies that revealed the substrate tolerance of THNS, we explored this enzyme for the biosynthesis of novel small molecules.[15,22] However, THNS prefers malonyl-CoA as a building block for both initiation and extension, which means that pyrone formation is secondary to flaviolin synthesis. Hence, we sought other type III PKSs the starter unit specificity of which were orthogonal to extender unit preference and would, therefore, not compete with malonyl-CoA.

At the outset of our investigations, proteins from two type III PKS genes, *sco7221* and *sco7671* had not yet been associated with the production of known metabolites. Recently, the role for SCO7221 was reported and the type III PKS was designated Gcs to reflect its role in generating the germicidin series of natural product pyrones (Scheme 1).[11] The physiological role for SCO7671 and its corresponding metabolite remain unidentified. In this study, we describe our investigation of the function, substrate specificity, and product profile of Gcs and SCO7671, two type III PKSs from *S. coelicolor*, including the notable ability of Gcs to accept an acyl-ACP as a starter unit.

Though the number of characterized bacterial type III PKSs continues to grow, there are still numerous family members with potentially novel activities yet to be described.[7] As expected, the amino acid sequence similarities of bacterial type III PKSs compared to other bacterial and plant chalcone-like synthases were found to be in the 20–35% range (see the Supporting Information). Analysis of these sequences revealed that SCO7671 is most closely related to a family of mycobacterial type III PKSs that includes PKS11 and PKS10. PKS11 has been studied in vitro, and found to produce 6-alkyl-4-hydroxy-2-pyrones in the presence of fatty acyl-CoAs.[23] PKS10 has been shown to play an as yet unidentified role in the biosynthesis of the cell wall lipid component dimycocerosyl phthiocerol—an important factor in the pathogenesis of *Mycobacterium tuberculosis*.[24] The only other known enzyme with significant similarity to Gcs was found in the pathogen *Streptomyces scabies* (http://www.sanger.ac.uk), which showed 86% sequence identity at the amino-acid level. However, no physiological or metabolic role has been reported for this type III PKS homologue.

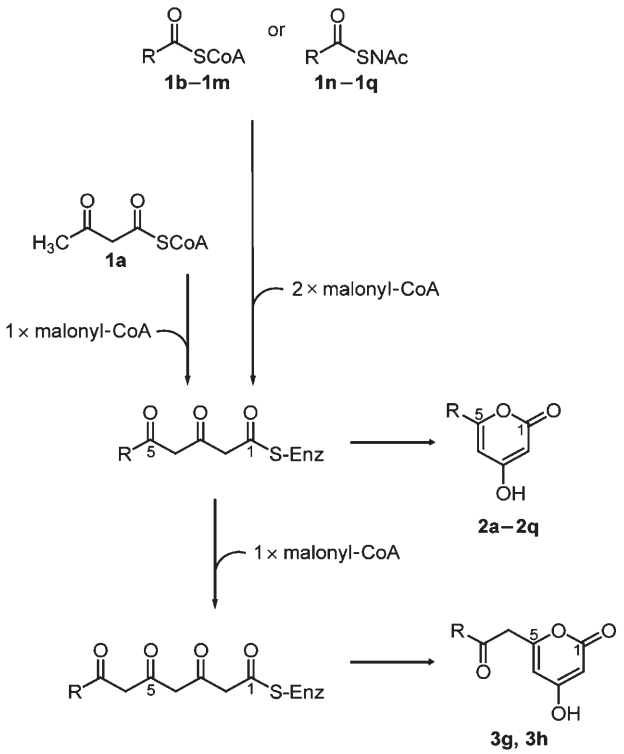
To confirm the presence of type III polyketide synthase catalytic activity in the purified proteins, both type III PKS genes *sco7221* (Gcs) and *sco7671* (SCO7671) were cloned into the pET-28b(+) expression vector, and the expressed proteins were purified in one step on Ni–NTA resin. Elution of the individual polypeptides from a size-exclusion column (see the Supporting Information) was consistent with a dimeric form for both enzymes. Next, acyl-CoA substrates were tested for their ability to function as priming units in the presence of malonyl-CoA extender units, and the yields of the individual reactions were determined (Scheme 2 and Table 1). For these studies, acyl-CoA (0.2 mM) starter unit, [2-^{14}C]malonyl-CoA (0.5 mM), and enzyme (4 μM) were incubated for 90 min at 30 °C before extraction with ethyl acetate followed by separation and visualization by using radio-TLC.

