

In Vivo Modification of Native Carrier Protein Domains

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Carrier proteins are central to the biosynthesis of primary and secondary metabolites in all organisms. Here we describe metabolic labeling and manipulation of native acyl carrier proteins in both type I and II fatty acid synthases. By utilizing natural promiscuity in the CoA biosynthetic pathway in combination with synthetic pantetheine analogues, we demonstrate metabolic labeling of endogenous carrier proteins with reporter tags in Gram-positive and Gram-negative bacteria and in a human carcinoma cell line. The highly specific nature of the

post-translational modification that was utilized for tagging allows for simple visualization of labeled carrier proteins, either by direct fluorescence imaging or after chemical conjugation to a fluorescent reporter. In addition, we demonstrate the utility of this approach for the isolation and enrichment of carrier proteins by affinity purification. Finally, we use these techniques to identify a carrier protein from an unsequenced organism, a finding that validates this proteomic approach to natural product biosynthetic enzyme discovery.

Introduction

The carrier protein (CP) is central to the biosynthetic pathways of many important primary and secondary metabolites.^[1] These small proteins, found either as discrete polypeptides or as small domains within larger synthases, facilitate biosynthesis by acting as a scaffold for growing natural products. Due to their key role in the biosynthesis of fatty acids,^[2] polyketides,^[3] and nonribosomal peptides,^[4] methods for the specific labeling of CPs in vivo would be useful for visualizing the localization and dynamics of these biosynthetic lynchpins, as well as to facilitate the proteomic identification of CPs from unsequenced natural-product-producing organisms.

In CP-mediated biosynthesis, the growing acyl chain is linked to the enzyme through a thioester bond to the terminal thiol of a coenzyme A (CoA)-derived 4'-phosphopantetheine (4'-PPant) prosthetic group. This post-translational modification is introduced by a phosphopantetheinyltransferase (PPTase, E.C. 2.7.7.7), which transfers the moiety from CoA to a conserved serine of the apo-CP.^[5] The finding that many PPTases are capable of accepting functionalized CoA analogues as substrates offers an opportunity for selective labeling of CP domains with fluorescence and affinity reporters.^[6,7] However, in order for this approach to succeed, reporter-labeled CoA analogues must be available to the PPTase, or else the PPTase will utilize endogenous CoA to produce *holo*-CPs, which are not easily visualized or detected. Due to the highly charged nature of CoA and its analogues, which precludes their use in intracellular-labeling approaches,^[8] our previous work has focused on studying the ability of reporter-labeled CoA precursors to cross the cell membrane and be transformed into fully formed CoA analogues in vivo via the endogenous CoA biosynthetic pathway (Scheme 1).^[9,10] Past studies have applied similar metabolic delivery strategies to the labeling of post-translationally lipidated and glycosylated proteins.^[11–13] However, while our earlier studies were essential to the identification of potentially useful CoA precursors, this work was only explored in the context of a single Gram-negative bacterial organism, *Escherichia coli*, and

only proved capable of labeling CPs when they were heterologously co-overexpressed with a promiscuous PPTase.

Here we report a significant advance in CP-labeling methodology by describing for the first time the labeling of endogenous CP domains in native bacterial systems by azide-labeled CoA precursor **1**. We demonstrate its utility in both Gram-positive and Gram-negative bacterium, as well as in a number of knockout strains, which probe its requirements for uptake and PPTase type. We also explore the activity of this analogue in a human carcinoma cell line, and perform an in-depth analysis of labeling of the human FAS in this cell line by **2**, a previously reported fluorescent CoA precursor. Finally, to evaluate the utility of this method for proteomic identification of CP-containing enzymes from unsequenced organisms, we demonstrate its application towards affinity purification of CPs, as well as for the de novo sequencing and genetic identification of a fatty acid biosynthetic CP from an unsequenced bacterium.

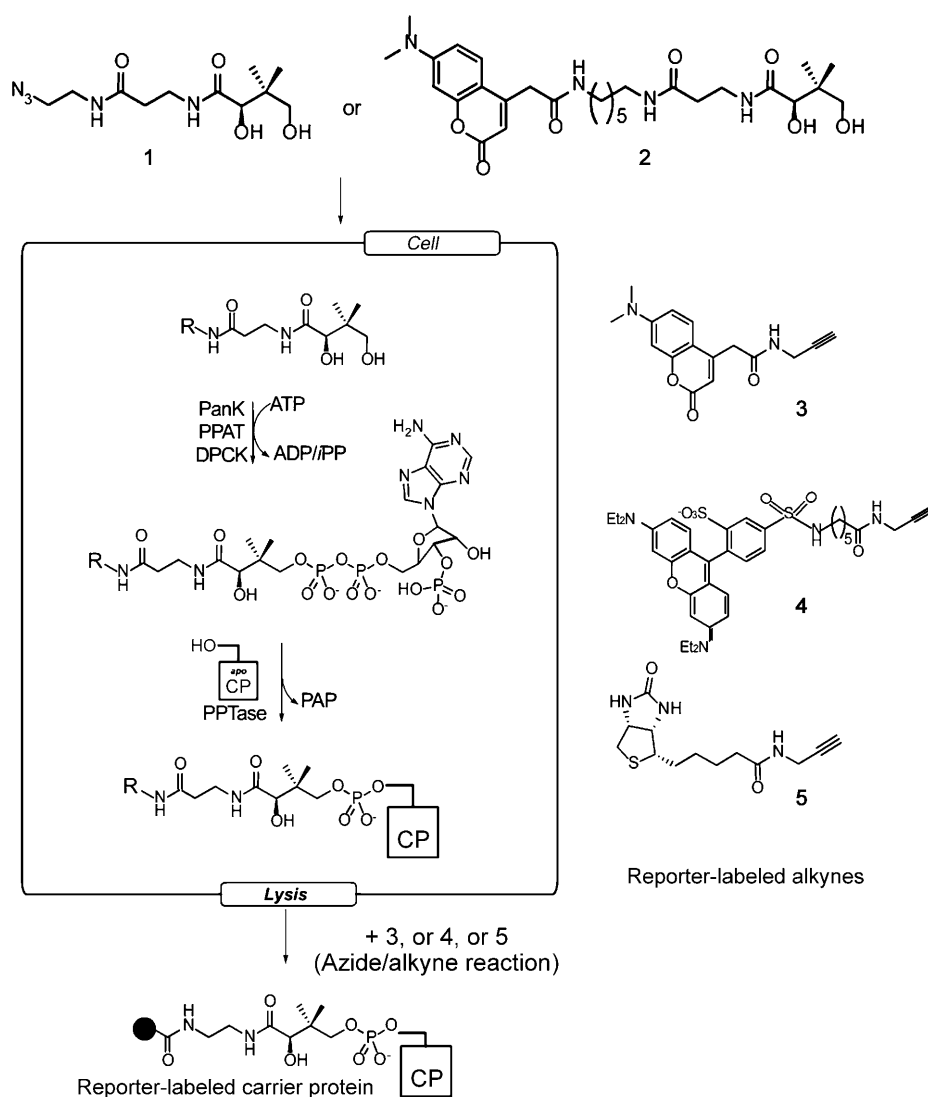
Results

In vivo labeling of native ACP

In our previous studies, pantetheine analogues **1** and **2** demonstrated cellular uptake and processing by the endogenous *E. coli* CoA biosynthetic enzymes pantothenate kinase (PanK, E.C. 2.7.1.33), phosphopantothenoyl adenyltransferase (PPAT, E.C. 2.7.7.3), and dephospho-CoA kinase (DPCK, E.C. 2.7.1.24) to form reporter-labeled CoA in vivo.^[9,10] The formation of these

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Scheme 1. In vivo labeling strategy. Cells are grown in the presence of azido-pantetheine **1** or fluorescent pantetheine **2**. After uptake, the native CoA biosynthetic enzymes convert the pantetheine analogues to CoA analogues, which then are appended to endogenous CPs by the native PPTase enzyme. After cell lysis azido-modified CPs can be detected by reaction with alkyne probes **3–5**.

