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# Dehydratase-Specific Probes for Fatty Acid and Polyketide Synthases

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Supporting Information

**ABSTRACT:** We targeted the development of a dehydratase (DH)-specific reactive probe that can facilitate detection, enrichment, and identification of DH enzymes in fatty acid synthases (FASs) and polyketide synthases (PKSs). The first reported mechanism-based inactivator, 3-decynoyl-*N*-acetylcysteamine (3-decynoyl-NAC), while active against the *Escherichia coli* $\beta$-hydroxydecanoyl thiol ester DH FabA, translates poorly to an activity-based probe because of nonspecific reactivity of the thioester moiety. Here we describe the design, synthesis, and utility of a DH-specific probe that contains a sulfonyl 3-alkyne reactive warhead engineered to avoid hydrolysis or nonenzymatic inactivation. When coupled with a fluorescent tag, this probe targets DH enzymes from recombinant type I and type II FAS and PKS enzyme systems and in whole proteomes. Activity studies, including FabA inactivation and antibiotic susceptibility, suggest that this sulfonyl 3-alkyne scaffold selectively targets a common DH mechanism. These studies indicate that the DH-specific mechanism-based probe can greatly accelerate both the functional characterization and molecular identification of virtually any type of FAS and PKS in complex proteomes.

Natural products have served as a primary resource in drug discovery, with a profound number of clinical antibiotics, anticancer, and immunosuppressive agents having been directly or indirectly derived from natural sources.[1] Many of these small molecules are biosynthesized by highly versatile and modular polyketide synthase (PKS) enzymes that are structurally and functionally analogous to fatty acid synthase (FAS) systems.[2] While the study of secondary metabolism has been extensively evaluated in a genetic context,[3] we have begun a program to develop proteomic techniques for isolating and identifying modular synthases that makes use of contemporary activity-based protein profiling methods.[4] This work aims to address those families of natural products that cannot be understood solely by genetic approaches.[5] Analogous in importance to the examination of the human proteome in context with the genome, proteomic study of natural product pathways remains a developing field with an urgent need for new small-molecule probes with which to interrogate the proteomes of diverse organisms. Multidomain PKS and FAS enzyme systems are particularly resistant to laboratory evaluation as recombinant enzymes, in part because of their high molecular weight and the general intractability of producer organisms to genetic manipulation and heterologous expression.[6] In addition, complicated organisms such as symbiotic ensembles are not amenable to most genetic sequencing methods that rely on pure, isolable strains. Direct profiling of microbial proteomes complements genetic approaches by allowing us to understand the activity, post-translational modification, and protein–protein interactions of these enzymes in their native and dynamic proteomic environments.

We recently developed an orthogonal active site identification system (OASIS) consisting of activity-based probes used to isolate and identify modular synthases.[4b] A current limitation of our original probe set is the limited number of domains targeted; a more complete set of probes would increase the sensitivity of the OASIS method (Figure 1). As not

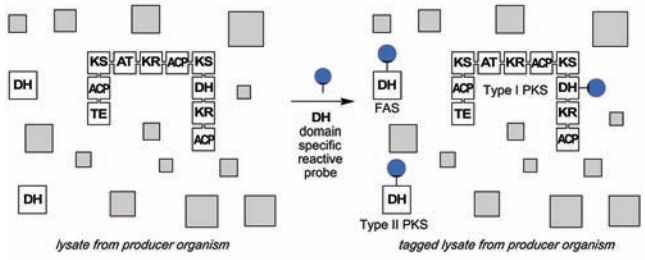


**Figure 1.** Methods for proteomic analysis of FAS and type I or type II PKS using a DH-specific reactive probe. Modules are comprised of acyl carrier protein (ACP), ketosynthase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR), and thioesterase (TE) domains. Application of a DH-specific probe is shown by the reactive coupling of a sphere.

all domains are present in each PKS and FAS biosynthetic protein, a more complete set of reactive probes may be critical to full identification.[7] The $\beta$-hydroxy acyl carrier protein (ACP) dehydratase (DH) found in all FAS pathways and any PKS whose product contains olefinic or saturated methylene units offers an attractive target for probe design. The enzyme FabA catalyzes two reactions in the *Escherichia coli* fatty acid biosynthetic pathway: dehydration of (*R*)-3-hydroxydecanoyl-ACP to (*E*)-2-decenoyl-ACP and isomerization of (*E*)-2-decenoyl-ACP to (*Z*)-3-decenoyl-ACP.[8] 3-Decynoyl-*N*-acetylcysteamine [3-decynoyl-NAC (**1**); Figure 2] has a historic place in the study of enzyme inhibition. Discovered by Bloch in 1967, **1** was shown to rapidly and irreversibly inactivate FabA in what constituted the first recognized instance of a "mechanism-based" or "suicide substrate" inhibitor,[9] where the enzyme