Table 1. Product yields for Gcs- and SCO7671-catalyzed reactions, in vitro.

	Starter unit	Pyrone	Percentage yield[a] Gcs	SCO7671
1a	acetoacetyl-CoA	**2a**	70–91	44–54
1b	acetyl-CoA	**2b**	–	trace
1c	propionyl-CoA	**2c**	1.2–1.8	4.2–7.4
1d	butyryl-CoA	**2d**	11–14	40–45
1e	hexanoyl-CoA	**2e**	36–40	55–70
1f	octanoyl-CoA	**2f**	1.0–1.1	26–34
1g	decanoyl-CoA	**2g**	–	2.2–5.5
		3g		0.9–2.1
1h	lauroyl-CoA	**2h**	–	trace
		3h		0.9–3.3
1i	benzoyl-CoA	**2i**	–	25–27
1j	*iso*-butyryl-CoA	**2j**	–	13–17
1k	malonyl-CoA	**2k**	–	trace
1l	(*R,S*)-3-hydroxy-3-methylglutaryl-CoA	**2l**	–	–
1m	(*R,S*)-β-hydroxybutyryl-CoA	**2m**	4.5–8.9	0.5–2.6
1n	(*R,S*)-β-hydroxybutyryl-SNAc	**2n**	1.9–2.0	0.4–0.5
1o	(*R*)-β-hydroxybutyryl-SNAc	**2o**	1.0–1.1	0.6–0.6
1p	(3*R*,2*S*)-hydroxy-2-methylvaleryl-SNAc	**2p**	0.9–1.3	–
1q	(3*R*,2*R*)-hydroxy-2-methylvaleryl-SNAc	**2q**	–	–

[a] The values shown are the ranges from at least two experiments. Yields are relative to the amount of starter unit and were determined by quantification of the amount of incorporated [^{14}C]-label and corrected for the number of extensions; yields refer to the triketide pyrones **2a**–**q** unless indicated otherwise.

UV spectroscopy and LC-ESI mass spectrometry results suggested the triketide pyrone product structures **2a**–**2p** (Scheme 2). Rigorous structural identification was performed for specific compounds by using NMR spectroscopy (see the Supporting Information). Pyrones with aliphatic C6-substituents exhibited characteristic UV absorption at 285–290 nm; the absorption peak shifted to 318 nm for the phenylpyrone, **2i**. In SCO7671-catalyzed reactions that were primed with the long-



Scheme 2. Reaction scheme for the formation of tri- and tetraketide pyrones; see Table 1 for R groups.

chain fatty acyl-CoA compounds, **1g** and **1h**, the corresponding tetraketide pyrones, **3g** and **3h**, were formed in addition to the triketide pyrone products, **2g** and **2h**. This assignment was based on UV absorption characteristics and the relative retention times on TLC and HPLC in comparison to previously characterized tri- and tetraketide pyrones.

Interestingly, no products (or only trace amounts) were observed upon incubation of enzyme with malonyl-CoA (**1k**) alone. The preferred starter unit substrates for Gcs were C_4–C_6 acyl-CoA esters, and the 3-oxo compound acetoacetyl-CoA gave the highest yield at 80% (Table 1). SCO7671 exhibited a broader substrate profile than Gcs and efficiently converted substrates with chain lengths between C_4 and C_8 to a series of pyrones **2d**–**f**. One striking difference in acyl chain specificity was found in the utilization of the α-branched acyl substrates *iso*-butyryl- and benzoyl-CoA, which were converted by SCO7671 in good yields but were not accepted by Gcs. The 26% product yield for phenylpyrone **2i**, which was obtained with SCO7671, is remarkable given that THNS processes benzoyl-CoA **1i** inefficiently.[15,18]

