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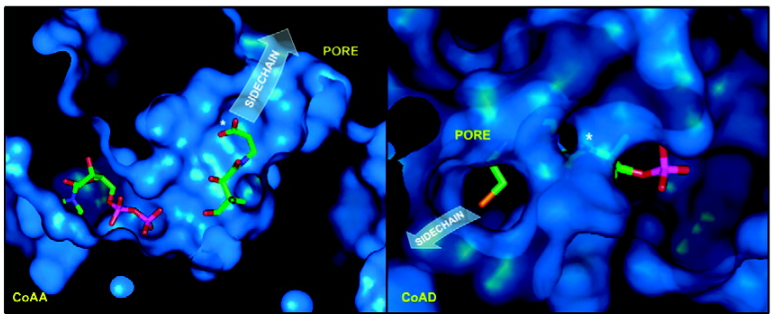


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# In Vivo Reporter Labeling of Proteins via Metabolic Delivery of Coenzyme A Analogues

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Selective chemical control of biochemical processes within a living cell enables the study and modification of natural biological systems in ways that may not be obtained through in vitro experiments.[1a,b] Accordingly, access to promiscuous metabolic pathways has provided a unique chemical entry into small molecule engineering in vivo.[1c] We recently introduced a method for covalent reporter labeling of carrier proteins using promiscuous phosphopantetheinyltransferase (PPTase) enzymes and reporter-labeled coenzyme A (CoA).[2a] To date, this method has been limited to in vitro and cell-surface protein labeling,[2,3] as CoA derivatives have not been shown to penetrate the cell. To overcome this obstacle, we demonstrate addition of labeled metabolic precursors to cell culture that results in cellular uptake and metabolic conversion into active, labeled CoA derivatives. We now establish a chemoenzymatic route to protein modification via a four-step enzymatic sequence in vivo.

The chemical synthesis and activity of CoA has been studied for well over a half-century, yet the full biosynthesis of the cofactor has only recently been elucidated in prokaryotes and eukaryotes.[4,5] CoA is biosynthesized by five enzymes in *Escherichia coli*, CoAA-CoAE, while eukaryotes contain a fusion of CoAD and CoAE, PPAT/DPCK. Knowledge of the substrate specificity of these enzymes remains incomplete, although some evidence points to promiscuity within this pathway. Early studies on CoAA indicated that the enzyme will also accept pantetheine as a substrate.[6] This ability has since been used in the chemoenzymatic synthesis of CoA analogues.[7] This permissiveness suggests using the CoA biosynthetic pathway to convert reporter-labeled pantetheine to reporter-labeled CoA in vivo. To this end, the synthesis of fluorescently labeled pantetheine analogues provides a direct link to reporter-labeled post-translational modifications.[8]

A key facet to any in vivo modification relies on access to a viable uptake mechanism. We chose the nonhydrolyzable, fluorescent pantetheine analogue **1** in an effort to probe its uptake and retention. In *E. coli*, excess pantetheine is exported from the cell, and a sodium-dependent symporter has been suggested to import pantetheine from the surrounding media.[9] Once inside the cell, pantetheine analogue **1** can be phosphorylated by CoAA and ATP to yield **2** (Figure 1). Further processing by CoAD and CoAE should proceed by stepwise adenylation of **2** to dephospho−CoA **3** and further phosphorylation to yield CoA analogue **4**. This analogue would now be captured within the cell and, therefore, remain viable for downstream processing events. Given the correct strain, this process could be used to selectively transfer the label in **4** onto a carrier protein domain (**5**).

