

# Chemoenzymatic Synthesis of Acyl Coenzyme A Substrates Enables *In Situ* Labeling of Small Molecules and Proteins

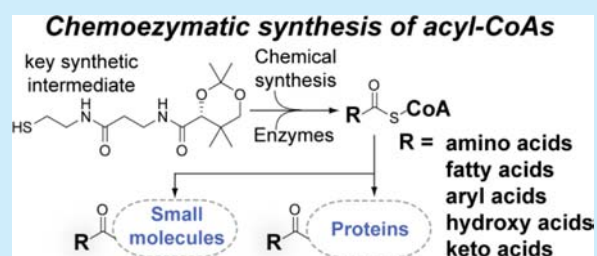
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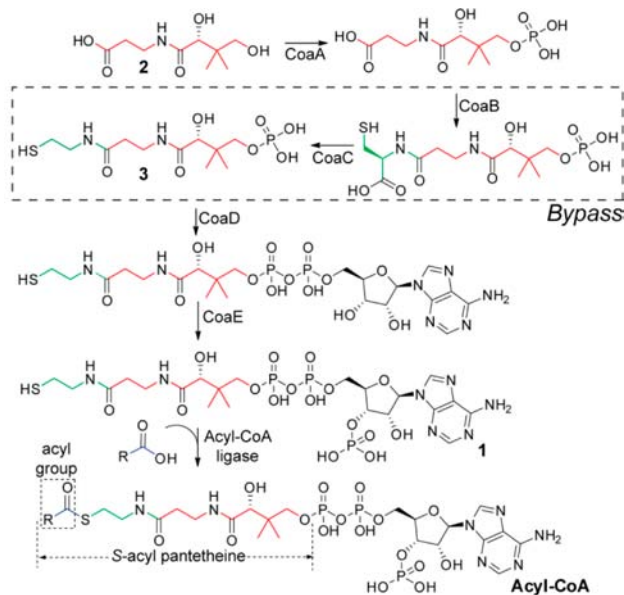
**S** Supporting Information

**ABSTRACT:** A chemoenzymatic approach to generate fully functional acyl coenzyme A molecules that are then used as substrates to drive *in situ* acyl transfer reactions is described. Mass spectrometry based assays to verify the identity of acyl coenzyme A enzymatic products are also illustrated. The approach is responsive to a diverse array of carboxylic acids that can be elaborated to their corresponding coenzyme A thioesters, with potential applications in wide-ranging chemical biology studies that utilize acyl coenzyme A substrates.



In biological chemistry, coenzyme A (CoA, **1**, [Scheme 1](#)) acts as a molecular shuttle for carboxylic acids linked to its terminal thiol. S-Acylated derivatives of **1** (acyl-CoAs, [Scheme 1](#)) participate in numerous enzymatic reactions, including primary energy metabolism, synthesis of biomolecules, post-translational modification of proteins, and other processes.

**Scheme 1. Natural Route for Biosynthesis of Acyl-CoAs<sup>a</sup>**



<sup>a</sup>Reactions catalyzed by CoaB and CoaC can be bypassed by synthetically appending the  $\beta$ -mercaptoethylamine moiety to **2**.

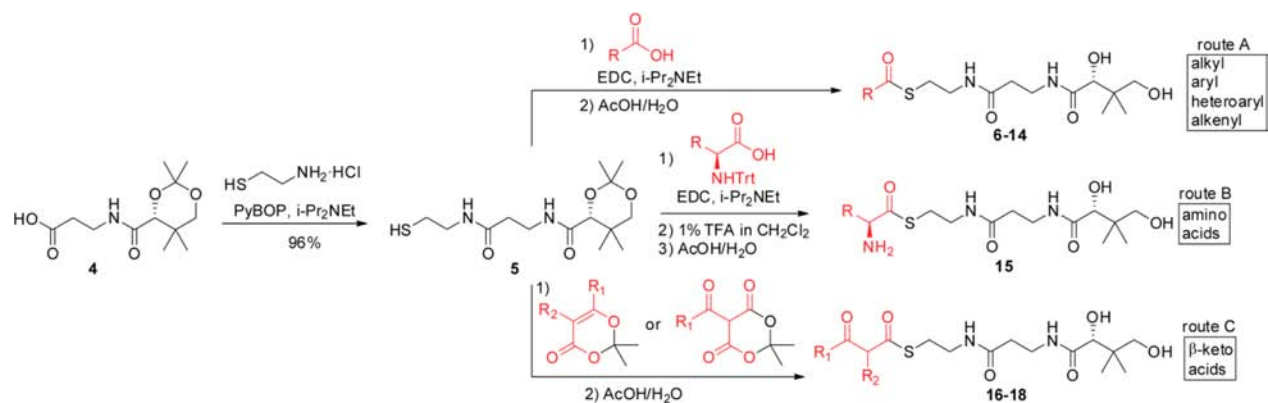
Acyl-CoAs are also preeminent in their participation in secondary metabolism, such as in the biosynthesis of polyketide and nonribosomal peptide antibiotics.<sup>1</sup> In each of the above-mentioned applications, the reactive thioester moiety of acyl-CoA is of primary importance, activating the attached carboxylic acid for participation in downstream enzymatic processes.

