CBI Student Sabbatical Proposal:  
Synthesis of Substrate Mimics to Probe Substrate Specificity in Polyketide Synthase Ketoreductase Domains

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Sabbatical Host: David H. Sherman  
Location: Life Sciences Institute, University of Michigan  
Duration: 10 weeks  
Timeframe: Spring or Summer 2012

Summary: Substrates that mimic late intermediates in the Tylosin and Erythromycin biosynthetic pathway will be synthesized as SNAC thioesters. These compounds will be used to probe substrate specificity in PKS ketoreductase domains and examine the impact of chain length on the stereochemistry of reduction.
Background

Natural products represent a significant source of pharmaceutical compounds (Newman and Cragg, 2007). 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 polyketide synthase (PKS) pathways has inspired efforts to engineer novel pathways or utilize portions of pathways for hybrid chemo-enzymatic synthetic routes. In order to effectively design pathways it is essential to understand the substrate specificity and regiochemistry of each reaction. These PKS modules, which have a similar architecture to mammalian fatty acid synthase, contain an acyltransferase (AT) domain that selects a new acyl building block and loads it onto an acyl carrier protein (ACP) domain. A ketosyntase (KS) domain performs a decarboxylative condensation between the upstream intermediate and the newly loaded ACP to extend the polyketide intermediate (Weissman et al, 1997). Additionally, many modules contain catalytic domains that modify the polyketide backbone at the β-position, through reduction or dehydration. Finally, the product of the PKS pathway is released by a thioesterase domain, producing either a linear or cyclized (macrolactone) product. Throughout the pathway, intermediates are covalently attached to an ACP domain via a phosphopantetheine prosthetic group derived from coenzyme A (CoA).

The ketoreductase (KR) domain of PKS pathways is responsible for the reduction of a β-carbonyl to an alcohol, thereby introducing a chiral center into the polyketide backbone (Holzbaur et al, 1998, Kao et al, 1998). There is also evidence that the KR domain may alter the stereochemistry of an α-substituent (Valenzano et al, 2009). Sequence alignments have implicated two sequence motifs in determining the stereochemistry of the alcohol product (Caffrey, 2003). However, the mechanism by which these residues influence the stereochemical outcome of reduction is not apparent from the structures of several KR domains in the absence of substrates (Keatinge-Clay and Stroud, 2006; Keatinge-Clay, 2007; Zheng et al, 2010). To date, biochemical experiments have utilized small molecule substrates and have provided a mixture of product stereochemistries (Zheng et al, 2010; Holzbaur et al, 2001). This may be because small molecule substrates lack the phosphopantetheine arm and acyl-carrier protein domain of native substrates that may play a critical role in properly orienting substrates in
the KR active site or that the acyl chains of model substrates are too short for selective positioning. It has been demonstrated that individual domains of PKS modules can be expressed and incorporate N-acetylcysteamine (SNAC) substrates with CoA extender units to generate products in a manner analogous to native pathways (Chen et al, 2007). I plan to synthesize substrates that can be used to perform experiments with substrates covalently attached to ACP domains.

Preliminary Work

Preliminary work in the Smith lab has focused on determining the structures of KR domains fused to substrate-loaded ACP domains to observe how substrates are positioned in the active site to generate specific stereochemical outcomes. For this investigation we will focus on the KR domains from 6-deoxyerthyonolide B synthase (DEBS), tylosin synthase (Tyl) and pikromycin synthase (Pik) because we can place these results in the context of existing literature on KR domains. Jamie Razelun in the Smith Lab cloned constructs of natural KR-ACP didomains. I have expressed, purified and loaded the KR-ACPs with a small substrate mimic prior to screening for crystallization conditions. Currently, I am using commercially available CoAs as the substrate or product analogue. I have demonstrated that the KR domains are active on CoA substrates through an assay that monitors consumption of NADPH. I would like to perform KR assays with natural ACP-linked substrates of varying length. In preparation for these experiments, I have cloned excised KR and ACP domains and am currently expressing and purifying them. I have planned experiments that will use commercially available CoAs with PKS domains to make substrates for KR domains that are carried by their cognate ACP domains. This approach presents substrates on ACP domains as they are in native pathways however; the commercially available CoAs lack the functional groups present on natural PKS intermediates. The synthetic substrates presented below will provide a means to biochemically investigate the KR domains with more precision than currently planned experiments.
Proposed Work