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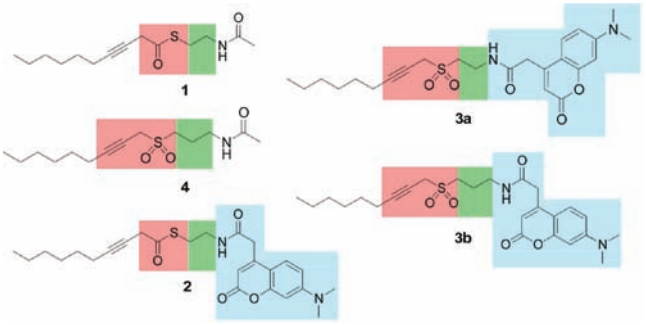


**Figure 2.** Structures of DH inhibitors. Fluorescent analogue **2** was designed as a direct mimic of 3-decynoyl-*N*-acetylcysteamine (**1**).[10] Second-generation probes **3a** and **3b** retained the mechanism-based activities of **1** and **2** with enhanced hydrolytic stability. The effects of the fluorescent tag were readily evaluated by comparison of **3a** and **3b** with **4**. The functional units in each inhibitor are indicated by color shading: the reactive warhead (red), the linker (green), and the fluorescent tag (blue).

target catalyzes the formation of a covalent bond between the small molecule and an active site residue, thus permanently inactivating the enzyme.

Our studies began with the preparation of fluorescent probe **2** (Figure 2) in order to adapt this technology to our OASIS system.[10] This probe was evaluated against the cell lysates of various microorganisms, but high levels of background fluorescence were observed (results not shown), presumably as a result of nucleophilic cleavage of the thioester bond in **2**, which would release a fluorescent thiol that in turn could form indiscriminate disulfide linkages with Cys residues in other proteins. This, along with the potential for nonenzymatic isomerization of **2** to its reactive allene followed by indiscriminate protein labeling, suggested the need for a new design.

In these studies, we found that mechanism-based inhibition of FabA by 3-decynoic acid analogues was strongly dependent on the p$K_a$ of the $\alpha$-proton of the suicide substrate, and substitution of the thioester linkage with more stable ester or amide linkages (replacement of the sulfur in **2** in Figure 2 with oxygen or nitrogen) greatly decreased or abolished the inhibition of FabA.[10] Replacement of the reactive thioester moiety with a nonhydrolyzable sulfone as in probe **3a** (Figure 2) not only eliminated the issue of nucleophilic cleavage but also provided an $\alpha$-proton with sufficient acidity to avoid nonenzymatic inactivation yet still facilitate enzymatic activation. To this end, fluorescent inhibitors **3a** and **3b** were synthesized from the convergent assembly of commercially available 2-nonyn-1-ol, cystamine for **3a** or 3-chloropropylamine for **3b**, and a fluorescent tag [see the Supporting Information (SI)]. In addition, acetylated **4** was prepared to provide an analogue of **1** for comparative activity studies.

Our biochemical studies began with an examination of the abilities of **3a** and **3b** to label recombinant FabA. Monitoring by SDS-PAGE analysis showed clear labeling of FabA by both inhibitors **3a** (Figure 3a) and **3b** (Figure 3b) that was abrogated by pretreatment of FabA with sodium dodecyl sulfate (SDS). Subsequent screening allowed us to identify an optimal pH for each labeling process. As shown in Figure 3a, probe **3a** underwent increased nonspecific labeling with increasing pH. In contrast, probe **3b** (Figure 3b) was less affected, suggesting that inclusion of a longer alkyl linker reduced the pH effects. We conducted time-course studies by

