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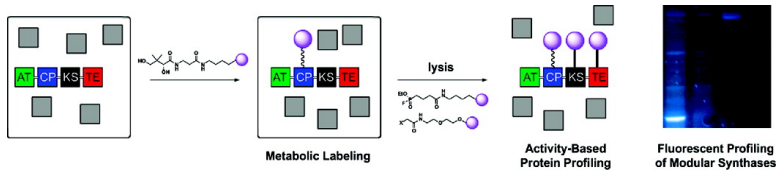

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Fluorescent Profiling of Modular Biosynthetic Enzymes by Complementary Metabolic and Activity Based Probes

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Natural products compose a wide-ranging milieu of antibiotic and anticancer drug leads, virulence factors, and signaling molecules. Many of these small molecules are produced by highly versatile and modular polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS), or PKS−NRPS hybrids[1] that are structurally and functionally analogous to fatty acid synthase (FAS) systems.[2] The past 25 years have seen significant progress in the isolation and structure elucidation of PKS and NRPS small molecule metabolites and the genetics and enzymology of the modular synthases which produce them.[3] Proteomic studies of natural product producers have lagged behind, in part because of increasingly streamlined genomic approaches which allow access to natural product gene clusters and recombinant enzymes. However, some of these enzymes, particularly multidomain PKS and NRPS systems, are resistant to study as recombinant enzymes both due to their large size and the intractability of their producer organisms to genetic manipulation and heterologous expression.[4] Direct profiling of microbial proteomes could prove highly complementary to genetic approaches by allowing us to understand the activity, transcriptional control, and post-translational modification of these enzymes in their native and dynamic proteomic environments.

A common feature of PKS, NRPS, and FAS systems is the use of carrier protein (CP) domains as a scaffold for the tethering and elongation of biosynthetic intermediates.[5] The site of this enzymatic tethering is the thioester of a post-translationally appended, coenzyme A (CoA) derived 4′-phosphopantetheine group. We have described two methods which utilize this unique post-translational modification for fluorescence/affinity labeling of CP domains in proteomic environments, first through an in vitro chemoenzymatic approach in which CoA analogues such as **1** along with the promiscuous phosphopantetheinyltransferase (PPTase) Sfp[6] are used to label and enrich CPs in crude cell lysate (Figure 1A), and more recently through the cellular uptake and in vivo metabolic pathway incorporation of fluorescent (**2**) or bioorthogonally tagged CoA precursors (Figure 1B).[7,8] While the specificity of these methods for the labeling of CP domains is ideal in many respects, it also restricts the information CP labeling methodologies can offer in terms of the type (PKS, NRPS, or FAS), identity, or alternate activities present in the labeled modular synthase. CP labeling methods are also reliant on a lack of either endogenous CP-phosphopantetheinylation or substrate promiscuity in an organism's CoA biosynthetic pathway, both of which represent potential limitations.

The focus of the present study is the supplementation of these CP based protein profiling approaches with activity based protein profiling (ABPP).[9] ABPP is a proteomic method which utilizes irreversible enzyme inhibitors, specific for a given enzyme class, labeled with fluorescent or affinity reporters to divide and classify a proteome based on enzyme activity. While such probes are not specific for PKS or NRPS enzymes, type I modular synthases of this type are uniquely susceptible to such a method of interrogation due to the presence of multiple active sites on a single polypeptide, each of which can be potentially targeted by an activity based probe (Figure 1C). The combination of CP-specific labeling methods with the proteome wide reactivity of activity based probes offers a powerful method for the identification, domain characterization, and inhibitor discovery of these biosynthetic enzymes.