As with other PKSs,[25] Gcs and SCO7671 were also capable of utilizing *N*-acetylcysteamine (SNAc) thioesters in place of CoA derivatives. As shown through the comparison of racemic β-hydroxybutyryl-CoA (**1m**) with its SNAc equivalent (**1n**), the yields were between two- and tenfold lower for the latter without an effect on the product profile. The capability of employing SNAc esters as substrates facilitates the expansion of the starter unit repertoire as they are readily accessible by using synthetic or enzymatic approaches.[26–28]

Tetraketide pyrones (**3g** and **3h**) were only observed when SCO7671 was primed with decanoyl- or lauroyl-CoA. This observation has been noted in at least two other bacterial type III PKSs. When the substrate specificity of PKS18 from *M. tuberculosis* was probed, it was found that larger substrates could give rise to a preference for additional rounds of extension.[29] In addition, the octanoyl-CoA primed THNS reaction resulted in a hexaketide product.[18] Access to an alternative binding tunnel that can accommodate long fatty acid chains has been suggested as an explanation for these data. Evidence for this tunnel can be seen in the PKS18 crystal structure,[29] and appears to result from subtle changes near the substrate-binding site. Sequence similarities are too low to accurately predict an analogous tunnel in SCO7671, but one would expect its presence based upon current biochemical data.

From the results described above it is evident that Gcs and SCO7671 type III PKSs can accept acyl-CoA starter units, extend them by one to three malonyl-CoA equivalents, and cyclize the linear polyketide to form pyrone natural products. However, recent reports led us to challenge the traditional view that all type III PKSs must exclusively use acyl-CoA starter units. Based upon sequence alignments, Austin and Noel suggested that perhaps bacterial type III PKSs, and not plant type III PKSs, might be able to accept starter units from ACPs.[5] Evidence for this prediction was obtained when the structure of a C-terminal type III PKS domain from *Dictyostelium discoideum* was reported.[12] In this system, the iterative type III PKS (Steely 1) is comprised of a much larger polypeptide that includes upstream type I fatty acid synthase (FAS) domains. One further suggestion came from the recent work of Challis and colleagues on the physiological role for Gcs.[11] Specifically, a fatty acid metabolite, 3-oxo-4-methyl-pentyl-ACP was proposed as the priming unit due to the role of this precursor as an intermediate in fatty-acid biosynthesis. This complex starter unit is extended only once by Gcs in vivo with an ethylmalonyl thioester to produce germicidin. It is possible that these two PKSs might accept either the ACP-tethered acyl chain or the acyl-CoA after offloading from the ACP. We, therefore, sought definitive biochemical evidence for acyl-ACP transfer in type III PKSs.

We designed a series of experiments to assess the ability of Gcs and SCO7671 to accept an acyl starter unit directly from a charged ACP. In particular, we chose a test set of four discrete ACPs that varied with respect to their biosynthetic context, and would be amenable to expression in *E. coli* in apo form. As hexanoyl-CoA gave good conversion efficiencies with both Gcs and SCO7671 *S. coelicolor* type III PKSs, this starter unit was used as a model substrate for the acyl-ACP transfer reactions. ACPs require post-translational modification of a conserved serine residue with the coenzyme A-derived phosphopantetheinyl (P-pant) group in order to form the requisite acyl-thioester linkage. The ACP synthases Sfp and Svp (from surfactin biosynthesis in *Bacillus subtilis* and bleomycin biosynthesis in *Streptomyces verticillus* ATCC15003, respectively) have been shown to convert a variety of noncognate ACPs from secondary metabolism to the holo form, both in vivo and in vitro.[30,31] Furthermore, these enzymes are able to convert apo-ACPs directly to their acylated holo forms in vitro when acyl-