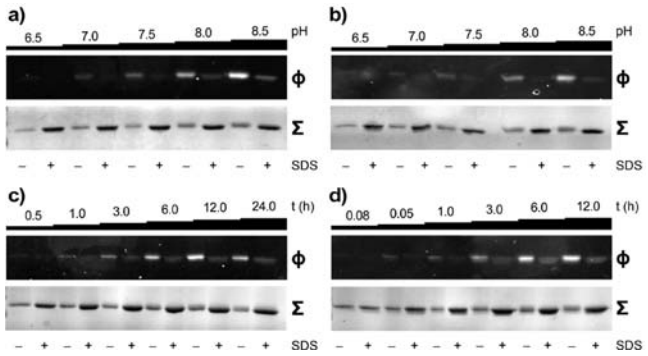


**Figure 3.** Labeling of recombinant FabA with probes **3a** and **3b**. (a, b) SDS-PAGE analysis denoting the labeling of 0.1 mg/mL FabA in 100 mM Tris-HCl for 12 h at 37 °C with (a) 25 $\mu$M **3a** or (b) 25 $\mu$M **3b**. (c, d) Time-course studies of the labeling of FabA with probe **3b**. SDS-PAGE analysis denoting the labeling of 0.1 mg/mL FabA in 100 mM Tris-HCl at 37 °C with 25 $\mu$M probe **3b** at (c) pH 8 or (d) pH 8.5. For each panel, Φ depicts the fluorescence observed with $\lambda_{ex}$ = 370 nm and $\lambda_{em}$ = 460 nm and Σ displays the total protein content by staining with colloidal Coomassie Blue.

evaluating the labeling of FabA at pH 8.0 (Figure 3c) and pH 8.5 (Figure 3d), where maximum labeling was observed at 12 and 6 h, respectively. In contrast, hydrolytically sensitive inhibitor **2** (Figure 2) showed greater nonspecific (denaturation-insensitive) labeling of FabA with increasing pH (results not shown). The evidence provided in Figure 3 indicates that the $\alpha$-protons in probes **3a** and **3b** were accessed by the enzyme and that the improved stabilities of these probes allowed successful labeling of FabA in basic media (pH $\geq$ 8). The relative labeling of FabA by probe **3b** was estimated as 32.6% at 12 h at pH 8.0 using a *crypto*-ACP with coupled fluorescent phosphopantetheine analogue as a standard of fluorescence intensity (see Figure S1 in the SI).[11]

To evaluate the specific activities of these probes against FabA, a spectrophotometric assay that monitored the decrease of crotonyl-CoA at 260 nm was used to determine $IC_{50}$ values.[12] Probes **3b** and **4** displayed dose-dependent activities against recombinant *E. coli* FabA, with calculated $IC_{50}$ values of 7.8 $\pm$ 1.1 and 5.6 $\pm$ 1.0 $\mu$M, respectively (Figure S2). In addition, initial rates of inactivation by probe **4** were measured.[13] As expected, the enzyme inactivation was irreversible. The kinetic parameters $k_{inact}$ and $K_i$ were evaluated to be 0.018 $\pm$ 0.004 min$^{-1}$ and 1.32 $\pm$ 0.43 mM, respectively (Figure S3). In light of the precise stereochemical and mechanistic details of FabA inactivation by **1**,[8] we propose that probes **3a** and **3b** act via the mechanism shown in Scheme 1. $\alpha$-Deprotonation of sulfonyl 3-alkyne **5** forms electrophilic allene **6**, which in turn is modified by an active-site histidine residue of FabA (**7** and **8**). The experiments here suggest that decreasing the chemical formation of the allenic intermediate by p$K_a$ elevation of the $\alpha$-proton of the sulfonyl alkyne scaffold successfully retains the activity while improving the stability against nucleophilic decomposition at pH 6.5–8.5 relative to inhibitor **2**.

Our work with the first-generation DH probe **2** showed cross-reactivity with FabB and FabF of *E. coli* FAS (two $\beta$-ketoacyl-ACP synthases) as well as nonselective labeling with proteins such as bovine serum albumin (BSA). In contrast, incubation of FabA, FabB, FabF, and BSA with 25 $\mu$M **3b** for 12 h at 37 °C at pH 8.0 or 8.5 resulted in preferential labeling of FabA with only modest labeling of FabB and FabF and