As shown in [Scheme 1](#), **1** is biosynthesized in bacteria via the action of the five enzymes CoaA–E.<sup>2</sup> Pantothenic acid (**2**, vitamin B5) is converted into phosphopantetheine (**3**) by the action of three enzymes (CoaA–C). Further adenylation, followed by phosphorylation of the ribose 3'-hydroxyl, affords **1**. Carboxylic acids are thioesterified to **1** by the action of ATP-dependent acyl-CoA ligases to afford acyl-CoAs. While a wide diversity of acyl-CoA ligases have been discovered, their limited substrate promiscuity has restricted their development as synthetically useful catalysts. As a result, the traditional synthesis of acyl-CoAs has relied on the chemical ligation of carboxylic acids to **1** with the aid of peptide coupling reagents to generate the thioester linkage. However, this approach suffers from several drawbacks, including the high cost of **1** as the starting material, poor yields for the coupling step, and labor-intensive HPLC-based purification. Acyl-CoAs are also known for their inherent instability by virtue of the chemically labile thioester and phosphoester linkages in the molecule. In the course of our previous studies<sup>3</sup> requiring the preparation of acyl-CoA substrates, we encountered these problems, often representing significant challenges. These difficulties prompted

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Scheme 2. Synthetic Strategy for the Preparation of S-Acyl Pantetheine Derivatives

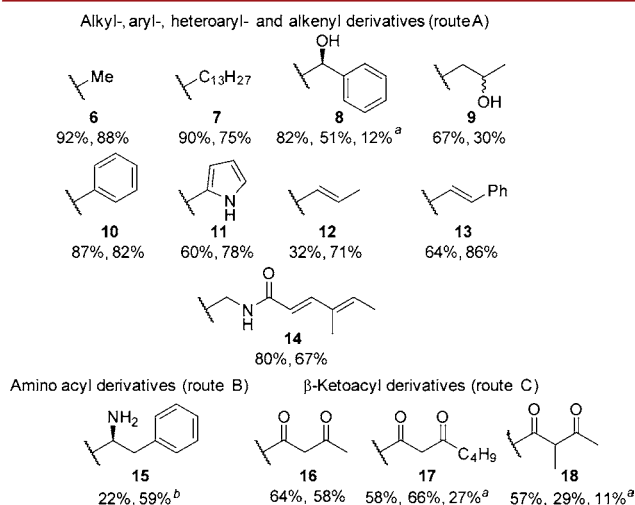


us to seek an alternative and more convenient access to acyl-CoAs.

We herein report a straightforward and efficient methodology for the preparation of a wide range of acyl-CoAs. Our strategy relies on the combination of an initial chemical preparation of S-acylated pantetheine derivatives, followed by a one-pot enzymatic transformation into the desired acyl-CoA end product. We also establish a mass spectrometric assay to characterize acyl-CoA products. Furthermore, we demonstrate the applicability of chemoenzymatically generated acyl-CoAs as acyl donors in acyl-transfer reactions.

Begley and co-workers have shown that *Escherichia coli* CoaA, CoaD, and CoaE enzymes are promiscuous, in that they can accept substrates modified either at the carboxy terminus of **2** or at the cysteamine moiety of **3**.<sup>4</sup> Burkart and co-workers reported that this observation allows for an access route to amide and ester analogues of acyl-CoAs that relies on bypassing the first enzymatic steps (Scheme 1) by employing appropriately designed derivatives of **2** that can be extended by CoaA, CoaD, and CoaE enzymes.<sup>5</sup> Despite these advances, it should be noted that functional acyl-CoAs with a thioester linkage that are capable of acting as physiological acyl-donors have not been previously elaborated. We have chosen a similar strategy to efficiently prepare thioester linked acyl-CoA derivatives by a chemoenzymatic route.