CoA instead of coenzyme A is used as substrate. Conversion of the four apo-ACPs MmcB (mitomycin biosynthesis),[32] TcmM (tetracenomycin biosynthesis),[33] Otc (oxytetracycline biosynthesis),[34] and SCOACP (*sco0549*, lipid metabolism)[16] into their acylated holo forms (acyl-ACP) was rigorously established by using electrospray mass spectrometry (see the Supporting Information). Following in vitro acylation, unreacted hexanoyl-CoA was eliminated by utilizing the His-tags on the ACPs. After elution from the resin, proteins were dialyzed into Bis-Tris buffer at pH 6.8 to limit hydrolysis of the thioester bond. MmcB and Otc were efficiently converted to their corresponding hexanoyl holo forms by using Sfp, whereas TcmM and SCOACP were poor substrates for this ACP synthase, and only yielded 10% and 30% acyl-ACP, respectively. However, when using Svp as the phosphopantetheinyl transferase all apo-ACP was converted to acyl-ACP. ESI-MS enabled estimation of the relative abundance of acylated species (see the Supporting Information).

ACP mutants were prepared by using primer-directed mutagenesis to replace the active-site serine (phosphopantetheine attachment site) with alanine. This ensured that transfer would result directly from the acyl-ACP and not unreacted acyl-CoA from the holo-ACP reaction. These ACP(Ser→Ala) mutants underwent mock phosphopantetheine modification in parallel to their corresponding native ACPs, which served as negative controls. Purification of the acyl-ACPs and ACP(Ser→Ala) mutants was performed by binding the His-tagged fusion proteins to Ni–NTA resin and thoroughly washing the resin with buffer. It was found that 50-resin volumes of wash buffer were required to eliminate carry-over of hexanoyl-CoA into the type III PKS assay as determined by the presence or absence of product in the ACP(Ser→Ala)-mutant containing reactions.

To perform the type III PKS conversion assay, equal amounts of hexanoyl-ACP, ACP(Ser→Ala) mutant, or hexanoyl-CoA (10 μM) were incubated with Gcs or SCO7671 (2 μM) in the presence of [^{14}C]malonyl-CoA (80 μM) as extender unit. Reaction products were analyzed by using radio-TLC (Figure 1). A single product corresponding to **2e** was observed in all hexanoyl-ACP primed reactions. For SCO7671 the amount of pyrone formed corresponded to approximately 10% of the amount obtained with hexanoyl-CoA. No significant difference between the individual ACPs was observed. In contrast, Gcs converted the hexanoyl-ACPs relatively efficiently. The reaction with the highest yield was with hexanoyl-TcmM as substrate and resulted in approximately 50% pyrone compared to the hexanoyl-CoA-primed reaction. The other tested hexanoyl-ACPs resulted in approximately 25% yield in pyrone compared to hexanoyl-CoA. A complete biochemical study comparing the K_m and k_{cat} values between acyl-CoA and acyl-ACPs has yet to be completed for the hexanoyl series of starter units with these enzymes. However, to the best of our knowledge, this is the first time that acyl transfer from ACP to type III PKS has been demonstrated in vitro. Furthermore, the ability of the type III PKSs used in this work to perform thioacyl transfer provides evidence that the enzyme is capable of accepting very large macromolecular substrates as acyl carriers.

In conclusion, we selected type III PKSs as the architecturally most simple system capable of forming a pyrone functional group to investigate the potential for removing these enzymes from their natural biosynthetic context and adapting them to

