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Sulfonyl 3-Alkynyl Pantetheinamides as Mechanism-Based Cross-Linkers of Acyl Carrier Protein Dehydratase

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Supporting Information

ABSTRACT: Acyl carrier proteins (ACPs) play a central role in acetate biosynthetic pathways, serving as tethers for substrates and growing intermediates. Activity and structural studies have highlighted the complexities of this role, and the protein−protein interactions of ACPs have recently come under scrutiny as a regulator of catalysis. As existing methods to interrogate these interactions have fallen short, we have sought to develop new tools to aid their study. Here we describe the design, synthesis, and application of pantetheinamides that can cross-link ACPs with catalytic β-hydroxy-ACP dehydratase (DH) domains by means of a 3-alkynyl sulfone warhead. We demonstrate this process by application to the *Escherichia coli* fatty acid synthase and apply it to probe protein−protein interactions with noncognate carrier proteins. Finally, we use solution-phase protein NMR spectroscopy to demonstrate that sulfonyl 3-alkynyl pantetheinamide is fully sequestered by the ACP, indicating that the *crypto*-ACP closely mimics the natural DH substrate. This cross-linking technology offers immediate potential to lock these biosynthetic enzymes in their native binding states by providing access to mechanistically cross-linked enzyme complexes, presenting a solution to ongoing structural challenges.

Acyl carrier proteins (ACPs) are highly conserved, small (~9 kDa), acidic proteins found in fatty acid synthase (FAS) and polyketide synthase (PKS) biosynthetic pathways.[1] The ACP serves as a central tether for all starting materials and intermediates in these pathways, mediating each chemical transformation in the elongation and elaboration of the metabolites. As the structures and activities of FAS and PKS enzymes continue to be refined, the role of the ACP has been subjected to further scrutiny.[2] Whether incorporated into a large megasynthase (type-I systems) or as discrete enzymes functioning independently (type-II systems), ACPs are highly flexible species that must interact with multiple catalytic enzyme partners through highly specific protein−protein interactions.[3] Complicating such binding events is the ability of type-II ACPs to sequester their elongating intermediates, shielding them from reactivity until favorable protein interactions are achieved.[4]

The need for new technologies to capture ACP dynamics and protein−protein interactions has become apparent in recent years.[5] Because of the inherent technical issues posed by the cloning and expression of large, multidomain synthases, ACP participation in these systems remains largely uncharacterized, and those systems that have been studied structurally show disordered ACPs.[6] To overcome the kinetic disadvantage of diffusion control, type-II enzymes are thought to associate in dynamic complexes, the compositions of which can change during the course of a particular biosynthetic pathway.[7] To serve as a covalent tether, an ACP must be post-translationally modified with a 4′-phosphopantetheine (PPant) prosthetic arm. It is in this covalent, protein-bound form that an ACP reacts with the active site of a catalytic partner protein.[8] The ACP must therefore functionally interact with all enzyme domains responsible for loading carbon units, condensing these units, modifying the condensation product, and releasing the final product from the synthase. Each step relies on specific protein−protein interactions to recognize and channel the biosynthetic intermediate to the proper partner enzyme.

The binding affinities of an ACP for its enzymatic partners are relatively weak ($K_d \sim 1-10\ \mu M$), allowing it to interact reversibly with each enzyme in the biosynthetic pathway and move on to the next. Therefore, attempts to visualize ACPs in complex with partner enzymes for structural analysis have been met with only modest success. To overcome this obstacle, we have devised a general methodology for covalent cross-linking of ACPs to partner domains by taking advantage of the unique post-translational modification of ACPs (Scheme 1).[8] In this approach, we synthesize analogues of pantetheine that contain mechanism-based inhibitors of FAS enzymes and append them to ACPs. When an inhibitor-loaded *crypto*-ACP interacts with its enzymatic partner through protein−protein interactions (Scheme 1a), the synthetic PPant side arm is allowed to enter the pocket of the catalytic protein (Scheme 1b), where it can react with an active-site residue and form a covalent bond that traps both the ACP and the enzyme in a bound conformation (Scheme 1c). The formation of the cross-linked species, which was initially applied to the study of ketosynthase domains,[8b] is highly dependent on ACP-mediated protein−protein interactions and has been applied as a tool in elucidating ACP−partner protein interactions. Similar strategies have also been successfully applied to probe peptidyl carrier protein interactions within nonribosomal peptide synthetase enzymes.[9]