CoA analogues was demonstrated by their ability to undergo reaction with a heterologously expressed CP and PPTase, which resulted in labeling of the overexpressed CP with a fluorescence or affinity tag. While valuable for method development, the heterologous PPTase and CP were present within the cells at levels that far exceeded those of endogenous proteins. To explore the utility of this method for the metabolic labeling of endogenous CPs in a native organism, we directed our efforts at the fatty acid ACP of *E. coli*. Fatty acid and CoA metabolism are well understood in this organism, and our previous reports indicated that the CoA biosynthetic pathway in BL-21 strains of *E. coli* is permissive enough to allow for the in vivo conversion of pantetheine analogues **1** and **2** to CoA analogues.

By using the wild-type K12 strain of *E. coli* as a model system, we set out to demonstrate the in vivo modification of the native fatty acid CP AcpP. Overnight growth in a 1 mM so-

lution of compound **1** in Luria-Bertani (LB) media followed by cell lysis and a copper-catalyzed cycloaddition reaction with fluorescent alkyne (**3** or **4**) allowed visualization of the modified CP with SDS-PAGE (Figure 1). Fluorescent pantetheine **2** showed no detectable labeling of native *E. coli* CPs under identical growth conditions. In addition, control cultures grown with vehicle (DMSO) showed no similar fluorescence after incubation with **3** or **4** under cycloaddition reaction conditions. The protein modified by **1** was found to run at the same apparent molecular weight as recombinantly purified and labeled AcpP on both denaturing and native PAGE gels, a compelling finding given that AcpP is readily separated from most other proteins on high-percentage native gels due to its small size and charge.^[14] To definitively identify the labeled protein, the fluorescent band was excised, proteolytically digested, and analyzed by LC-MS/MS. The results confirmed that the labeled band was indeed the AcpP protein from *E. coli* (Figure S1 in the Supporting Information).

Because a traditional obstacle to the use of in vivo labeling techniques has been the ability of the small molecule to penetrate the cell, we also examined in detail the method of uptake of **1** by *E. coli*. While *E. coli* are capable of synthesizing pantothenate de novo, they also express a pantothenate transport system. The *panF* gene encodes pantothenate permease, a sodium-dependant pantothenate symporter.^[15] If implicated in the uptake **1**, PanF ac-

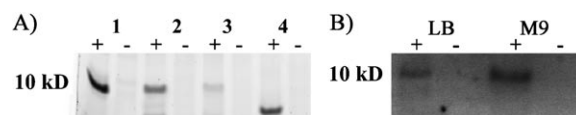


Figure 1. Detection of in vivo modified carrier proteins. A) Cultures were grown with or without compound **1** and reacted with RX-alkyne **4** after lysis. Distinct CP labeling is seen in *E. coli* (**1**), *B. subtilis* 168 (**2**), *B. subtilis* 6051 (**3**), and *S. oneidensis* (**4**) as compared to negative controls B) Labeling of *E. coli* ACP by **1** with visualization by DMAC-alkyne **3** was effective in both LB (lane **1**) and M9 minimal media (lane **2**) as compared to negative controls (lanes **2**, **4**).

tivity could represent a caveat to the general applicability of this labeling method to organisms that lack PanF homologues. To decouple this dedicated pantothenate-uptake system from the metabolic-labeling protocol, we performed metabolic labeling in *E. coli* strains that contain an in-frame deletion in the *panF* gene to knock-out activity.^[16] The result of these labeling experiments were qualitatively indistinguishable from those performed with wild-type *E. coli* (Figure S6); this suggests that pantetheine analogues such as **1** enter *E. coli* by passive diffusion or an alternate uptake mechanism. Additionally, strains of *E. coli* with the pantothenate biosynthetic genes *panD* and *panC* knocked out showed no difference in CP labeling by **1** relative to wild-type *E. coli*; this indicates the presence or absence of active pantothenate biosynthesis has no effect on labeling efficiency.

Finally we examined the toxicity of metabolic labeling by **1** to growing *E. coli*. An ideal metabolic label would allow for the study of CPs in live organisms with minimal perturbation to their natural growth and metabolism. However, past studies have associated the in vivo modification of the fatty acid acyl carrier protein (ACP) by CoA analogues with growth inhibition in a number of bacteria, including *E. coli*.^[17,18] To examine the toxicity of the metabolic-labeling procedure we determined minimum inhibitory concentrations for **1**, **2**, and N5-Pan, a prototypical bacteriostatic pantetheine analogue (see Figure S15 for structure), towards *E. coli* grown in nutrient-rich and minimal media. As opposed to N5-Pan, which inhibits *E. coli* growth in minimal media at 30 μM , the compounds used in this study (**1**, **2**) showed no growth inhibition of *E. coli* at concentrations up to 500 μM . This lack of growth inhibition was not due to a lack of ACP modification under these conditions as growth of *E. coli* supplemented with **1** in minimal media showed modification of ACP at similar levels to those observed in LB (Figure 1B). To reconcile the nontoxic ACP labeling of **1** with the proposed mode of action of bacteriostatic pantetheine analogues, we have since performed a full study of the effects that secondary structural characteristics have on the toxicity of CoA precursors.^[19] These findings indicated that the more polar reporter labels incorporated in **1** and **2** might result in decreased toxicity relative to N5-Pan-modified ACP due to an inability of ACPs modified by **1** and **2** to interact with partner enzymes of the fatty acid biosynthetic pathway; this is an optimal property for non-toxic, in vivo labeling of CPs.

In vivo modification of ACPs from other bacteria

Having demonstrated in vivo CP modification in wild-type *E. coli*, we sought to next test the generality of this methodology for labeling of CP domains in other bacterial species whose CoA and fatty acid biosynthetic pathways have been less well studied (Figure 1). Accordingly *Bacillus subtilis* 6051 and *Shewanella oneidensis* MR-1, and *Bacillus brevis* 8246 were grown with 1 mM pantetheine azide **1** overnight. Lysis followed by chemical conjugation with fluorescent alkyne **4** allowed low-molecular-weight proteins corresponding to the fatty acid ACPs in each of these strains to be visualized by SDS-PAGE

(Figures 1A and 4). Once again, this tentative identification was verified by band excision and LC-MS/MS analysis. Database searching allowed identification of the fatty acid ACPs from *B. subtilis* and *S. oneidensis*. This demonstrates that **1** possesses properties compatible with cellular uptake and processing by the CoA biosynthetic pathways in both Gram-positive and Gram-negative bacterial species, which together constitute a large number of known natural-product-producing organisms.