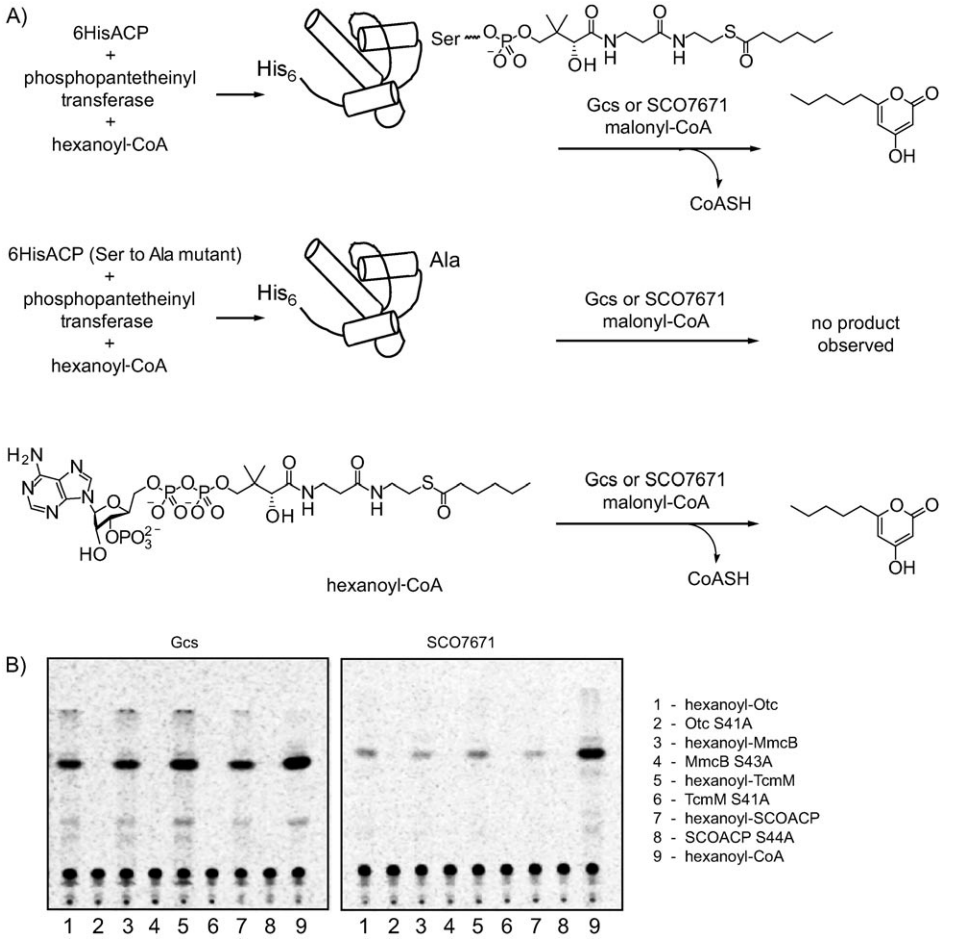


Figure 1. A) ACP-transfer assays: reaction mixtures contained starter ACP or starter CoA (10 μM), [2-^{14}C]malonyl-CoA (80 μM), type III PKS (2 μM), and were incubated for 40 min before reactions were stopped by addition of hydrochloric acid. B) Radio-TLC analysis of products obtained from Gcs- or SCO7671-catalyzed reactions. Relative to hexanoyl-CoA, percentage conversions for Gcs after 40 min incubation were: Otc 25%, MmcB 25%, TcmM 50%, SCOACP 25%, CoA 100%.

accept unnatural substrates. Studies on the substrate tolerance and product profile of two type III PKSs (Gcs and SCO7671) from *S. coelicolor* revealed that both enzymes are capable of converting a range of substrates into α-pyrones with moderate to good yields. Our search for additional methods to deliver complex starter units has provided definitive biochemical evidence that Gcs can utilize charged ACPs as substrates. To our knowledge, this is the first direct demonstration that acyl-ACPs are suitable for presenting starter units to type III PKSs, and it is consistent with recent in vivo studies[11,12] that suggest that acyl-ACP transfer occurs in select prokaryotic and eukaryotic type III PKS systems. Futhermore, it is notable that in vitro Gcs is capable of utilizing acyl groups carried by either the small molecule, acyl-CoA, or the small protein, ACP.

Experimental Section

Bacterial strains and chemicals: [2-^{14}C]Malonyl-CoA (55 mCi mmol^{-1}) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and was diluted with unlabeled malonyl-CoA (10 mM) to give a concentration of 5 mM for use in all reactions. All acyl-CoAs were purchased from Sigma. All reagents were used without further purification. *E. coli* strains DH5α and BL21(DE3) from Novagen were used as hosts for cloning and gene expression, respectively.