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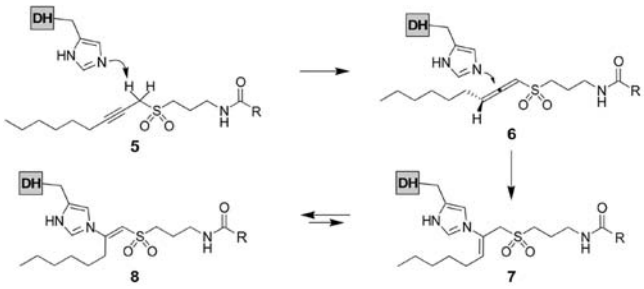
**Scheme 1. Predicted Mechanism of Enzyme Inhibition**[a]




[a]On the basis of the known activity of 3-decynoyl-NAC, FabA should be inhibited by **5**, which would irreversibly inhibit FabA through formation of an electrophilic allenic intermediate **6**. Alkylation of an active-site histidine would yield **7** and **8**. R denotes the position of a tag such as in **3b**.

virtually no labeling of BSA (Figure S4). With knowledge of the differential active-site chemistry, this result indicates improved targeting of the DH domains.

We next asked whether **3b** could be used to probe endogenous dehydratase activity in proteomes. We began with an analysis of the conditions using probe **3b** incubated with unfractionated *E. coli* lysates to which recombinant FabA had been either added (spiked) or omitted (native) for 12 h at 37 °C at pH 8. Proteins were precipitated with trichloroacetic acid (TCA), washed with cold acetone to remove unreacted probe, and subsequently resolubilized and analyzed by in-gel fluorescence. Concentration-dependent labeling of FabA was seen in the proteomic environment (Figure 4a) that closely

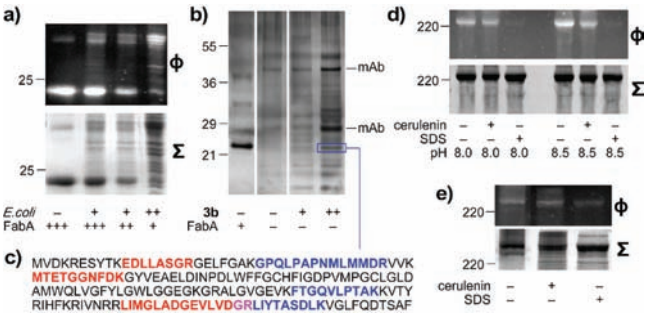



**Figure 4.** Proteomic applications of DH-specific reactive probe **3b**. (a) Labeling of K12 *E. coli* lysate spiked with recombinant FabA by **3b**. (b) Silver-stained SDS-PAGE gel depicting immunoprecipitation of FabA from K12 *E. coli* lysate (right two lanes), with purified FabA standard (left lane) and increasing concentrations of probe **3b**. (c) LC/MS–MS analysis identified the isolated band (blue box) as FabA with over 40% peptide coverage. Amino acids of the identified peptides are colored either in red or blue. (d) Labeling of recombinant mycocerosic acid synthase (MAS) from *M. tuberculosis*. (e) Labeling of the DH domain of type I FAS in human SKBR3 lysate. For each panel, Φ depicts the fluorescence observed with $\lambda_{ex}$ = 370 nm and $\lambda_{em}$ = 460 nm and Σ displays the total protein content by staining with colloidal Coomassie Blue. Full gels (Figures S5 and S12) and experimental procedures are provided in the SI.

resembled the results with isolated FabA. Furthermore, pretreatment of FabA by SDS resulted in an absence of protein labeling (Figure S12b). Taken together, these experiments indicate that **3b** is a true mechanism-based inactivator that shows little to no cross-reactivity with other enzymes in the proteome. Also, the increasing specificity and markedly low background of **3b** show that the sulfonyl alkyne scaffold is an optimal design for applications involving specific labeling of endogenous dehydratase activity from crude cellular lysates.

To isolate the cellular target of probe **3b**, we performed immunoprecipitation (IP) using an antibody targeted to the coumarin tag (XRI-TF35 mAb) linked to Affi-gel resin.[14] *E. coli* lysate was incubated with **3b**, and labeled proteins were isolated by purification with antibody-immobilized resin. SDS-PAGE of the pulldown eluant revealed a band at 23 kDa with silver staining (Figure 4b). This observation was dependent on the concentration of **3b**. A sample of the excised band was submitted for LC–MS/MS protein identification and found to contain the endogenous *E. coli* FabA with over 40% peptide coverage (Figure 4c). These results validate the use of **3b** as a selective isolation and identification tool for DHs within the proteomic environment.