In addition to demonstrating metabolic labeling of the fatty acid ACPs from these organisms, we explored in further detail the biosynthetic capabilities necessary for transfer of the 4'-phosphopantetheine arm from CoA to the conserved serine residue of ACP by a PPTase (Scheme 1). There are two classes of bacterial PPTase enzymes: the AcpS type, which is associated with primary metabolism and necessary for modification of the fatty acid ACP, and the Sfp type, which is associated with secondary metabolism and is involved in the modification of CPs in NRPS and PKS systems.^[6,20,21] While some cross-reactivity exists, AcpS-type PPTases are less permissive of variation in CP or CoA analogue identity.^[22] Conversely, the Sfp type shows relaxed specificity in terms of CP or CoA identity.^[6] We had previously observed CP labeling in *B. subtilis* 6051, which carries a functional copy of Sfp. To ensure that CP labeling by **1** would not be hindered at the last step in organisms lacking a Sfp-type PPTase we also tested this method in *B. subtilis* strain 168, which contains an in-frame deletion in its Sfp gene and therefore contains only the less-permissive AcpS-type PPTase. Metabolic labeling by **1** followed by chemoselective ligation to fluorescent alkyne **4** demonstrated labeling at similar levels to those observed by using strain 6051 (Figure 1A). This indicates the azido-CoA analogue formed from **1** is compatible with less-promiscuous AcpS-type PPTases; this is a desirable feature for the labeling of CPs from disparate biosynthetic systems.

Labeling of human type I FAS in living cells

Fatty acid, polyketide, and nonribosomal peptide synthases can be classified as type I or II depending on whether the active domains required for product biosynthesis are located on discrete polypeptides (type II) or are housed on multimodular megasynthases (type I).^[1] Bacterial fatty acid synthases are type II and as such have discrete active domains, including the ACP. In animals, most fatty acids are biosynthesized by type I synthases. Our success in labeling ACPs of type II fatty acid biosynthesis in bacterial organisms with **1** lead us to test whether this approach would also be feasible in eukaryotic cells, particularly in the labeling of type I fatty acid ACP domains.

We chose as a model system the SKBR3 cell line, which is derived from human breast cancer cells.^[23] SKBR3 cells were plated at 25% confluency and allowed to grow for 24 h at 37 °C before introduction of the pantetheine analogue. Incubation of the cells with compound **1** for up to 60 h did not result in detectable labeling of the FAS after cell lysis and reaction with fluorescent alkyne **4**. This was surprising, because we had expected labeling in eukaryotic cells to be enhanced compared

to bacteria because mammalian cells cannot produce endogenous pantothenate.^[24] The hypothesis that this difference could be due to differential pantothenate import mechanisms in eukaryotic and prokaryotic cells led us to test another pantetheine analogue, fluorescent pantetheine **2**, which had shown good compatibility with the CoA biosynthetic pathway in other studies.^[25,26] Interestingly, this compound, which had proven ineffective in modifying fatty acid ACPs from endogenous bacterial systems effectively labeled the type I FAS in human cells (Figure 2A). Growth of SKBR3 cells with **2** for between 40 and 60 h resulted in visibly bright staining of the cells, and an increased amount of observable cell death compared to incubation with **1** or DMSO. To quantify the level of uptake, cell cultures grown with **2** or vehicle DMSO were subjected to analysis by flow cytometry (FACS, Figure 2B). Of cells grown with **2**, 100% of the cells could be identified with FACS as containing significant fluorescence. Lysis of the cells and visualization of the fluorescent labeled FAS by using SDS-PAGE gel confirmed labeling of the FAS protein (Figure 2A). However,

this labeling was relatively modest compared to expectations based on the qualitative observation of strong uptake. To reconcile these results, lysate from the cells was examined by HPLC along with chemoenzymatically prepared standards of the four possible intermediates in the processing of pantetheine **2** by the CoA biosynthetic pathway. The major fluorescent peak found in SKBR3 lysate was observed to co-elute with the product of **2** and PanK, leading us to identify it as the singly processed phosphopantetheine analogue. (Figure 2C) This is consistent with previous reports on the processing of pantetheine prodrugs by human cells that indicated the bifunctional PPAT/DPCK as the major point of blockage for formation of CoA analogues *in vivo*.^[27] While we have previously shown that recombinant human PPAT/DPCK can be used to convert fluorescent pantetheine analogues into CoA analogues for CP labeling *in vitro*,^[26] in living cells these enzymes are most likely present at far lower levels and only process the most efficient 4'-phosphopantetheine substrates. Additionally the human FAS is cytosolic, but CoA is known to be seques-

