CBI Student Sabbatical Proposal:
Characterization of Two Type I Polyketide Sythases Methyltransferase Domains from the Curacin A Biosynthetic Pathway

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Sabbatical Host: David H. Sherman
Location: Life Sciences Institute, University of Michigan
Duration: 8 weeks
Timeframe: TBD

Summary: I will use a new enzymatic approach to generate acyl carrier protein (ACP) linked substrates from commercially available fatty acids. These substrates will be used to investigate the timing of the C-methylation in the CurJ module and the substrate specificity of the CurL O-methyltransferase.
Background

Natural products represent a significant source of pharmaceutical compounds (1). The large number of chiral centers and similarly reactive chemical groups in many natural products make them challenging targets for traditional synthetic methods. The modular nature of type I bacterial polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) pathways has inspired efforts to engineer novel pathways or utilize portions of pathways for hybrid chemo-enzymatic synthetic routes (2,3). In order to effectively design pathways it is essential to understand the substrate specificity and regiochemistry of each catalytic domain. Most type I PKS modules contain, minimally, an acyltransferase (AT) domain to select a new acyl building block and load it onto an acyl carrier protein (ACP) domain and a ketosyntase (KS) domain to extend the polyketide intermediate by decarboxylative condensation of the upstream intermediate and the new ACP-loaded building block. Similarly, in NRPS pathways an adenylation (A) domain selects a new amino acid and loads it onto the neighboring peptidyl carrier protein domain. Additionally, many modules contain catalytic domains that modify the backbone through reduction (KR, ER), dehydration (DH), methylation and epimerization. Finally, the product of the pathway is released by a thioesterase domain, producing either a linear or cyclized product. Throughout the pathway, intermediates are covalently attached to an ACP domain via a phosphopantetheine prosthetic group derived from coenzyme A (CoA) (4).

Curacin A, produced by the marine cyanobacterium *Moorea producens*, is a small molecule with anticancer properties (5). The curacin biosynthetic pathway contains both PKS and NRPS modules (6). Two of the PKS modules contain methyltransferase (MT) domains not commonly observed in PKS pathways. The C-methyltransferase in the CurJ polypeptide methylates the \( \alpha \)-carbon of the intermediate. The CurJ module also contains KR and DH domains (Figure 1A). The order of canonical modification reactions in PKS is known (KR then DH) but when the methylation event occurs in the CurJ reaction sequence has not been investigated, *i.e.* the MT substrate could be the \( \beta \)-keto, \( \beta \)-hydroxy or \( \alpha \)-\( \beta \)-unsaturated intermediate. The CurL module contains an \( O \)-methyltransferase domain that methylates the \( \beta \)-hydroxyl produced by the ketoreductase domain (Figure
As there are no other modification domains in the module, the order the reactions in CurL is clear but the substrate specificity of the CurL O-MT has not been investigated.

Recently, Eli Eisman, a graduate student in David Sherman’s group, has characterized a novel acyl-activating (AA) domain from an olefin synthase (OLS) in *Synechococcus sp.* PCC 7002. This domain is responsible for selecting, activating and loading a fatty acid onto an ACP for further modification by a PKS-like module to generate a hydrocarbon (Figure 1C). This enzymatic domain is a promising new tool for characterizing PKS modules, as it may be able to load hydrophobic carboxylate substrates onto carrier proteins to generate substrate mimics. I propose to use my sabbatical to develop this tool for the production of ACP linked substrates to investigate the reaction order of the CurJ module and the substrate specificity of the CurL O-methyltransferase.

**Preliminary Work**

I have identified the domain boundaries for the CurJ C-MT and CurL O-MT through sequence alignments and sub-cloned the isolated MT domains and their cognate carrier protein domains. These constructs have been expressed and purified in the Smith lab. The isolated MT domains are currently in crystallization trials. I have also demonstrated that the carrier proteins can be loaded using a recombinant phosphopantetheinyl transferase (Svp) to transfer acyl groups from commercially available CoA-linked substrates. The MT domains are not active on the commercially available substrate analogs, which are short four carbon chains.

**Proposed Experiments**

I would like to use my sabbatical to characterize the substrate specificity of the CurJ and CurL mehyltransferase domains. The acyl-activating domain will be used to load fatty acid substrate analogues onto the CurJ and CurL ACP domains that can be assayed as substrates for the respective methyltransferases. These long chain substrates will likely be better analogues than the previously tested substrates. For the investigation of the CurJ C-MT, 3-oxododecanoic acid, 3-hydroxyoctanoic acid, 2-octenoic acid and octanoic acid can be used as mimics for the KS, KR, DH and ER products respectively.
The substrate specificity of the CurL O-MT will be investigated similarly. The Sherman lab has recently acquired a library of 3-hydroxy fatty acids. I will load fatty acid substrates of varying lengths including 3-hydroxyoctanoic, 3-hydroxydodecanoic and 3-hydroxypalmitic acid to test the chain length preference of the CurL O-MT (Figure 2B). The fatty acid substrates will be incubated with the AA domain, ACP domain and ATP, excess substrate removed by buffer exchange and then incubated with the methyltransferase domain and S-adenosylmethionine cofactor (Figure 2C). The progress of both the loading reaction and the methylation assay can be measured using intact protein mass spectrometry in the University of Michigan Mass Spectrometry Core.

In the event that the AA domain cannot be used to prepare ACP substrates I will use an established chemoenzymatic approach to generate ACP substrates. The fatty acids described previously can be linked to CoA using established conditions (7). The CoA-linked substrates will then be loaded onto purified ACP domains using the recombinant phosphopantetheinyl transferase Svp from *Streptomyces verticillus* (8). This approach has been used in the Sherman lab.

Relationship to Thesis and Training

My previous research project involved the structural characterization of a post-PKS sugar methytransferase. This work included protein crystallization, site directed mutagenesis, HPLC based activity assays and binding experiments. The proposed sabbatical is tangential to my previous work and involves characterization of methyltransferases within the context of non-canonical PKS modules. This work will include new biochemical and analytical techniques and may involve some chemical synthesis. The proposed sabbatical includes the investigation of biological questions and the development of a new tool, the AA domain, for investigating PKS and NRPS systems. This method will be useful for future experiments in both the Sherman and Smith groups.

References


Figure 1: Polyketide synthase modules described in the text.
Figure 2: Proposed substrates and enzymatic synthesis of ACP linked substrates.