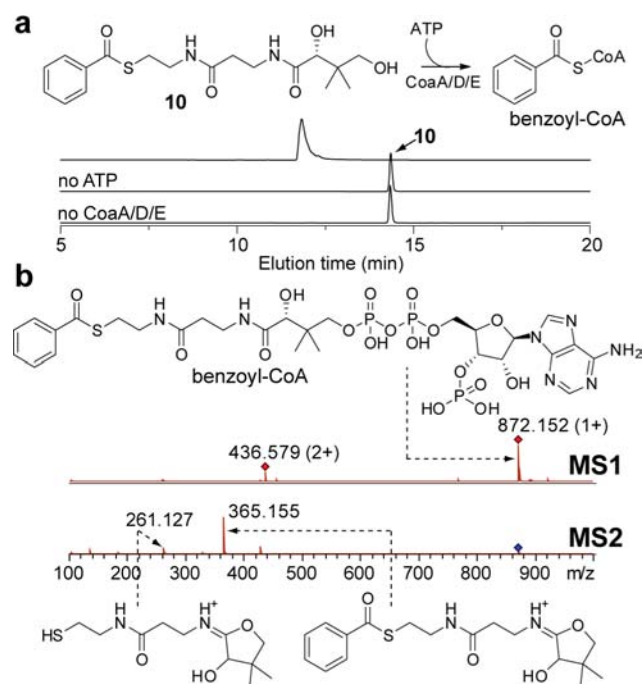
We first sought to identify an efficient synthetic strategy for the preparation of functionally diverse S-acyl pantetheines. As shown in Scheme 2, we chose acetonide **4** as a starting point.<sup>6</sup> Peptide coupling of cysteamine to **4** delivered thiol **5** in 96% yield.<sup>7</sup> Intermediate **5** can now be used to attach a variety of carboxylic acids (Figure 1) to the thiol headgroup elaborating the reactive thioester linkage. For alkyl, aryl, heteroaryl, and alkenyl carboxylates, we identified an EDC-based protocol as the most efficient and general.<sup>8</sup> In particular, some challenging substrates were successfully coupled to **5** in synthetically useful yields. These include crotonic acid (**12**),<sup>9</sup> pyrrole carboxylic acid (**11**), and  $\alpha$ - and  $\beta$ -hydroxyacids (**8**, **9**) (Figure 1). Subsequent removal of the acetonide protecting group proceeded smoothly using AcOH/H<sub>2</sub>O (route A, Scheme 2).<sup>10</sup> This mild deprotection protocol did not affect sensitive functional groups, such as  $\beta$ -hydroxy carbonyls (**9**) or alkenes (**12–14**).<sup>11</sup> Furthermore, no HPLC purification step was needed for most products. For the synthesis of an aminoacyl-S-pantetheine derivative, we found that a trityl-protected precursor was the most convenient among several tested.<sup>12</sup> Despite the lower coupling yield, mild conditions were sufficient for the additional deprotection step required to



**Figure 1.** Substrate scope for the synthesis of S-acyl pantetheine derivatives (R groups shown). Yields given correspond to the coupling reaction (first yield) followed by the deprotection (second yield) as outlined in Scheme 2. <sup>a</sup>Yields following HPLC purification. <sup>b</sup>Combined yield for both deprotection steps.

access this compound class (**15**, 1% TFA in CH<sub>2</sub>Cl<sub>2</sub>, route B, Scheme 2). Initial attempts to couple  $\beta$ -ketoacids to **5** remained unsuccessful. Gratifyingly, we were able to prepare derivatives **16–18** by reaction of **5** with masked ketoacids (route C, Scheme 2) in refluxing toluene.<sup>13</sup> Using this protocol, the corresponding  $\beta$ -keto thioesters were obtained in acceptable yields (Figure 1).