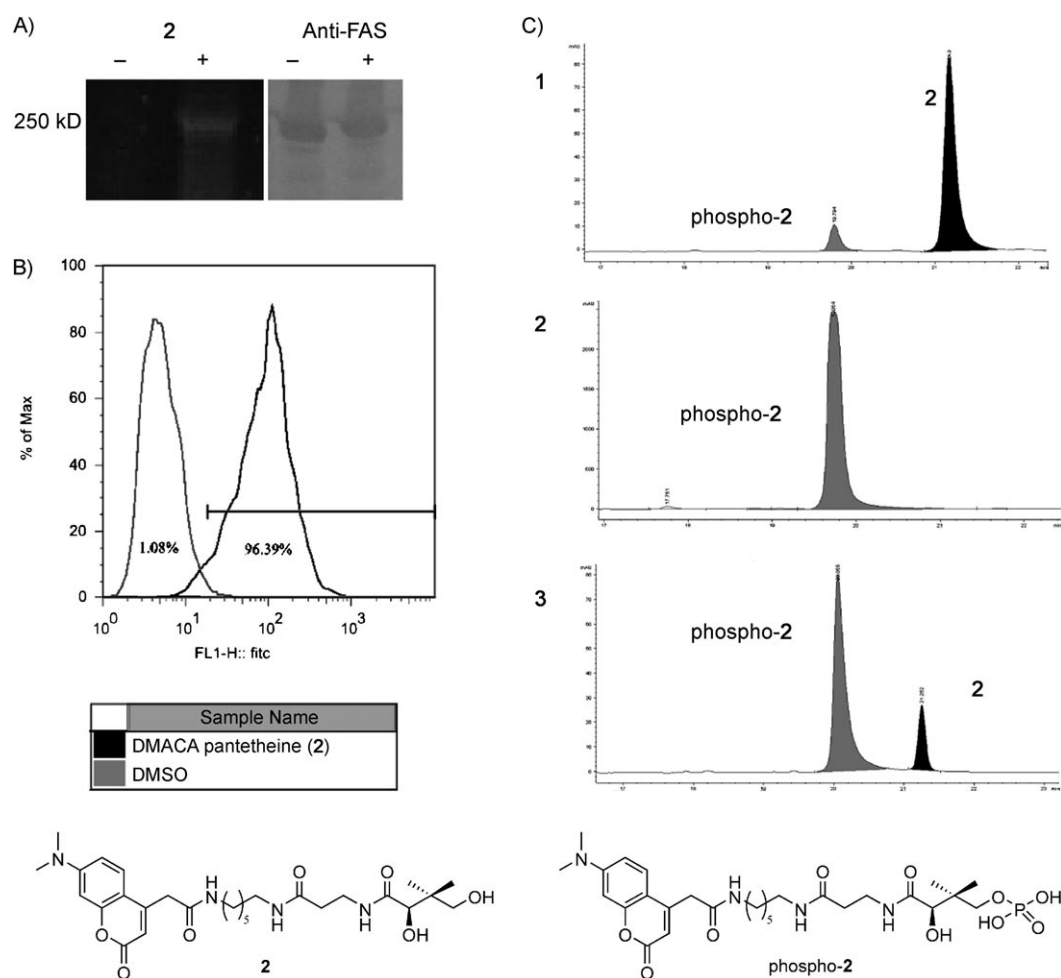


Figure 2. Analysis of type I FAS ACP labeling in SKBR3 cells A) *In vivo* modification of the human type I FAS. Lysate from cultures grown with compound **2** (+) show fluorescent modification of the FAS megasynthase as compared to negative controls (left). Blot with anti-FAS antibody confirms the presence and location of FAS on the gel (right). B) FACS analysis of SKBR3 cells grown with compound **2** shows 99% of the SKBR3 cells grown with **2** took up the compound. C) (panel 1) Compound **2** (black) incubated with PanK+ATP for 15 min results in addition of a phosphate on the 4'-hydroxyl and a shift to lower retention time (grey). (panel 2) After 120 min the reaction is complete, with all of **2** converted to the phospho analogue. (panel 3) Lysate from SKBR3 cells grown with **2** contains mostly phospho analogue. No further conversion to the CoA analogue in the SKBR3 cells could be detected (Supporting Information).

tered at its highest intracellular levels in the mitochondria; this makes localization of the CoA analogue of **2** another complicating factor.^[28] Efforts are currently ongoing to develop an analogue that shows uptake similar to **2** and demonstrates improved compatibility with the human CoA biosynthetic enzymes.

Affinity purification of a metabolically labeled bacterial carrier protein

One of the major goals of this research has been to isolate and identify new modular biosynthetic enzymes with these techniques. The studies described above had shown that metabolic labeling in genetically unmodified cultures by **1** or **2** allowed for the visualization and identification of fatty acid ACPs from human, *E. coli*, *B. subtilis*, *S. oneidensis*, and *B. brevis* lysates. Here, labeled CPs from sequenced organisms were readily identified by proteolytic digest and MS analysis of excised gel bands, followed by comparison of the observed peptides to genomic databases. However, these analyses also commonly identified background proteins that migrated at the same molecular weight on SDS-PAGE. In unsequenced or unannotated organisms, this phenomenon could complicate de novo sequencing efforts and hinder identification of novel synthases by diluting the pool of labeled CP peptides or identifying false positives based on sequence homology. To remove contaminating proteins and also to verify the specificity of CP modification by metabolic labeling, we tested the utility of a biotin-streptavidin affinity purification method to isolate modified fatty acid ACPs.

This technique was demonstrated by using *S. oneidensis* MR-1, a bacterial strain noted for its production of polyunsaturated fatty acid natural products.^[29] The finding that metabolic labeling by **1** is not toxic to growing bacteria suggested that it modifies only a fraction of the total cellular ACP content because it allows for at least a basal level of vital cellular processes such as fatty acid biosynthesis. Therefore, in order to facilitate the recovery of labeled CPs we performed the metabolic labeling on a large scale by using one-liter cultures. *S. oneidensis* MR-1 was grown overnight in the presence of 1 mM pantetheine analogue **1**, centrifuged to a pellet, washed, and lysed to yield azide-labeled CPs within the crude cell lysate. The lysate was then subjected to a copper-catalyzed [3+2] cycloaddition reaction with the biotin alkyne **5**. After removal of excess **5** by a desalting column, followed by incubation with streptavidin agarose beads, the affinity-purified proteins were recovered by the addition of SDS-PAGE running buffer and boiling. Figure 3 shows the results of enrichment of lysate from *S. oneidensis* that was metabolically labeled with **1** (lanes 1–6) in contrast to a DMSO-treated control (7–9). Comparison by SDS-PAGE shows enrichment of an approximately 10 kDa protein from the lysate of MR-1 grown with **1** and treated with biotin **5** (lane 3). This protein is not enriched when alkyne **5** is not added (lane 6), and is also not present in non-metabolically labeled, DMSO-treated cultures of *S. oneidensis* (lane 9). This indicates that the enriched band does not result from endogenous biotinylation or non-specific reaction

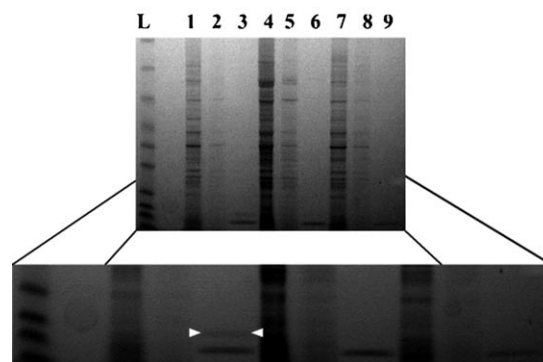


Figure 3. Affinity purification of ACP. Lysate from cultures of *S. oneidensis* MR1 that were grown with compound **1** and then treated with biotin-alkyne **5** were incubated with streptavidin agarose, which allowed for the purification of modified CP (crude lysate lane 1, desalting column flow lane 2, recovered ACP band—between white arrows—10 kDa lane 3). Cultures grown with compound **1** but not treated with biotin-alkyne **5** (lanes 4–6) or grown without compound **1** (lanes 7–9) showed no protein enrichment after incubation with streptavidin agarose. The strong band below ACP is a contaminant from the resin and was seen under all elution conditions.

of biotin-probe **5** in cell lysates. To our satisfaction, excision, tryptic digest, and MS analysis of this band resulted in the identification of the fatty acid ACP. This experiment shows that in vivo CP-labeling techniques can be used to specifically recover labeled CPs from their native systems.

Identification of a carrier protein from an unsequenced organism

B. brevis is a Gram-positive bacterium responsible for the production of the natural products gramicidin and tyrocidine.^[30] We chose the nonproducing strain (ATCC 8246) of this unsequenced organism to test whether this chemical proteomic approach was capable of identifying CPs without the aid of genomic sequence data. As shown above with *S. oneidensis*, native CPs labeled by **1** and **2** can be reliably identified through database searches of known genomes after tryptic digest and MS. The ability to extend this methodology to the large number of unsequenced natural-product-producing organisms would further validate this approach for studying CP-based natural product biosynthetic enzymes.

Accordingly, *B. brevis* 8246 was grown overnight in the presence or absence of compound **1**. The cultures were lysed and prepared as in the other in vivo labeling experiments, followed by reaction with cognate fluorescent alkyne **4**. As can be seen in Figure 4, *B. brevis* 8246 cultures grown in the presence of **1** show distinct labeling of a protein with an apparent M_w of 15 kDa as compared to control cultures. Although this organism is unsequenced many other *Bacillus* species have been well studied, and these lead us to the expectation that this labeled protein was the type II fatty acid ACP, as we had seen in previous work with *B. subtilis*. To verify this hypothesis, we again excised the fluorescent band and submitted it to MS/MS analysis (Figure 4B). Because no database was available for this unsequenced organism, we constructed a data set comprising all known bacterial fatty acid ACPs against which to search.