In the present study, we extended the repertoire of chemoenzymatic ACP cross-linking tools to study the interactions of ACPs and dehydratase (DH) domains in FAS. β-Hydroxy-ACP DH enzymes are found in all FAS pathways and

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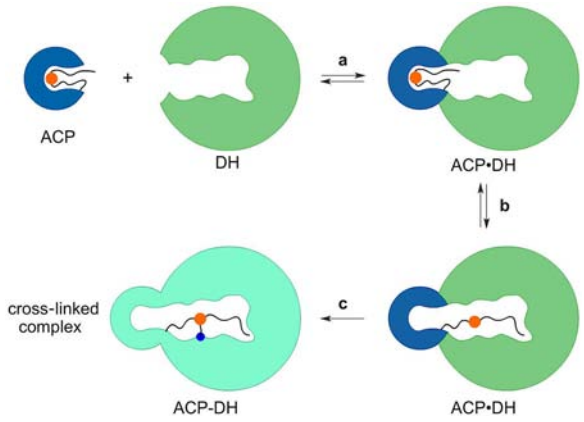
Scheme 1. General Representation of the Cross-Linking Strategy for a FAS Dehydratase (DH) and Its ACP Loaded with a Cross-Linking Pantetheine Probe: (a) Protein−Protein Interaction; (b) Switchblade Mechanism; (c) Mechanism-Based Inactivation and Cross-Linking



in any PKS whose product contains olefinic or saturated methylene units. The enzyme FabA catalyzes two kinds of chemical reactions in the *Escherichia coli* fatty acid biosynthetic pathway: dehydration of (*R*)-3-hydroxydecanoyl-ACP to give (*E*)-2-decenoyl-ACP and isomerization of (*E*)-2-decenoyl-ACP to give (*Z*)-3-decenoyl-ACP.[10] 3-Decynoyl-*N*-acetylcysteamine (**1**) (Figure 1), which was first reported by Bloch to inactivate

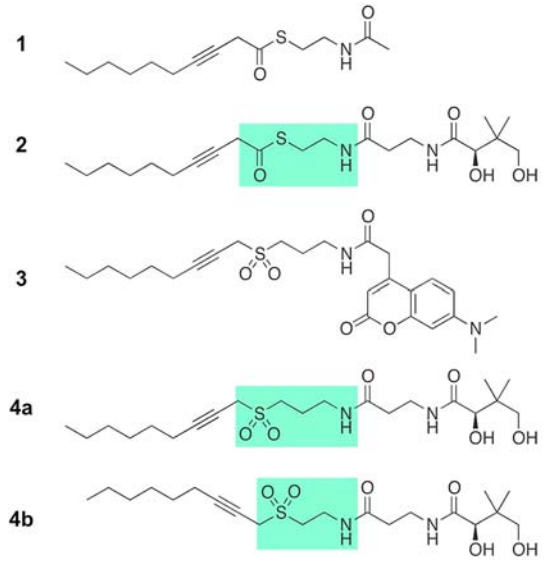


Figure 1. Structures of DH probes and cross-linking agents.

FabA rapidly and irreversibly,[11] is the first instance of a "mechanism-based inhibitor". Here the enzyme target catalyzes the formation of a covalent bond between the inhibitor and an active-site residue, thus permanently inactivating the enzyme. In this work, we expanded the use of pantetheinamides by installing a sulfonyl 3-alkyne reactive functionality to facilitate specific cross-linking of the ACP and DH domains.

Our studies originally began with the preparation of cross-linker **2** to expand the use of this historical inhibitor scaffold and guide specific cross-linking of ACP and DH domains.[12] However, when this pantetheine analogue was investigated, only low levels of cross-linking were observed. This observation was attributed to rapid release of the reactive functionality from the *crypto*-ACP by nucleophilic cleavage of the thioester bond in **2** before the formation of the ACP−DH complex or to the decomposition of **2** itself. We therefore began investigating potential replacements of this warhead for increased stability.

To this end, we recently reported the design, synthesis, and utility of DH-specific probe **3** containing a sulfonyl 3-alkyne reactive warhead (Figure 1).[13] Replacement of the reactive thioester moiety of **1** with the nonhydrolyzable sulfone in **3** not only eliminated the instability of nucleophilic cleavage but also provided an α-proton with low enough acidity to avoid nonenzymatic allene formation while still facilitating DH-specific labeling. In addition, our previous studies suggested that mechanism-based inhibition of FabA by 3-decynoic acid analogues is dependent upon the pK_a of the α-proton of the suicide substrate scaffold (thioester > oxoester ≫ acid/amide),[13] which led us to focus on the use of the sulfonyl 3-alkyne scaffold to obtain second-generation ACP−DH cross-linking reagents. To this end, pantetheine analogues **4a** and **4b** were synthesized from the convergent assembly of commercially available 2-nonyn-1-ol, cysteamine for **4a** or 3-chloropropylamine for **4b**, and a *p*-methoxybenzyl-protected pantothenic acid (synthetic methods are provided in the Supporting Information).