In selecting analogue **1**, we chose to eliminate the thioester linkage found in natural CoA metabolites, thereby avoiding potential side reactions due to hydrolysis and oxidation in vivo. To create the non-natural connectivity, we began by protecting the 1,3-diol of pantothenic acid (**7**) as *p*-methoxybenzylacetal **8** (Scheme 1). Acetal **8** was coupled to fluorescent amine **6**,[10] providing the

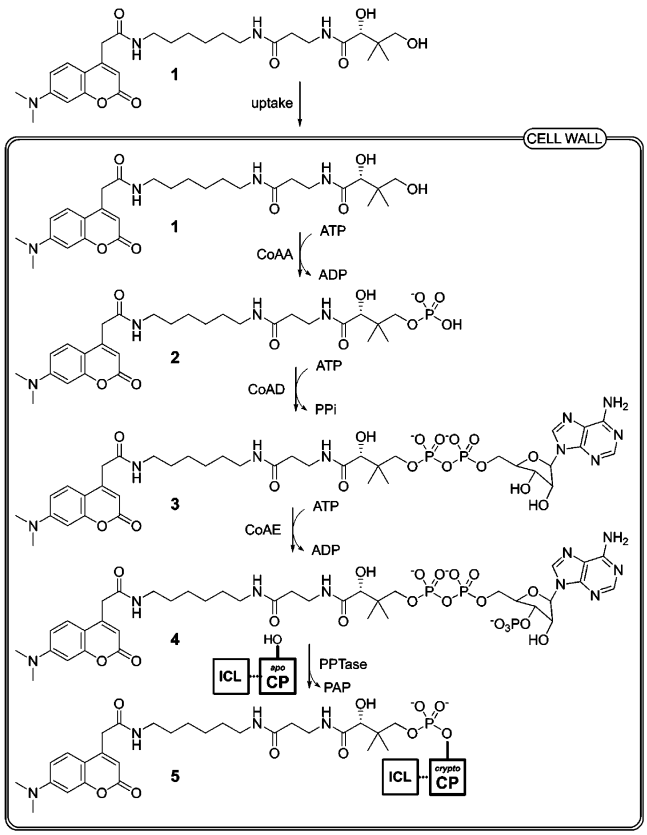
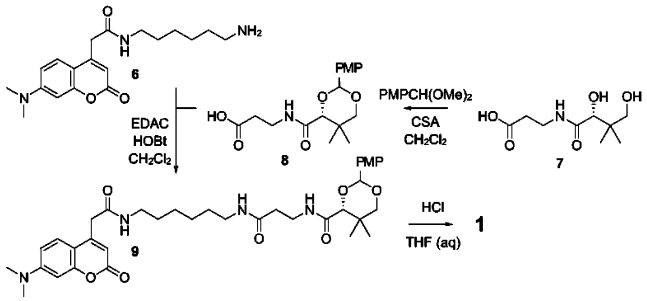



***Figure 1.*** In vivo metabolic labeling of a carrier protein (CP) via cellular uptake of pantetheine analogue **1** and conversion to CoA analogue **4** by CoAA, CoAD, and CoAE. This process is followed by reaction of a PPTase with **4** and a carrier protein yielding labeled protein **5**. VibB, a natural fusion, is depicted as a carrier protein fused with isochorismate lyase (ICL).

***Scheme 1.*** Synthesis of Pantetheine Mimic **1**




protected pantetheine mimic **9** in 77% yield. Final deprotection under acidic conditions provided analogue **1** in an overall yield of 37% from **6** and **7**.

To test if analogue **1** would be an acceptable substrate for *E. coli* CoAA, CoAD, and CoAE, we reconstituted the CoA pathway



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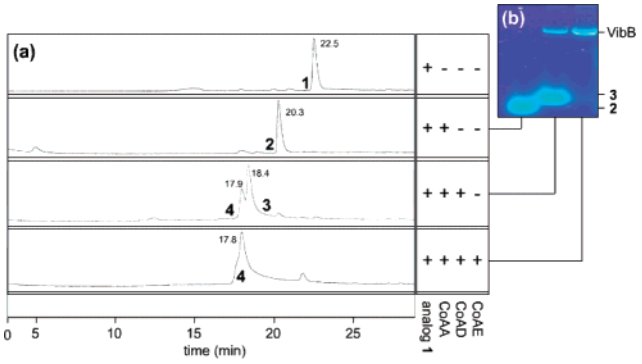


***Figure 2.*** In vitro enzymatic reconstitution of the metabolic-labeling process. (a) HPLC analysis of the stepwise conversion of **1** to fluorescent CoA analogue **4**. (b) Fluorescent SDS−PAGE gel depicting the labeling of VibB by **4** with Sfp. Note intermediates **2** and **3** can also be visualized through gel analysis.