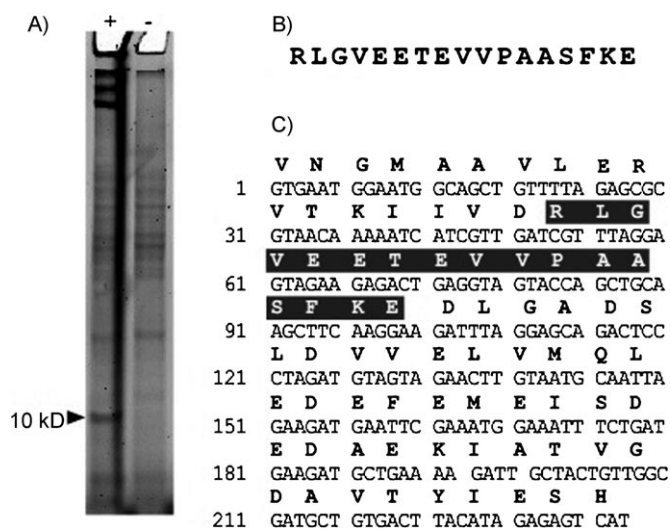


Figure 4. Identification of a carrier protein from an unsequenced organism. A) Metabolic labeling of *B. brevis* 8246 by **1** gives a band around 10 kDa that is not present in the negative control. B) Tryptic MS analysis of this band by using a database of 200 known bacterial ACP sequences identified a peptide fragment matching an ACP sequence. C) Degenerate and arbitrary PCR allowed for sequencing of the gene that encodes this peptide and its identification as the fatty acid ACP.

Searching the observed peptide fragments against this database returned a single hit that matched a tryptic fragment from the ACP in *B. cereus*. Because of the small size of type II fatty acid ACPs, only a small number of tryptic peptides are generated on proteolytic digest, and a single matching peptide represents a significant percentage of sequence coverage.

To complete the identification of the CP from *B. brevis* 8246 we constructed a set of degenerate primers from the identified peptide. By using degenerate and arbitrary PCR techniques, we were able to generate PCR fragments from genomic DNA.^[31]

Sequencing confirmed that we had amplified the ACP gene from 8246. As seen in Figure 5, the sequence is nearly identical to the ACP gene from *B. cereus* and has high homology to the ACP genes from *B. thuringiensis* and *B. anthracis*. It differs considerably from the sequence for *B. subtilis* ACP. The taxonomy of *B. brevis* has been reclassified a number of times, but this result concurs with recent reports indicating *B. brevis* and *B. cereus* are closely related.^[32] While this analysis relied on the relatively good sequence homology among small CP domains, the application of this method in combination with affinity tags targeting alternate active sites on PKS and NRPS multi-enzymes^[33] should facilitate de novo sequencing of a variety of CP-containing systems from unsequenced organisms.

Discussion

Previous attempts to label CPs in vivo with functionally useful labels have been hindered by the inability of charged CoA analogues to permeate the cell membrane. In our prior studies we addressed this issue by developing uncharged pantetheine analogues and studying the ability of the native CoA biosynthetic pathway to convert them into reporter-labeled CoA analogues

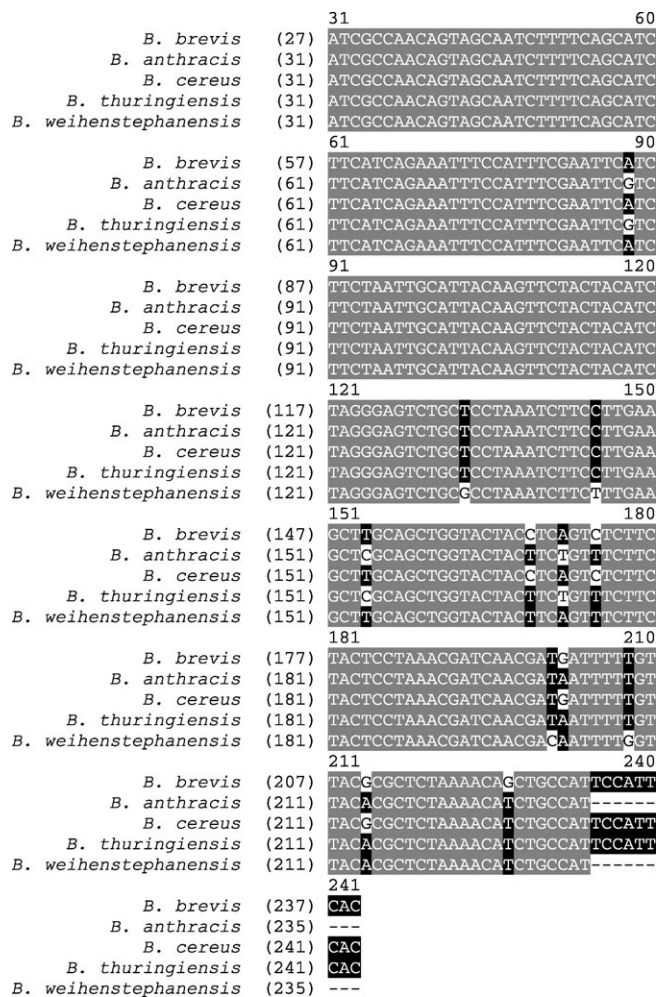


Figure 5. Alignment of *Bacillus* ACP sequences. Alignment of the new ACP sequence from *B. brevis* shows high homology with known *Bacillus* ACP sequences.

in living cells.^[25,34] Although these investigations provided insight into the structural features necessary for in vivo production of CoA analogues from synthetic pantetheine analogues, all of these experiments were conducted with heterologously expressed CPs and PPTases in a single Gram-negative bacteria, *E. coli*. Here we have extended this technique to metabolically deliver CoA analogues to apo-CPs at native levels. By using this strategy, we have modified CPs from both Gram-positive and Gram-negative bacteria. The modified type II fatty acid ACPs in these organisms are detectable after cell lysis by bio-conjugation of fluorescently tagged alkynes with azido-pantetheine **1**. After visualization by PAGE, these labeled proteins can be readily identified from LC-MS/MS analysis of excised gel fragments. Alternatively, cell lysate from organisms that were grown with **1** can be subjected to copper-catalyzed reaction with biotin-alkyne **5**, and their CPs can be isolated. The metabolic incorporation of **1** shows minimal toxicity and is compatible with CoA and PPTase pathways from a variety of different natural-product-producing organisms. This new ability to selectively label and affinity purify endogenous CPs from

native organisms should allow for the discovery of novel CPs as well as their associated biosynthetic systems.