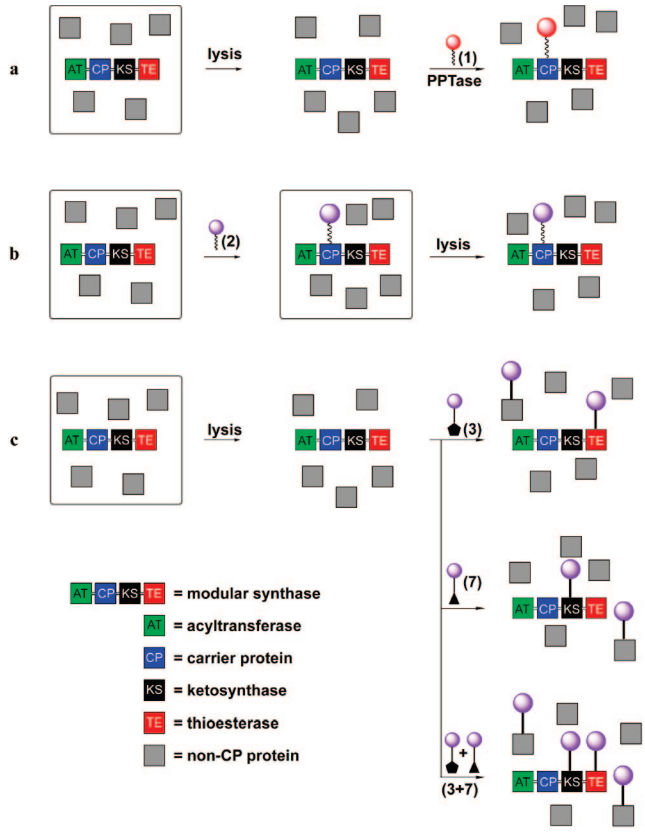

Figure 1. Methods for the proteomic analysis of FAS, PKS, and NRPS enzymes (PKS pictured). For probe structures, see Figure 2. (A) Chemoenzymatic labeling of apo-CP domains by CoA analogue **1** and Sfp in crude cell lysate. (B) Metabolic labeling of CP domains by uptake, biosynthetic processing, and in vivo labeling by fluorescent pantetheine analogue **2**. (C) Activity based protein profiling of KS and TE domains by probes **3** and/or **7**.

To test the feasibility of this approach, we first generated a panel of fluorescently labeled activity based probes (Figure 2, **3**–**8**) and tested them for in vitro reactivity against a number of purified PKS, NRPS, and FAS hydrolytic enzymes. The fluoro-

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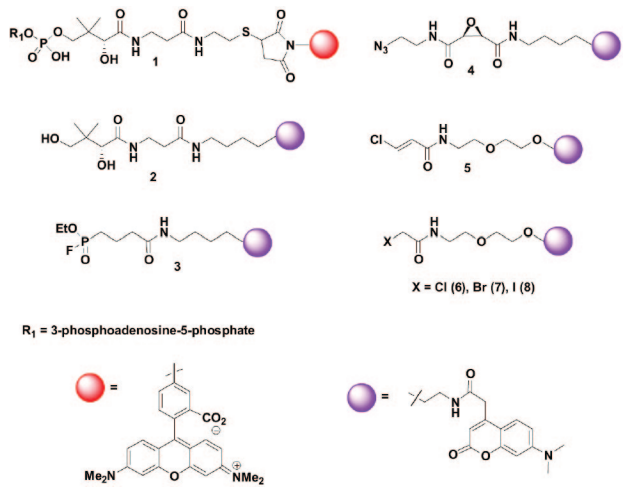

Figure 2. Chemoenzymatic tag (**1**), metabolic label (**2**), and activity based probes (**3**−**8**) utilized in this study.

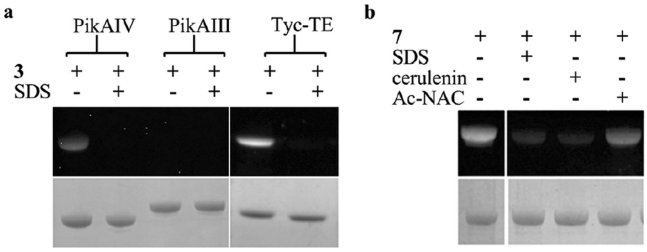

Figure 3. (A) In vitro labeling of purified recombinant PKS and NRPS acyltransferase (AT) and thioesterase (TE) domains by fluorophosphonate **3** (25 uM). (B) In vitro labeling of purified recombinant PikAIV PKS by bromoacetamide **7** (12.5 uM). PikAIII = module 3 of pikromycin PKS containing AT, CP, KR, and KS domains. PikAIV = module 4 containing AT, CP, KS, and TE domains. Tyc-TE = excised tyrocidine NRPS TE domain.

phosphonate warhead of probe **3** is a specific inhibitor of serine hydrolases which has been extensively applied toward a multitude of ABPP applications in eukaryotic systems.[10] Multimodular PKS enzymes utilize two distinct serine hydrolases during biosynthesis, an acyltransferase (AT) domain for substrate loading, and a thioesterase (TE) domain for chain termination. To test the specificity of **3**, we incubated it with recombinant enzymes from the pikromycin PKS and found it showed strong labeling of only TE containing domains (PikAIV, Figure 3A).[11] Probe **3** also showed strong labeling of the excised NRPS TE domain from the tyrocidine synthase. In both cases, this signal was lost upon active-site disruption by addition of detergent (SDS). This indicates the reactivity of **3** with modular biosynthetic enzymes should be limited to the labeling of terminal TE containing modules (Figure 3A).