A substrate that mimics the product of Tyl module 6 and DEBS module 5 (6) will be synthesized as an N-acetylcysteamine derivative. Using the same synthetic strategy we can also generate a short-chain substrate analog (5) to examine the impact of chain length on stereochemical outcome (Figure 1). These compounds can be used to generate a free-standing ACP substrate for the KR domains of the downstream modules in an enzymatic fashion using the native ketosynthase-acyltransferase and acyl carrier protein domains and (methyl)-malonyl CoA (Figure 2). The sabbatical with Dr. David Sherman will give me the opportunity to synthesize specific substrates with proper chirality, and provide me with tools to carry out a biochemical investigation of KR domains. Additionally, the Sherman lab has expertise in the extraction and analysis of small molecules required for the proposed experiments.

The diketone product mimic for Tylosin module 6/DEBS module 5 can be synthesized from (4S,5R)-4-methyl-5-phenyl-1,3-oxazolidin-2-one, propanoyl chloride and decanal using an Evans aldol reaction to generate intermediate 3 (Wilkinson et al, 1998; Evans et al, 1981). The chiral auxiliary group can then be removed by hydrolysis.
with LiOH to generate carboxylic acid intermediate 4, which can be coupled to N-acetylcysteamine to generate final product compound 6 (Wilkinson et al., 1998; Mortison et al., 2009). A shorter diketide product analogue 5 can be synthesized in a similar fashion by substituting propionaldehyde for decanal in the aldol condensation (Figure 3).

![Proposed Synthetic Scheme](image)

The synthetic route I have proposed here is based on my reading of natural products synthesis literature. After consultation with Rafay Shareef in the Sherman lab I have become aware of some of the challenges of handling reagents required for this synthesis. An alternative synthetic route can be conceived that utilizes proline as a chiral catalyst to generate the desired products (List, 2002). This strategy may be a more appropriate way to ensure that my sabbatical is both a significant learning opportunity and a productive endeavor. The synthetic route proposed above would be my first effort in chemical synthesis and would require learning many new techniques including handling reagents in an inert atmosphere, working with strong Lewis acids, workup and purification steps. Additionally, I will need to verify that I have the desired product.

In an effort to directly examine KR domains with ACP loaded substrates, KS-AT, KR and ACP domains will be combined in a single pot with substrates and cofactors. Specifically a KS-AT domain will be incubated with a downstream ACP and methylmalonyl CoA. The substrate mimic SNAC will then be added to the reaction. The progress of these reactions can be monitored by observing a shift in the retention time of the ACP using HPLC. Once the KS has condensed the two acyl substrates on the free ACP domain, the KR domain will be added to the reaction with NADPH to perform
reduction of the newly formed β-carbonyl. The reaction will be quenched with potassium hydroxide, to hydrolyze the final product from the ACP, neutralized with hydrochloric acid and analyzed using chiral HPLC. The expression and purification of the proteins, HPLC based examination of substrates on ACP domains, and KR activity assays have been performed in the Smith lab, however organic extraction and analysis of small molecules products will present an opportunity to further broaden my training.

Relationship to Thesis and Training

The work proposed herein will provide me with mentorship in chemical synthesis, for which I have no previous experience, and is outside the purview of the Smith lab. I will then use these substrates to directly investigate the impact of chain length on the stereochemical fidelity of excised ketoreductase domains. Originally we had designed experiments to test the role of substrates in determining stereochemistry of reduction by the KR domains that used general substrates. This simplification was motivated by our lack of expertise in chemical synthesis and the commercial availability of substrates. Through this sabbatical we will generate substrates that provide a better approximation of native substrates that will allow us to make the most direct conclusions from our data.

References


ketosynthase domain in epimerisation and demonstration that ketoreductase domains can have altered product specificity with unnatural substrates. Chemistry Biology 8, 329-340.