Our biochemical studies began with an evaluation of the ability of **4a** and **4b** to modify the *E. coli* FAS ACP (AcpP). Using the CoA biosynthetic enzymes CoaA, CoaD, and CoaE along with 4′-phosphopantetheinyl transferase (Sfp), we were able to confirm loading of the analogues **4a** and **4b** onto the *apo*-ACP by means of conformationally sensitive urea polyacrylamide gel electrophoresis (urea-PAGE) analysis.[14] A characteristic increase in electrophoretic mobility was observed upon AcpP loading of fatty acid pantetheines **4a** and **4b** to give the *crypto*-ACPs (Figure 2a). The conversion of the *apo*-ACP to the *crypto*-ACPs[14b] was accomplished in quantitative yield at 37 °C for 1 h. Upon addition of FabA to the corresponding *crypto*-ACPs obtained using **4a** and **4b**, clear bands indicating irreversible covalent cross-linking between the ACPs and the DH domain were observed after 12 and 24 h at 37 °C. These products resulted in observed gel shifts of the FabA band from ∼20 kDa for FabA to ∼45 kDa for the cross-linked AcpP−FabA complex upon analysis by sodium dodecyl sulfate PAGE (SDS-PAGE). The observation of FabA consumption at 12 and 24 h (Figure 2b) clearly showed that **4a** efficiently facilitated cross-linking between the *crypto*-AcpP and FabA, suggesting that the longer alkyl linker more closely mimicks the native substrate (*R*)-3-hydroxydecanolyl-ACP and correctly aligns a His residue with the α-proton of the sulfonyl 3-alkyne in the FabA active site. A slight gel shift due to nonspecific probe labeling during SDS-PAGE sample preparation was seen in all bands within probe-containing lanes. Subsequent screening allowed us to investigate the pH dependence of the cross-linking process (Figure 2c). While the maximum labeling of probe **3** was observed at pH 8.0 or 8.5,[13] the cross-linking reaction to form **7** (Scheme 2) was unaffected by pH. As an ultimate test of the cross-linking efficiency, we conducted time-course studies using **4a** as the ACP−DH cross-linking reagent at 37 °C and pH 7.0 (Figure 2d). This study indicated that the improved stability of **4a** allowed successful cross-linking between the *crypto*-AcpP and FabA. Using pantetheinamide **4a**, we observed greatly improved cross-linking efficiency in yields greater than 90%, as estimated by the band intensity after 36 h at pH 7.0.

On the basis of the precise stereochemical, mechanistic, and structural studies of FabA inactivation by **1**,[10] we propose that **4a**

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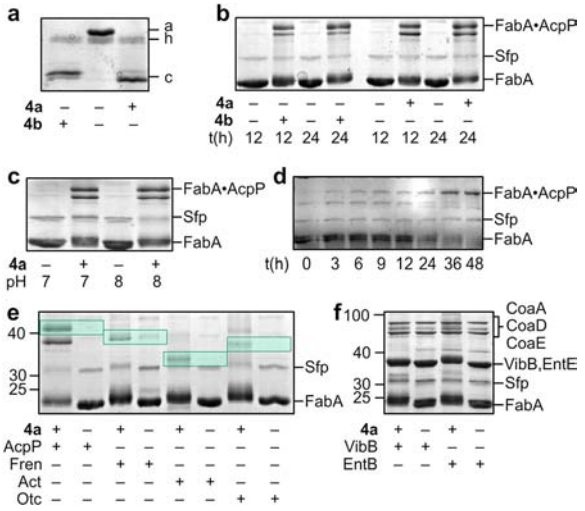
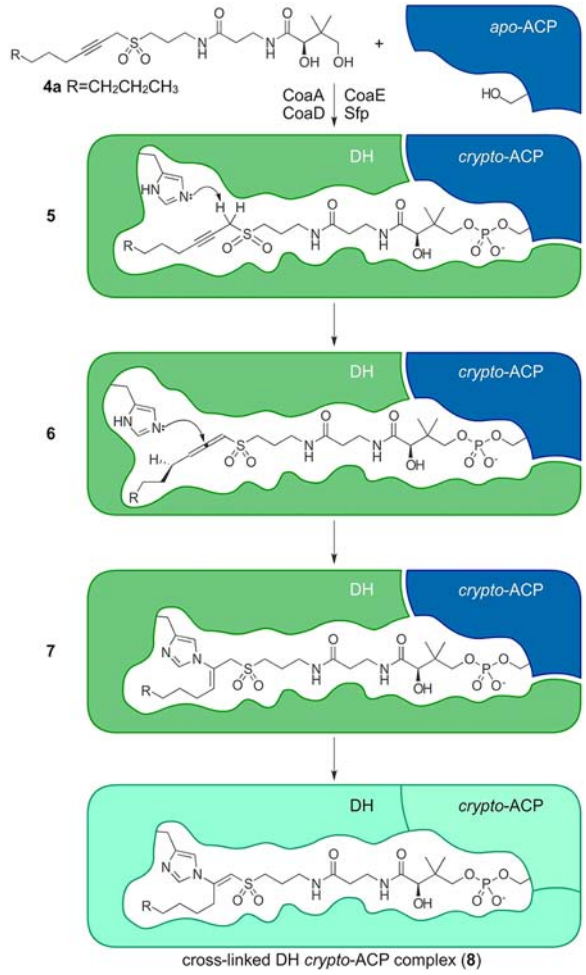