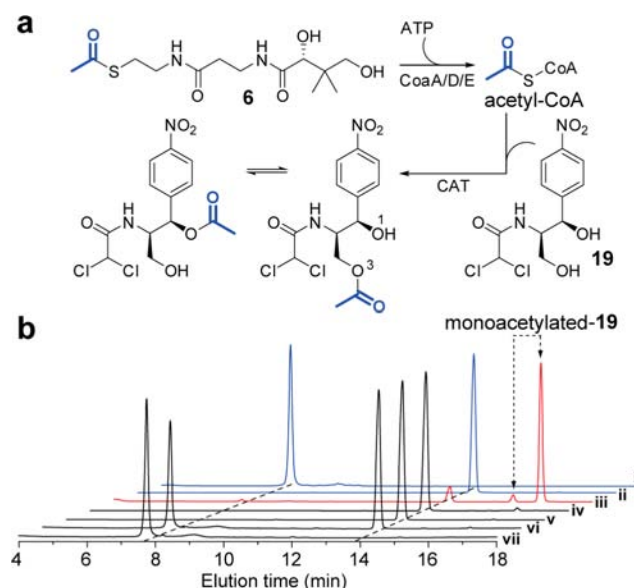
In order to elaborate pantetheine thioesters **6–18** into the corresponding acyl-CoAs, we next cloned, expressed, and purified recombinant *E. coli* CoaA, CoaD, and CoaE enzymes. Substrates **6–18** were assayed using a premixed enzyme cocktail of purified CoaA, CoaD, and CoaE as the catalyst. In addition to the substrate and the enzyme cocktail, the only other component that needed to be provided was freshly prepared ATP (see Supporting Information for detailed assay procedures). Using a 3-fold molar excess of ATP, the enzyme cocktail catalyzed the stoichiometric conversion of a 1000-fold molar excess of substrates to their corresponding acyl-CoA products in 3 h at 30 °C, while no conversion was observed in the absence of either ATP or the enzymes (Figure 2a). The identity of the enzymatically synthesized benzoyl-CoA (Figure 2a), generated using **10** as the substrate, was verified by NMR (see Supporting Information).



**Figure 2.** Enzymatic synthesis and characterization of acyl-CoAs starting from *S*-acyl pantetheine substrates. (a) Illustratively, HPLC analysis of reaction substrate **10** and the product benzoyl-CoA at 280 nm demonstrates that product formation is dependent on the presence of ATP and CoaA/D/E enzyme catalysts. (b) Mass spectrometric characterization of the enzymatic product benzoyl-CoA. In the MS1 spectra, dominant  $[M + H]^{1+}$  and  $[M + 2H]^{2+}$  ions (labeled with red diamonds) are observed that correspond to the molecular formula of benzoyl-CoA ( $C_{28}H_{40}N_7O_{17}P_3S$ ). Upon fragmentation of the  $[M + H]^{1+}$  ion, characteristic benzoyl-(cyclo)pantetheine and (cyclo)pantetheine MS2 product ions are observed. A proposed chemical route for the generation of the observed (cyclo)pantetheine MS2 product ions is shown in Figure S29.

Next, we developed a mass spectrometric assay to query the identity of the acyl-CoA products in order to circumvent their preparative isolation and NMR characterization. Guided by our previous reports describing ‘*phosphopantetheine ejection*’ MS2 product ions,<sup>14</sup> we observed characteristic acyl-(cyclo)pantetheine and (cyclo)pantetheine MS2 product ions upon fragmentation of the acyl-CoA  $[M + H]^{1+}$  parent MS1 ion (Figure 2b and Figures S1–S13). Note that the observation of the (cyclo)pantetheine MS2 ion is indicative of the thioester linkage present in the acyl-CoA enzymatic product. Modulation of MS/MS parameters demonstrated that, with increasing fragmentation energy, the abundance of the (cyclo)pantetheine MS2 product ion increased relative to that of the acyl-(cyclo)pantetheine ion (Figure S6).

Having verified the chemoenzymatic production of acyl-CoAs, we next verified their viability to perform their physiological roles, that is, to act as donors in acyl transfer reactions. To illustrate, we employed *chloramphenicol acetyltransferase* (CAT), an enzyme that catalyzes the acetylation of chloramphenicol (**19**), using acetyl-CoA as the acetyl donor (Figure 3a).<sup>14</sup> Starting from **6**, in a single-pot assay, we produced acetyl-CoA that was then used as a substrate by CAT to generate acetylated **19**. Two monoacetylated **19** products were observed (Figure 3b, trace iii), consistent with the slow noncatalytic transfer of the acetyl group from 3-acetyl-**19** to the 1-hydroxyl of **19**.<sup>15</sup> This then