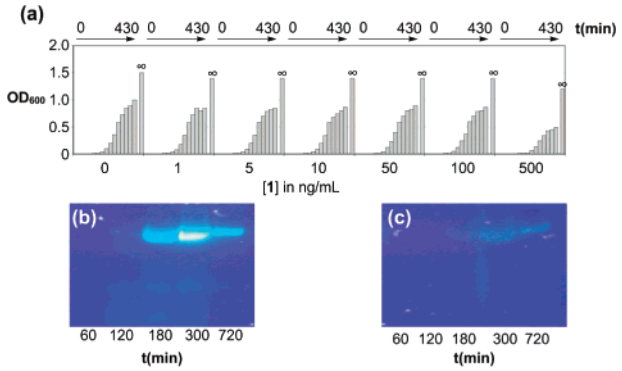


***Figure 3.*** In vivo tagging of carrier protein fusion (VibB) within *E. coli*. (a) Growth of *E. coli* culture ($OD_{600}$) over a range in concentrations of **1**. ∞ was measured at 1360 min. (b) In vivo formation of *crypto*-VibB following a time course following addition of 1 mM **1** to culture. (c) In vivo formation of *crypto*-VibB following a time course following addition of 100 $\mu$M **1** to culture.

with recombinant proteins in vitro and followed the progress of each enzymatic reaction, as illustrated in Figure 2. The conversion of analogue **1** to **4** was followed by HPLC in a stepwise manner, and **1** was shown to be an acceptable substrate for the pathway (Figure 2a). Additionally, carrier protein modification was accomplished in vitro with the addition of recombinant, purified VibB, a carrier protein fusion from *Vibriobacter cholerae*, and Sfp, a PPTase from *Bacillus subtilis*. As shown by SDS−PAGE (Figure 2b),[2] VibB becomes fluorescently labeled as in **5**. We found that VibB was also tagged without the addition of CoAE, due to contaminating native CoAE that co-purifies with overexpressed CoAD.

As a practical concern, reagents for in vivo labeling should be nontoxic. As illustrated by *E. coli* growth (Figure 3a), minimal bacteriostatic activity was seen at concentrations above 180 $\mu$M. This result is in accord with prior studies of *N*-pentylpantothenamide,[11] in which bacteriostatic activity was observed only in minimal media. When greater concentrations are required, introduction of **1** may coincide with protein induction, thereby minimizing toxic effects. Comparable growth studies were conducted to determine uptake. Analogue **1** was added to media during IPTG induction of *E. coli* co-transformed with *vibB*- and *sfp*-harboring expression vectors.[2] The outcome of these studies (Supporting Information) indicated that up to 4% of **1** is uptaken when exposed to 1 mM of **1** in LB media.

While the percentage uptake of **1** is modest, the intracellular concentration is sufficient to allow the labeled precursor to incorporate into the CoA pathway. Following incubation of the co-transformed *E. coli* with 1 mM **1** and IPTG, lysate from the isolated cells was visualized via fluorescent SDS−PAGE (Figure 3b), indicating post-translational modification of VibB by Sfp and fluorescent CoA analogue **4**. Fluorescent intensity reaches a maximum at 5−8 h post-induction (Figure 3b). Lower intensity is also readily visualized when incubation is carried out in 100 $\mu$M **1** (Figure 3c). As a control, no labeling was seen from addition of **1** to cell lysate from induced, untreated cells.

The flux of pantetheine through *E. coli* is high due to constituent biosynthesis and rapid export.[9] We anticipate that other organisms that do not produce pantetheine precursors or lack export mechanisms may be effective hosts for this protein-labeling technique. The fact that **1** was viable in *E. coli* provides strong support for the general application of these tools to natural product pathway elucidation, fusion protein localization, and the analysis of complex expression patterns. The tolerance to modification throughout this pathway should allow delivery of a wide variety of chemical motifs to in vivo processes. We are currently investigating the use of these methods to visualize and manipulate heterologous proteins, fusion constructs, and natively expressed carrier protein domains.

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**Supporting Information Available:** Experimental procedures, NMR data for compounds **1** and **6**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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