Figure 2. Evaluation of ACP–DH cross-linking reagents **4a** and **4b**. (a) Pantetheinamides **4a** and **4b** were appended enzymatically to *apo*-AcpP using a one-pot chemoenzymatic method. The conversion of *E. coli apo*-AcpP to the corresponding *crypto*-ACP when treated with 500 μM **4a** or **4b** was visualized by urea-PAGE (a = *apo*, h = *holo*, c = *crypto*). (b) SDS-PAGE analysis of the cross-linking of AcpP and FabA upon treatment with 500 μM **4a** or **4b** at 37 °C for 12 or 24 h. (c) SDS-PAGE gel showing the pH dependence of the cross-linking reaction for 12 h at 37 °C. (d) Time-course studies depicting the cross-linking of AcpP to FabA with 500 μM **4a**. The cross-linking reaction of **4a** loaded onto the ACP was accomplished in high yield (>90%) after 36 h at 37 °C. (e, f) Effect of carrier protein (CP) identity on FabA cross-linking. Pantetheinamide **4a** was used to modify (e) the FAS and PKS CPs AcpP (FAS), Fren (PKS), Act (PKS), and Otc (PKS) and (f) the NRPS CPs VibB and EntB. The results indicate the clear preferential interaction of FabA with AcpP from FAS over the other ACPs from PKS and the peptidyl CPs from NRPS.

Scheme 2. Schematic Representation of the Cross-Linking of ACP and DH Domains Using Probe 4a



and **4b** loaded onto ACP act via the mechanism shown Scheme 2. When the DH and *crypto*-ACP interact (**5**), the reactive 3-decynoyl unit is positioned precisely in the FabA active site via protein–protein interactions, allowing the active-site residue His_{70} of FabA to deprotonate the acidic α-sulfone proton, generating electrophilic allene **6**. This in turn reacts with the His_{70} residue to form AcpP–FabA cross-linked products **7** and **8**.

Next, we tested the ability of the ACP–DH cross-linking reaction to visualize protein–protein interactions between native and non-native ACP·DH pairs.[8] In addition to the FAS carrier protein AcpP, the type-II PKS carrier proteins FrenACP (from *Streptomyces roseofulvus*), ActACP (from *Streptomyces colicolor*), and OtcACP (from *Streptomyces rimosus*) as well as the NRPS carrier proteins VibB (from *Vibrio cholerae*) and EntB (from *E. coli*) were loaded with **4a** and incubated with FabA.[8] Cross-linking with FabA was seen only with FrenACP, ActACP, and OtcACP, while VibB and EntB remained un-cross-linked (Figure 2e,f). A clear preference for *E.coli* AcpP could be seen by comparison of the intensities of the cross-linked bands. This result reflects the sequence and activity-based homology between type-II FAS ACPs and type-II PKS ACPs.[15] Indeed, DH-specific fluorescent probes based on this sulfonyl alkyne scaffold labeled DH enzymes from recombinant type-I and type-II FAS and PKS systems.[13] The lack of cross-linking between FabA and the NRPS ACPs indicates a distinct specificity in protein–protein interactions that are not satisfied with these pairs. These experiments indicate that, as seen with ACP–KS cross-linking,[7] the formation of ACP·DH complexes is driven by the native protein–protein interactivity within the ACP·DH pairing.