We have also demonstrated the metabolic labeling of an ACP from a type I FAS. This indicates that our methodology is also applicable to type I modular biosynthetic systems and could provide a means for isolation and identification of the analogous type I PKS and NRPS megasynthases, which are responsible for the production of diverse natural products. Interestingly, whereas **1** was effective in modifying type II fatty acid ACPs in prokaryotes, it did not appear to be efficiently processed by the same pathway in the human SKBR3 cell line. Our previous work has demonstrated that turnover of an analogue in both the CoA biosynthetic pathway and the phosphopantetheinylation reaction can be greatly influenced by small changes in the structure of the particular analogue, and it has been observed previously that the human PanK isoform shows a very low sequence homology to those that are used by many prokaryotes.^[35] One explanation for the extremely robust uptake of fluorescent pantetheine **2**, as was observed by FACS and HPLC analysis, could be that **2** possesses greater activity with the human PanK enzyme than **1**. Efficient processing of **2** by the PanK enzyme would lead to production of a phosphorylated, cell-impermeable intermediate. Because there are no currently annotated import or export mechanisms for pantetheine analogues in human cells, continued passive diffusion of the compound into the human cells could result in increasing accumulation of this cell-impermeable intermediate, a finding consistent with the increased cell death observed in cells grown with **2** for longer periods of time. With such a concentration mechanism in place, it seems credible that despite its low turnover by the PPAT/DPCCK enzyme, a small amount of **2** is converted to CoA and shuttled to the ACP by the human PPTase; this leads to the observed modest labeling of human FAS. This preliminary observation of the differential activity of pantetheine analogues **1** and **2** in bacteria and humans could eventually prove useful for the specific tuning of the CP-labeling method to different organisms, or for the *in vivo* labeling of specific CPs in complex mixtures of organisms. With further refinement this technique may prove practical for the study of symbiotic natural-product-producing communities by allowing for time-dependent CP-labeling in an organism-specific fashion.^[36]

In addition to possessing compatibility with several different biosynthetic enzymes, a useful metabolic label must also compete with the natural substrate without inducing cell death. In this study pantetheine analogues **1** and **2** have been shown to be compatible with three CoA biosynthetic enzymes and the CP post-translational modification process in a full spectrum of prokaryotic and eukaryotic organisms, including Gram-positive and Gram-negative prokaryotes and eukaryotes. These analogues show model properties in terms of their ability to successfully compete with the natural substrates for the CoA biosynthetic enzymes and PPTases without any genetically or media-based advantages. One disadvantage of this technique from the perspective of its use in proteomic identification of CPs is that this competition likely results in only intermediate to low-level labeling of CPs by our unnatural CoA precursors.

However, metabolic labeling of CPs offers an advantage compared to genetic methods (RT-PCR) or post-lysis protein identification techniques in its ability to detect proteins throughout all stages of growth, rather than representing a single temporal snapshot of the dynamic proteome.^[33] This characteristic should prove valuable to the study of the synthesis and degradation of biosynthetic enzymes.

Finally, the use of native metabolic pathways as a means of *in vivo* modification of CPs has enabled the identification of a previously unknown CP. Especially in systems with large or unsequenced genomes, a protein-based approach allows for a directed search for CP-dependent biosynthetic pathways without the use of laborious techniques such as genomic library construction and screening. No genetic information is required, and complications arising from silent biosynthetic gene clusters are removed. Furthermore, because this method depends only on the activity of a protein to be phosphopantetheinylated, it should hold no obvious bias against CP-containing systems that are not readily identifiable by homology alone, including those belonging to orphan gene clusters.^[37] We have initially developed this methodology by using type I and II fatty acid biosynthetic CPs that are constitutively expressed in growing cultures. In these studies no effort was made to correlate polyketide or nonribosomal peptide production with CP labeling by **1** or **2**. However, the demonstrated compatibility of this method with uptake, CoA biosynthesis, and PPTase activity in a wide range of organisms should allow for its similar application to PKS and NRPS-producing organisms when correlated with small-molecule production or antibiotic activity. In the future, we believe that *in vivo* labeling of native CPs should prove highly complementary to existing methods and add greatly to the knowledge of natural product producing synthases.

Conclusions

A significant number of these biologically relevant natural products are produced in systems that tether the growing product to a 4'-phosphopantetheine modified CP. Selective labeling of these enzymes could allow for a better understanding of these proteins in their native environments and provide a platform for discovery of natural product synthases from unsequenced organisms. Here we have demonstrated a new set of tools for investigation and discovery of natural-product-producing systems. Using native metabolic pathways as a means of *in vivo* modification of endogenous CPs has allowed for the identification of a previously unknown CP. Because CPs and CP domains are found in fatty acid, polyketide, nonribosomal peptide, and other metabolite biosynthetic pathways, the ability to specifically modify these peptides *in vivo* allows one to probe numerous biochemical pathways. We have demonstrated for the first time the modification of native CPs by reporter-labeled CoA precursors in both prokaryotic and eukaryotic organisms; this gives access to the full range of CP-dependent systems.

Experimental Section

Experimental procedures

See Table 1.

Table 1. Strains and cell lines used in this study.		
Organism	Medium	Ref.
<i>E. coli</i> K12	LB/M9	[38]
<i>B. subtilis</i> 168	LB	[39]
<i>B. subtilis</i> 6051	LB	ATCC 6051
<i>B. brevis</i> 26A1	RM	ATCC 8246
<i>S. oneidensis</i> MR-1	RM	[40]
human SKBR3	McCoy's 5A	[41]

Primers used in this study:

ARB1	5'-GGCCACGCGTCGACTAGTACNNNNNNNNN-GATAT-3'
ARB2	5'-GGCCACGCGTCGACTAGTAC-3'
ARB6	5'-GGCCACGCGTCGACTAGTACNNNNNNNNN-NACGCC-3'
Internal frag	5'-CGCCTGGGCGTAGAAGAAACGGAA-3'
External frag	5'-CGCCTGGGCGTAGAAGAAACGGAA-3'
<i>B. brevis</i> acpp F	5'-ATGGAATGGCAGCTGTTTATAGAGCGCGT-3'
<i>B. brevis</i> acpp R	5'-TAGATGACTCTCTATGTAAGTCACAGCATCGC-3'

In primers ARB1 and ARB6 N is an equimolar mix of A/T/G/C bases.

Media: Luria Broth (LB) was purchased from Fisher Scientific (Waltham, MA). M9 was made according to the standard procedure.^[42] Rich media (RM) contained 1% bactopectone, 1% beef extract, 0.25% NaCl. McCoy's 5A media (Invitrogen) was modified to contain a final concentration of 10% fetal bovine serum. *E. coli* knock-out strains were grown in LB.