For the labeling of PKS ketosynthase (KS) cysteine esterases, we synthesized **4**, a duel bioorthogonal/fluorescently labeled analogue of the well-known KS inhibitor cerulenin.[12] Unfortunately, this probe showed low levels of labeling of KS enzymes at concentrations up to 300 uM, as did a similarly labeled chloroacrylamide[13] **5**, making them technically unfeasible for use as activity based reagents (Supporting Information Figure S1). While chloroacetamide **6** showed slightly higher levels of labeling, it was haloacetamides **7** and **8** which showed the greatest SDS and cerulenin-sensitive labeling of KS enzymes at low (<25 μM) probe concentrations.[14] Although these reagents are known as nonspecific cysteine alkylating reagents at high concentrations, we found that performing our labeling

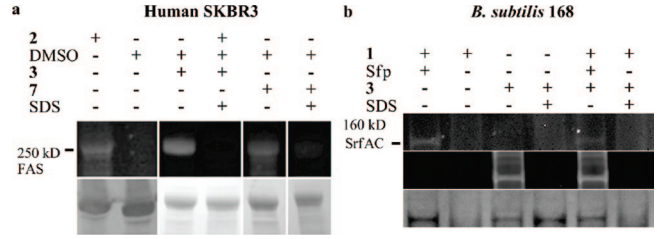

Figure 4. Metabolic labeling, chemoenzymatic labeling, and ABPP of eukaryotic and prokaryotic type I modular synthases. (A) FAS labeling of SKBR3 by metabolic tag **2**, probe **3**, or probe **7**. Top: fluorescence. Bottom left: anti-FAS blot. Bottom middle/right: Coomassie. (B) Chemoenzymatic and activity based labeling of *Bacillus subtilis* strain 168 by **1**/Sfp and **3**. Top: 532 nM excitation of probe **1**. Middle: UV excitation with 437 nM emission filter for visualization of probe **3**. Bottom: Coomassie. Note the SrfAC signal observed on coadministration of Sfp, **1**, and **3**.

reactions with 12.5 μM **7** or **8** in the presence of high (10 mM) concentrations of the scavenging nucleophile DTT led to active-site-directed labeling of the KS domain of PikAIV, as inferred from SDS-sensitive labeling and the decrease in labeling intensity observed upon preincubation of PikAIV with KS-reactive agents cerulenin or diacetyl cystamine (Ac-NAC) (Figure 3B).[15]

With these ABPP tools in hand, we first tested the compatibility of our duel labeling strategies in a proteomic context by applying them to a model system for PKS labeling, the eukaryotic FAS. The human breast cancer cell line SKBR3 is known to produce FAS at high levels, a characteristic phenotype associated with aggressive tumor growth.[16] SKBR3 cells were grown under standard tissue culture conditions in the presence of either 1 mM metabolic label **2** or vehicle DMSO for 48 h. Workup and analysis of the cytosolic fraction showed that, in contrast to the DMSO treated control, cells grown in the presence of fluorescent pantetheine analogue **2** show specific labeling of a high molecular weight (HMW) band corresponding to the approximate size of the human FAS (Figure 4A). Fluorophosphonate **3** and haloacetamide **7** showed SDS-sensitive labeling of the same band, confirmed as FAS by LC MS/MS analysis and treatment with an anti-FAS antibody, although **7** suffered from higher levels of nonspecific background than **2** and **3** (Figure 4A). Notably, FAS was the only protein which appeared to possess labeling with **2**, **3**, and **7**, demonstrating the complementarity of the probe set. We also performed competitive ABPP[10b] by preincubating SKBR3 lysate with known FAS inhibitors orlistat and cerulenin prior to administration of **3** or **7**. The results showed that orlistat decreased FAS labeling by **3** but not **7**, while cerulenin decreased FAS labeling by **7** but not **3** (Figure S2).[17] This is indicative of the ability of these probes to delineate the domain specificity of inhibitors of modular synthases in complex proteomes.