Next, we evaluated the ability of probe **3b** to identify a model type I PKS. While the above characterization of **3b** focused on a type II DH in FabA, the dual-activity dehydratase/isomerase FabA in *E. coli* FAS, the DH domains in type I PKSs serve only a dehydratase function and therefore exhibit different active-site structures.[15] We chose to investigate the labeling of mycocerosic acid synthase (MAS), which produces methyl-branched fatty acids in *Mycobacterium tuberculosis*. MAS is an iterative type I PKS producing C27–C32 mycocerosic acids from straight-chain precursors and contains the domain structure KS-AT-DH-ER-KR-ACP.[16] Recombinant MAS (Figure S6) was successfully labeled by treatment with **3b** (Figure 4d). Treatment of MAS with the KS inhibitor cerulenin prior to administration of **3b** did not result in loss of labeling.[17] The relative maximal labeling of MAS by probe **3b** was estimated as 48.2% at pH 8.0 and 68.1% at pH 8.5. Since DH in type II FAS and type I PKS possess distinct active-site structures, the similar labeling patterns suggest that **3b** is sensitive to a shared catalytic mechanism, namely, proton abstraction by an active-site His-Asp dyad. This sulfonyl alkyne scaffold provides a general activity-based probe for common DH-like enzymes, further suggesting utility for the product template (PT) domain in fungal nonreducing PKSs[18] and the thioester hydrolase (TH) domain in fungal type I iterative PKSs.[19]

As an ultimate test of proteomic activity, we evaluated the availability of our dual-labeling strategies in a proteomic context by applying them to a model system for type I FAS. The human breast cancer cell line SKBR3 is known to produce FAS at detectable levels, a characteristic phenotype associated with aggressive tumor growth.[20] To determine the optimal labeling conditions and provide additional evidence, SKBR3 cell lysates were adjusted to pH 8.0 or 8.5 and probed with **3b** for 12 h at 37 °C. SDS-PAGE analysis of these reactions showed no distinguishable signal for a high-molecular-weight (HMW) band at pH 8.0 but specific labeling of a HMW band corresponding to the size of the human FAS at pH 8.5 (Figure S7). Preincubation with cerulenin did not disrupt the labeling (Figure 4e, middle lanes), while denaturation by treatment with SDS greatly decreased the labeling (Figure 4e, right lanes). Notably, the HMW FAS protein was the only labeled species in this proteome. This finding corresponds identically to our previous results with a labeled protein from SKBR3 cell lysate using ACP, KS, and TE-specific probes.[4a]

Finally, since **1** has been shown to possess antibiotic activity,[9c] we evaluated the antimicrobial activity of probe **3b** and acetyl derivative **4** (Figures S8 and S9). In liquid culture, both **3a** (MIC of 501 μM, or 238 μg/mL) and **4** (MIC of 1235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$\mu$M, or 355 $\mu$g/mL) delivered antibiotic activity comparable to that of **1**.[9c] These results suggest that **3b** and **4** share a common mode of action in reducing bacterial growth by targeting DHs in live bacterial cells. This, along with the specificity studies demonstrated here, suggests that these probes may also have utility for *in vivo* as well as *in vitro* applications.

In summary, we have demonstrated new tools for the study of DH enzymes in FASs and PKSs based on the development of a DH-specific fluorescent probe with a novel sulfonyl alkyne scaffold. We have demonstrated that this scaffold shows antibiotic activity through selective targeting of DH enzymes. The synthesis of this probe is straightforward, offering easy access to synthetic derivatives, and the scaffold itself is stable toward a variety of *in vitro* and *in vivo* conditions. Fatty acid biosynthesis remains an attractive target for the development of novel antimicrobial chemotherapeutics,[21] and this scaffold could offer immediate development of antibiotic analogues. In addition, we have determined the optimal conditions for DH labeling and visualization by in-gel fluorescent analysis and established the activity of this probe both in purified proteins and with proteomic samples. Applications of this technique include gel-based inhibitor screens,[22] identification of active-site residues from uncharacterized enzymes,[23] and selective isolation and identification of DH-containing enzymes. Combinations of this DH probe with other domain-specific probes should improve the detection, enrichment, and high resolution of low-abundance proteins in proteomic samples, and we are currently applying such combinations to the discovery of natural product pathways in sequenced and unsequenced organisms.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Supporting figures; procedures for the syntheses of **3a**, **3b**, and **4**; complete gel images; and full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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