In vivo carrier protein labeling: Bacteria were grown in the above-noted media with or without **1** (1 mM) for 12–17 h in an orbital shaker at 37°C. Cells were harvested by centrifugation at 20000 rpm for 5 min and resuspended in lysis buffer (100 mM NaCl, 25 mM potassium phosphate pH 7.0). For small cultures (<10 mL), cells were lysed by addition of lysozyme (3 mg mL⁻¹, Worthington Biochemical Corporation, Lakewood, NJ) incubated at 25°C for 1 h and sonicated (3×30 s with a microtip at low power). For large cultures (>10 mL) cells were resuspended in lysis buffer (10 mL per liter of culture) with lysozyme (0.1 mg mL⁻¹). After 1 h incubation with shaking on ice, cells were lysed by two passes through a French pressure cell. The lysate was then subjected to reaction with fluorescent alkyne **3** or **4** as previously reported.^[34,43]

Determination of kinetic parameters of pantetheine analogues with PanK: Analogues were assayed for turnover with *E. coli* PanK in a coupled enzyme assay as previously described.^[34]

Identification of *B. brevis* ACP: Cultures of *B. brevis* were grown in the presence of **1** as described above. MS identification of a labeled protein gave a single peptide that matched the *B. cereus* AcpP protein. By using this sequence, primers were designed (ARB1,2,6, internal frag, external frag), and arbitrary PCR was carried out by following the procedure of Caetano-Anolles.^[31] Amplified bands were ligated into *Taq*-amplified (TA) vectors (Invitrogen) and sent for sequencing. The resulting sequence allowed for the design of specific primers and the cloning of the full acpp gene (*B. brevis* F and R). (Table 1).

Tissue culture: SKBR3 human breast cancer cells were grown in McCoy's 5A medium (Invitrogen) supplemented with FBS (10%,

HyClone, Logan, UT, USA) and penicillin–streptomycin–glutamine (2%, Invitrogen). Cells were plated at ~25% confluency on day 0. On day one, the media was changed to a media that contained either fluorescent pantetheine **2** (1 mM), or DMSO (0.5%) as a control. On day three cells were trypsinized and washed twice with cold PBS. Cells were lysed by incubation in lysis buffer and Triton-X 100 (0.1%) on ice. Protein was recovered by centrifugation.

FACS analysis: 10⁶ cells were trypsinized and washed once in cold PBS containing 1% FBS (HyClone). Cells were resuspended in PBS containing 1% FBS (1 mL) and analyzed by flow cytometry. Live cells, as determined by forward and side scatter profile, were analyzed by using a FACScalibur (Becton Dickinson) for uptake of compound **2**.

Large-scale in vivo carrier protein labeling and affinity purification: Lysate from MR-1 culture (1 L) grown with or without compound **1** (1 mM) was subjected to the bioconjugation reaction as previously reported with alkyne **5** as the conjugate probe.^[34,43] Reactions (1 mL) were diluted to 3 mL in RIPA buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1% TritonX 100) and run over two desalting columns (Econo-Pac 10 DG, Bio-Rad) to remove unreacted **5**. The reaction was eluted in lysis buffer and incubated with streptavidin agarose resin (Thermo Sci, Waltham, MA, USA) for 2 h at room temperature. The resin was collected by centrifugation and washed in RIPA buffer (5×). The bound protein was then recovered from the resin by the addition of SDS-PAGE loading buffer and incubation at 95°C for 10 min. After brief centrifugation to remove the melted agarose, the supernatant was loaded onto a SDS-PAGE gel (12%) for analysis.

Mass spectroscopic analysis, 1D SDS-PAGE, and in-gel digestion: The proteins were separated by 1D SDS-PAGE by using Bis-Tris NuPAGE gels (10%, Invitrogen). The gel was fixed overnight in MeOH (50%)/H₂O (43%)/AcOH (7%), washed twice with H₂O (200 mL) for 10 min, and stained overnight with Gel Code Blue (Pierce, Rockford, IL, USA). Subsequently, the gel was washed with H₂O (200 mL) for 1 h. Excised gel bands were washed with 50% acetonitrile (MeCN) and 50% DTT (5 mM)/NH₄HCO₃ (25 mM) (2×200 µL) with vortexing for 10 min, and finally washed with MeCN (200 µL). The dehydrated gel piece was rehydrated by addition of ice-cold trypsin (20 µL, 10 ng µL⁻¹, Promega) in DTT (5 mM)/NH₄HCO₃ (25 mM), and incubated on ice for 30 min, and the remaining trypsin solution was removed and replaced with fresh DTT (5 mM)/NH₄HCO₃ (25 mM). The digestion was allowed to continue at 37°C overnight. The peptide mixture was then acidified with trifluoroacetic acid (2%, 2 µL; TFA; Sigma), vortexed for 30 min, and the supernate was extracted. Finally, MeCN (20%)/TFA (0.1%) (20 µL) was added, and the mixture was vortexed to extract the remaining peptides and combined with the previous fraction. The combined extractions are analyzed directly by nanobore LC–MS/MS.

Mass spectrometry: The samples were analyzed by electrospray ionization by using a QSTAR-Elite hybrid mass spectrometer (Applied Biosystems/MDS Sciex) interfaced to a Tempo nanoscale reversed-phase HPLC (Eksigent/Applied Biosystems) by using a 75 µm×15 cm column (Grace Davison, Columbia, MD, USA) that was packed with Vydac MS C18 (300 Å, 5 µm) packing material. The buffer compositions were as follows: buffer A: H₂O (98%), MeCN (2%), acetic acid (0.1%, Fluka), heptafluorobutyric acid (0.005%, Fluka); buffer B: MeCN (98%), H₂O (2%), acetic acid (0.1%), heptafluorobutyric acid (0.005%). Samples (10 µL) were injected by the Tempo autosampler onto a C18 PepMap pre-column

(5 mm×300 µm, LC Packings, Sunnyvale, CA, USA) by using the channel 1 loading pump at a flow rate of 15 µL min⁻¹ buffer A. After washing for 5 min, the peptides were transferred onto the analytical column and eluted directly into the mass spectrometer with a 25 min linear gradient from 5 to 40% buffer B at a flow rate of 300 nL min⁻¹ by using channel 2. LC-MS/MS data were acquired in a data-dependent fashion by selecting the most intense peak with charge state 2–4 that exceeds 40 counts with exclusion of former target ions set to “always” and the mass tolerance for exclusion set to 100 ppm. TOF MS were acquired at *m/z* 500–1800 Da for 0.5 s with 20 time bins to sum. MS/MS are acquired from *m/z* 65–2000 Da by using “enhance all” and 20 time bins to sum, Dynamic Background Subtract, Automatic Collision Energy, and Automatic MS/MS Accumulation with the Fragment Intensity Multiplier set to 12 and Maximum Accumulation set to 3 s before returning to the survey scan.

Database search: The MS/MS were analyzed by Analyst 2.0 (Applied Biosystems) and subjected to database search by using Mascot 2.2.1 (Matrix Science, Boston, MA, USA) with Mascot Daemon 2.2 (Matrix Science) data import filter parameters set as follows: default precursor charge state 2–4; precursor and MS/MS data centroiding by using 50% height and 0.05 amu merge distances. MS/MS peaks with intensity less than 1% of the base peak were discarded, as were MS/MS spectra with fewer than 22 peaks remaining. Data were searched against the Swissprot database obtained at <ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/> containing 237 168 sequences. The search identified tryptic peptides with up to two missed cleavages and used mass tolerances of 100 ppm (MS) and 0.10 Da (MS/MS), with variable modifications as follows: deamidation (NQ), oxidation (M), pyro-Glu (N-term Q). The search results indicated that individual ion scores > 42 indicate identity or extensive homology (*P* < 0.05).

Growth-inhibition assay: Assays were modified from reported procedure.^[35] A single colony of K12 was picked into M9 media for overnight growth and the resulting culture was diluted 1:10 000. This diluted culture was plated into 96-well tissue culture plates that contained dilutions of pantetheine analogues in M9 medium. The plate was incubated overnight at 37 °C, and the wells were assessed for growth.