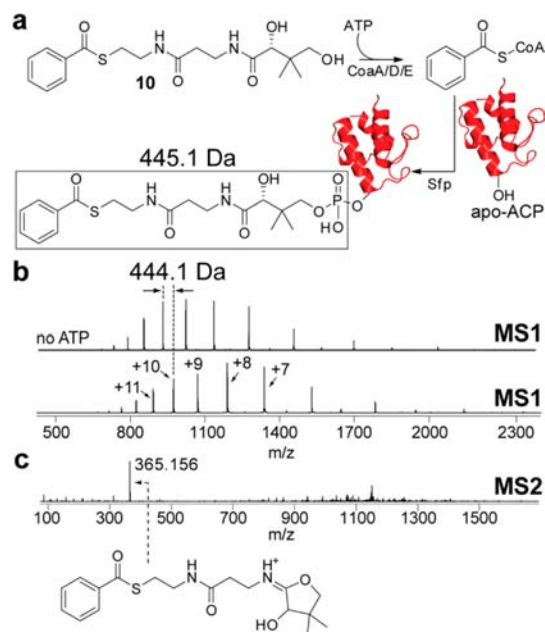


**Figure 3.** Chemoenzymatically synthesized acyl-CoAs are acyl donors for *in situ* labeling of small molecules. (a) Scheme for the conversion of **6** to acetyl-CoA, followed by the transfer of the acetyl group to **19**. (b) HPLC characterization at 280 nm of standards of acetyl-CoA (i) and **19** (ii) and the enzymatic reaction (iii) demonstrating the formation of two acetyl-**19** products (also see Figure S14). Absence of ATP (iv) or CoaADE enzymes (v) abolished acetylation of **19**, while absence of CAT (vi) or **19** (vii) led to the production of acetyl-CoA but no acetylated-**19** products.

facilitated a second acetylation event at the 3-hydroxyl position, leading to production of diacetylated **19** (Figure S14).

A second physiological role of **1** is to donate its phosphopantetheine moiety, such as in the conversion of apo-acyl carrier proteins (-ACPs) to their holo forms. Substrate promiscuity of the phosphopantetheinyl transferase Sfp, which allows for the transfer of the acyl-phosphopantetheine moiety from acyl-CoAs to generate acyl-ACPs, has been widely used to study assembly line biosynthesis of natural products, among several other biochemical transformations.<sup>16</sup> We next queried whether the chemoenzymatic acyl-CoA synthetic scheme described above can be used to drive *in situ* production of acyl-ACPs using Sfp.

Illustratively, in a single-pot reaction starting from **10** and apo-ACP as substrates, and CoaA/D/E and Sfp as catalysts (Figure 4a), we observed the ATP-dependent stoichiometric formation of benzoyl-S-ACP as the product. The mass difference between the MS1 ions observed in the absence and presence of ATP in the reaction correspond to the benzoyl-phosphopantetheine moiety that is transferred by Sfp (Figure 4b). Furthermore, fragmentation of the peptidyl MS1 ions demonstrated identical benzoyl-(cyclo)pantetheine MS2 product ions that we previously observed for the benzoyl-CoA enzymatic product (Figure 4c). Hence, without intermediary purification of the acyl-CoAs, we could achieve *in situ* labeling of protein substrates by acyl-CoAs. Additionally, each of the *S*-acyl pantetheine substrates **6–18** was responsive to CoaA/D/E-Sfp loading onto apo-ACP, thus underscoring the vast chemical space that can be explored by the above-mentioned methodology (Figures S15–S27). Furthermore, a preparative scale synthesis of acyl-ACP using the scheme described above, followed by purification by size exclusion chromatography,



**Figure 4.** *In situ* labeling of ACPs with enzymatically synthesized acyl-CoAs. (a) Reaction scheme for the conversion of **10** to benzoyl-CoA and concomitant transfer to an ACP molecule (in cartoon representation colored red). (b) LC/MS ESI-ToF characterization of benzoyl-ACP. As shown for the  $[M + 10H]^{10+}$  ion, the deconvoluted mass difference between apo-ACP (observed in the negative control reaction with no ATP added) and acylated-ACP corresponds to the benzoyl-phosphopantetheine moiety that is transferred to a conserved serine residue of the ACP (note that the difference of 1 Da between the masses is due to the serine side chain hydroxyl proton that is lost upon transfer). (c) Further conformation for ACP acylation is provided by the observation of the characteristic benzoyl-(cyclo)pentanethine MS2 product ion, identical to the MS2 product ion shown in Figure 2b.

yielded several milligrams of purified and fully acylated ACP product (Figure S28).

In conclusion, we have developed a workflow to generate a multitude of *S*-acyl pantetheine molecules from a key synthetic intermediate and demonstrated the enzymatic elaboration of structurally diverse acyl-pantetheines to fully functional acyl-CoA molecules that can participate in acyl transfer and acyl-phosphopantetheine transfer reactions to small molecules and proteins. The chemoenzymatic schemes, together with the mass spectrometry based assays described in this study, are expected to be broadly applicable in biochemical investigations involving acyl-CoA substrates and intermediates. In particular, biochemical and structural studies requiring large amounts of highly pure acyl-CoAs and acyl-ACPs will benefit from the methodology described herein.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02113.

Experimental details, synthetic schemes, and NMR spectroscopy and mass spectrometry data (PDF)

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### Author Contributions

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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