Acylation of ACPs: A typical reaction mixture contained apo-ACP or ACP(Ser→Ala) mutant (30 μM), Sfp (1 μM for MmcB and Otc) or Svp (1 μM for TcmM or scoACP), hexanoyl-CoA (0.3 mM), $MgCl_2$ (10 mM), Tris buffer (0.1 M, pH 8.1) in a total volume of 0.6 mL. After incubation at 30 °C for 45 min Ni–NTA (60 μL) resin was added, and the proteins were allowed to bind at 4 °C for 30 min with gentle agitation. The resin was washed with lysis buffer (3.2 mL; 250 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole, pH 7.9) and the proteins were eluted with elution buffer (10% glycerol, 0.2 M EDTA, pH 8.0, 0.1 M NaCl, 50 mM bis-Tris, pH 6.8). Purified ACPs were dialyzed, overnight, at 4 °C into dialysis buffer (10% glycerol, 0.1 M NaCl, 50 mM bis-Tris, pH 6.8) by using Slide-A-Lyzer (Pierce, MWCO 3500) minidialysis units. Protein concentrations were determined by using the Bio-Rad Bradford protein assay with BSA as the standard. Hexanoyl-ACPs were analyzed with electrospray mass spectrometry by using a ThermoFinnigan LTQ linear ion trap instrument (capillary temperature 250 °C, capillary voltage 32 V, tube lens 95 V). Mass spectra were deconvoluted by using ProMass™ for Xcalibur (Novatia, Princeton, USA).

Type III PKS starter unit assays: To test for substrate specificity and determine yields, type III PKS (4 μM in 0.1 M HEPES, pH 7.4) was preincubated at 30 °C for 5 min. The reaction was started by adding acyl-CoA (0.2 mM) and [2-^{14}C]malonyl-CoA (0.5 mM), and stopped after 90 min by adding concentrated hydrochloric acid (20%, *v/v*). Reaction products were resolved by using TLC (silica 60 F_{354}; Merck) with formic acid (1%) in dichloromethane/methanol (92:8) as mobile phase. The TLC plate was exposed to a phosphoimager screen, overnight, alongside [2-^{14}C]malonyl-CoA standards of known concentration. Radioactivity was quantified by using ImageQuant TL v2003 software (Amersham Biosciences).

Type III PKS ACP-transfer assays: Hexanoyl-ACP primed reactions were performed in dialysis buffer with the pH adjusted by addition of Tris buffer, pH 8.1 (40 mM final concentration) in a total volume of 50 μL. The reaction mixture contained type III PKS (2 μM in 0.1 M HEPES, pH 7.4), [2-^{14}C]malonyl-CoA (80 μM), and hexanoyl-ACP (10 μM). As negative controls, hexanoyl-ACPs were replaced with ACP(Ser→Ala) mutants that had been carried through the acylation reaction as described above. Reactions that contained hexanoyl-CoA (10 μM) were used as positive control. Samples were incubated at 30 °C for 40 min, and products were extracted and analyzed by radio-TLC as described above.

Synthesis of (*R*,*S*)-*N*-acetylcysteaminyl-3-hydroxybutyrate (1 n) and (*R*)-*N*-acetylcysteaminyl-3-hydroxybutyrate (1 o): In a typical procedure, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; 1.1 equiv), 4-dimethylaminopyridine (DMAP; 0.2 equiv) and SNAc (1.1 equiv) were added to a stirred solution of 3-hydroxybutyric acid (1 equiv) in CH_2Cl_2 (5 mL) at 23 °C. The reaction was stirred for 18 h and then concentrated under reduced pressure. Flash chromatography (silica, 2% MeOH in CH_2Cl_2) afforded the title compounds **1 n** (43 mg, 21%) and **1 o** (136 mg, 89%) as yellow oils. $R_f = 0.15$ (silica, 5% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta =$ 1.24 (d, $J = 6.2$ Hz, 3H), 1.97 (s, 3H), 2.70–2.74 (m, 2H), 3.02–3.07 (m, 2H), 3.42–3.49 (m, 2H), 4.21–4.29 (m, 1H), 5.79 ppm (br s, 1H); MS (ESI, +ve): $m/z = 228.0$ $[M+Na]^+$ ($C_8H_{15}NNaO_3S$ requires 228.1).

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