Finally, we wished to understand the relevance of studying pantetheinamide probe **4a** as a true surrogate of ACP–DH interactions. The suitability of this probe to mimic sequestration of the natural substrate within the hydrophobic cavity of *E. coli* AcpP was determined by solution-phase protein NMR spectroscopy. We linked **4a** to ^{15}N-labeled AcpP, purified the *crypto*-AcpP, and collected the heteronuclear single-quantum coherence (HSQC) spectrum. A comparison with the *holo*-AcpP spectrum under identical conditions is shown in the HSQC overlay and corresponding chemical shift perturbation plot in Figure 3. In particular, major perturbations of residues Asp_{35}, Thr_{39}, and Glu_{47} within helix II and Ala_{59} and Glu_{60} in helix III are indicative of substrate sequestration, as previously demonstrated for AcpP and other ACPs within type-II FAS and PKS pathways.[2d,4,16] Of particular interest are the large perturbations of Thr_{63} and Tyr_{71} in helix IV, which display interactions with the sulfonyl 3-alkynyl moiety. These results demonstrate that association with FabA allows the release of the probe from the ACP binding pocket into the active site of the enzyme and indicate that comparison of the ACP in the free and cross-linked states could shed light on the molecular details of this "switchblade" mechanism.

In summary, we have demonstrated new tools for the study of protein–protein interactions between ACPs and DHs in FAS and PKS based on the development of a pantetheinamide with a

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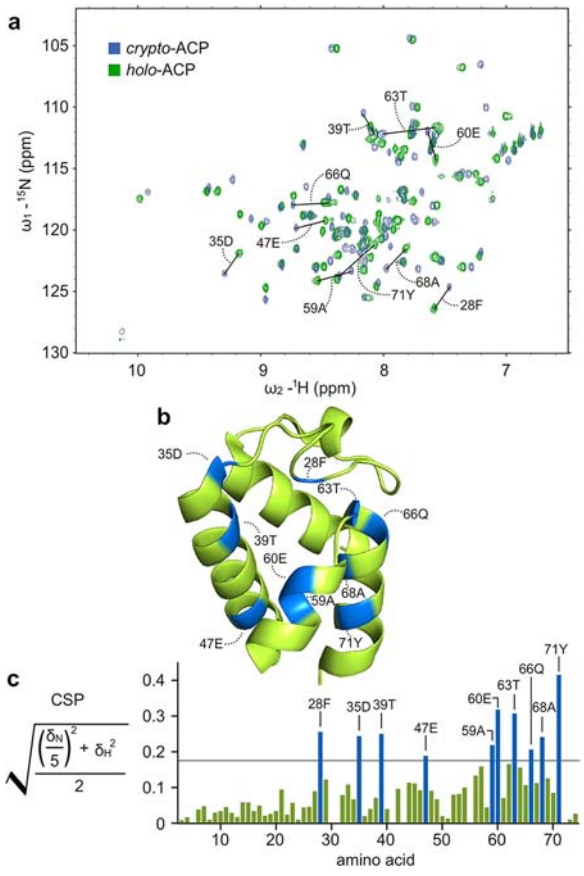

Figure 3. Solution-phase NMR spectra of *crypto*-ACP demonstrating probe sequestration. (a) $^{1}H,^{15}N$-HSQC spectra of purified *crypto*-ACP containing ^{15}N-enriched *E. coli apo*-AcpP[16b] modified chemoenzymatically with **4a** (blue) and *holo*-AcpP (green). Significant shifts are annotated by residue number. (b) Structure of AcpP highlighting the residues that showed the largest chemical shift perturbations. (c) Chemical shift perturbation plot of *crypto*-AcpP vs *holo*-AcpP.

sulfonyl alkyne scaffold. The synthesis of this probe is straightforward, offering easy access to synthetic modulation. The probe itself is stable toward one-pot chemoenzymatic methodology, is sequestered within the hydrophobic cleft of *crypto*-AcpP, and forms the designed AcpP−FabA cross-linked product in high yield. This scaffold could offer optimized pantetheine analogues for use in investigating protein−protein interactions for type-I and type-II PKS biosynthetic pathways. This sulfonyl 3-alkynyl pantetheinamide provides a general approach for elucidating interactions of ACPs with other DH-like enzymes, such as the product template (PT) domain in fungal nonreducing PKSs[17] and the thioester hydrolase (TH) domain in fungal type-I iterative PKSs.[18]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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