HPLC analysis of SKBR3 lysate: Cytosolic extract from the growth of SKBR3 cells with **2** was precipitated by trichloroacetic acid, centrifuged, and the supernatant was analyzed by reversed-phase HPLC by using a Burdick and Jackson OD5 column (4.6 mm by 25 cm, Morristown, NJ, USA) with monitoring at 254 and 360 nm. The solvents that were used were TFA (0.05%) in H₂O (solvent A) and TFA (0.05%) in MeCN (solvent B). Compounds were eluted at a flow rate of 1 mL min⁻¹. The method used an isocratic step from 0 to 5 min with 100% A, followed by a linear gradient to 45% B over 15 min, followed by an increasing gradient with solution B until at 25 min the solvent composition was 100% solution B. Under these conditions, **2** and its CoA analogue intermediates eluted between 17 and 21 min. For comparison of the fluorescent compounds observed in SKBR3 lysate to authentic CoA analogue intermediates of **2**, **2** was incubated in a stepwise manner with the recombinant *E. coli* biosynthetic enzymes PanK, PPAT, and DPCK as previously described.^[25] The product of the reaction of **2** and PanK was combined with a small amount of SKBR3 lysate to verify co-elution of the two peaks.

General synthetic procedures and materials: All commercial reagents (Sigma–Aldrich, Alfa Aesar, TCI America) were used as provided unless otherwise indicated. The 5-isomer of Rhodamine-

Red X NHS Ester was purchased from Molecular Probes (Carlsbad, CA, USA). Azido-pantetheine **1**, fluorescent pantetheine **2**, biotin alkyne **5**, and 7-dimethylaminocoumarin-4-acetic acid were prepared as previously described.^[25,34] All reactions were carried out under an argon atmosphere in dry solvents with oven-dried glassware and constant magnetic stirring unless otherwise noted. Triethylamine (TEA), and ethyl-*N,N*-diisopropylamine (DIPEA) were dried over Na and freshly distilled. ¹H NMR spectra were taken at 300, 400, or 500 MHz and ¹³C NMR spectra were taken at 100.6 or 75.5 MHz on Varian NMR spectrometers and standardized to the solvent signal as reported by Gottlieb.^[44] TLC analysis was performed by using silica gel 60 F254 plates (EM Scientific) and visualization was accomplished with UV light (λ = 254 nm) and/or the appropriate stain (iodine, 2,4-dinitrophenylhydrazine, cerium molybdate, ninhydrin). Silica gel chromatography was carried out with Silicycle 60 Å 230–400 mesh according to the method of Still.^[45] TLC prep plate purification was performed with EMD silica gel 60 F₂₅₄ precoated plates. Electrospray (ESI) and fast atom bombardment (FAB) mass spectra were obtained at the UCSD Mass Spectrometry Facility by Dr. Yongxuan Su by using a Finnigan LCQDECA mass spectrometer and a ThermoFinnigan MAT-900XL mass spectrometer, respectively.

Synthesis of fluorescent 2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)-*N*-(prop-2-ynyl)acetamide (3): 7-dimethylaminocoumarin-4-acetic acid (250 mg, 1.01 mmol), DIPEA (440 µL, 2.53 mmol), propargyl amine (70 µL, 1.01 mmol), and 1-hydroxybenzotriazole (484 mg, 3.16 mmol) were dissolved in DMF (25 mL) with stirring and cooled to 0 °C. *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide (485 mg, 2.53 mmol) was added in one portion, and the reaction was allowed to slowly warm to RT and followed by TLC. After 20 h the solvent was removed under reduced pressure and the sparingly soluble crude reaction mixture was taken up in EtOAc with heating. After filtration the soluble portion was purified by column chromatography (1:1 EtOAc/hexanes to EtOAc to 5% MeOH/CH₂Cl₂). This procedure was repeated several times on the EtOAc-resuspended filter cake to afford propargyl-DMC **3** (87 mg, 30%) as an orange solid. ¹H NMR (500 MHz, CDCl₃): δ = 7.43 (d, *J* = 9.0 Hz, 1H), 6.61 (dd, *J* = 2, 9.0 Hz, 1H), 6.51 (d, *J* = 2 Hz, 1H), 6.05 (s, 1H), 5.92 (brs, 1H), 4.02 (dd, *J* = 2.0, 4.5 Hz, 2H), 3.64 (s, 2H), 3.06 (m, 7H); ¹³C NMR (75.5 MHz, [D₆]DMSO): δ = 168.4, 161.4, 156.1, 153.5, 151.7, 126.7, 110.1, 109.7, 108.8, 98.2, 81.5, 74.0, 41.0, 39.1, 28.9; HRMS (EI): *m/z* calcd for C₁₆H₁₆N₂O₃: 284.1155 [*M*]⁺; found: 284.1154.

Synthesis of fluorescent alkyne 2-(6-(diethylamino)-3-(diethyliminio)-3H-xanthen-9-yl)-5-(*N*-(6-oxo-6-(prop-2-ynylamino)hexyl)sulfamoyl)benzenesulfonate (4): Rhodamine Red-X NHS Ester (10 mg, 0.013 mmol), DIPEA (10 µL, 0.06 mmol), and propargyl amine (5 µL, 0.07 mmol), were dissolved in DMF (0.5 mL), covered with aluminum foil, and allowed to stir for 1 h. After removal of the solvent under reduced pressure, the bright-red residue was redissolved in MeCN (1.4 mL) and purified by RP-HPLC. The solvents used were TFA (0.05%) in H₂O (Solvent A) and TFA (0.05%) in MeCN (solvent B). Compounds were eluted at a flow rate of 1 mL min⁻¹ with monitoring at 560 nm. The method used an isocratic step from 0 to 5 min with 40% B, followed by a linear gradient to 58% B over 15 min, followed by an increasing gradient with solution B until at 16 min the solvent composition was 100% solution B. Under these conditions **4** eluted around 11.3 min. Pooling and lyophilization of multiple HPLC runs yielded **4** (6.5 mg, 72%) as a red solid. ¹H NMR (500 MHz, [D₆]DMSO): δ = 8.39 (s, 1H), 8.20 (brt, *J* = 5.0 Hz, 1H), 7.91 (d, *J* = 8.0 Hz, 1H), 7.90 (brs, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 7.03 (d, *J* = 9.5 Hz, 2H), 6.94 (d, *J* = 9.5 Hz, 2H), 6.91

(s, 2H), 3.79 (d, $J=2.5$ Hz, 2H), 3.62 (q, $J=7.5$ Hz, 8H), 3.01 (s, 1H), 2.83 (q, $J=6.5$ Hz, 2H), 2.04 (t, $J=7.5$ Hz, 2H), 1.42 (p, $J=8.0$ Hz, 2H), 1.37 (p, $J=7.5$ Hz, 2H), 1.19 (t, $J=7.0$ Hz, 12H), 1.15 (t, $J=7.0$ Hz, 2H). MS (ESI): m/z calcd for $C_{36}H_{44}N_4O_7S_2$: 708.26 $[M]^+$; found: 731.25 $[M+Na]^+$.

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