Finally, we sought to apply these probes directly to the analysis of natural product producer proteomes. *Bacillus subtilis* was the first sequenced Gram-positive bacteria and represents a model organism which is known to produce small molecule natural products characteristic of multidomain PKS and NRPS synthases.[18] Strain 168 of this organism contains a mutation in the allele coding for its secondary metabolism PPTase, lowering the amount of endogenous CP-phosphopantetheinylation and making it susceptible to our chemoenzymatic CP labeling technique (Figure 1A). Strain 6051 contains the wild-type allele, whose endogenous PPTase activity is necessary for metabolic

labeling by CoA precursor **2** (Figure 1B). Both strains in turn should be accessible by our activity based labeling strategy (Figure 1C).

Accordingly, *B. subtilis* 168 and 6051 were grown up to late log phase, lysed, and labeled chemoenzymatically by **1** and Sfp. The Sfp-dependent labeling of several HMW bands was observed in strain 168 but not strain 6051 (Figure 4B and Figure S3). Since labeling by our chemoenzymatic method can be blocked by endogenous phosphopantetheinylation in wild-type strain 6051, we also grew 6051 in the presence of metabolic label **2** and observed no fluorescent labeling of the HMW bands characteristic of multidomain modular synthases (Figure S4).

Having thus profiled the carrier proteome of *B. subtilis*, we sought to use ABPP to further characterize the CP labeling in strain 168. Significantly, none of the HMW bands showed active-site-dependent labeling by KS probe **7**, leading us to tentatively assign them the role of NRPS proteins. Administration of **3** to strain 168 showed strong denaturation-sensitive labeling of a ~150 kD protein corresponding to a band also labeled by **1** (Figure 4B). Inspection of the *B. subtilis* genome[19] led us to identify this protein as SrfAC, the terminal module of the surfactin synthase, on the basis of size, presence of CP and TE domains, and apparent absence of a KS domain. Band excision and MS/MS analysis confirmed this hypothesis, demonstrating 22% sequence coverage of the 144 kD polypeptide. MS analysis also provided evidence as to the site-specific nature of these probes as S1003, which represents the unmodified catalytic serine of the CP domain, and was found in MS spectra of samples treated with **3** but not **1**. Applying **3** to wild-type 6051 lysate, we did not observe similar labeling of this ~150 kD band, a finding which combined with the lack of CP labeling and differences in the Coomassie stain (Figure S5) led us to conclude this strain did not produce Srf proteins under the two growth conditions examined.

In summary, we have shown that metabolic and chemoenzymatic methods of CP labeling can be used in combination with ABPP to probe inhibitor specificity, assign domain structure, and assign identity of natural product producing modular synthases in vitro and in vivo. Of particular significance is the highly complementary nature of the methods used, which together provide a level of information not available to either of these methods of analysis alone. The results of our protein profiling of *B. subtilis* indicate this technology should be immediately useful for the analysis of natural product gene expression at the protein level. Using ABPP metabolic labeling as a link between genomic analysis and natural product isolation would help obviate one of the common difficulties of natural product genome mining by providing a simple and general assay for the expression of PKS and NRPS gene clusters.[20] While this is commonly followed by RT-PCR, since ABPP probes are not specific for PKS and NRPS enzymes, they may be useful in identifying other enzymes upregulated during natural product expression and delineating signaling pathways involved in secondary metabolite production. Such associations could prove highly valuable in terms of engineering biosynthetic pathways and providing new targets for the inhibition of production of PKS and NRPS virulence factors.[21] A current limitation of our activity based probe set is the small number of domains (KS and TE) targeted, which are absent in many modular biosynthetic enzymes; the design and synthesis of ABPP reagents targeting additional domains should be aided by growing knowledge of structure and inhibition of FAS and NRPS proteins.[22,23] The methods presented here should also lend themselves readily to enrichment and coupling to online tandem MS strategies which have demonstrated significant advantages in the detection and resolution of low-abundance proteins in proteomic samples.[9,24] Such technology has already been shown to be a powerful and highly compatible method for the analyses of purified modular synthases.[25] We are currently applying these complementary methods toward studies of secondary metabolism in a variety of natural product producer organisms.

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Supporting Information Available: Supplementary figures, procedures for the synthesis of probes **1**–**8**, 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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