CBI STUDENT SABBATICAL PROPOSAL COVER SHEET

# Isolation of Natural Product Intermediates Narbonolide and 10-deoxymethynolide & Biological Evaluation of PikC Unnatural Substrates: PikC Oxidation Studies and Antibacterial Assays

March 1st, 2011

**CBI Trainee:** Solymar Negretti-Emmanuelli, Medicinal Chemistry

**Advisor:** John Montgomery, Department of Chemistry

**Sabbatical Host:** David Sherman

**Host Location:** University of Michigan Life Sciences Institute

**Duration of Sabbatical:** 10 weeks

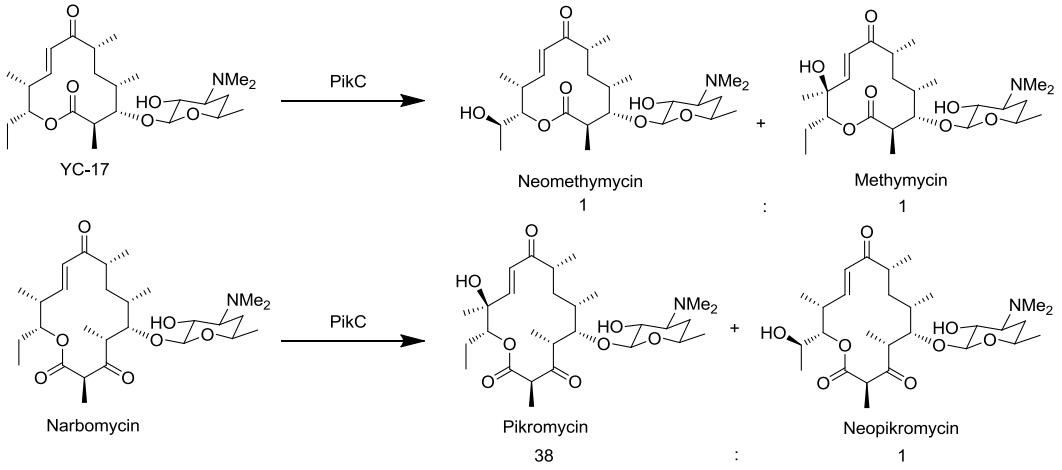
**Time Frame:** Starting February or March 2011

**Proposed activities:** Aglycone intermediates narbonolide and 10-deoxymethynolide from the Pikromycin and Neomethymycin/Methymycin biosynthetic pathway will be isolated from *Streptomyces venezuelae* mutant strain for synthetic manipulations. Gene expression, protein purification, PikC oxidation experiments, and antibacterial assays will be conducted with samples of unnatural substrates previously synthesized in the Montgomery Laboratory.

## Synthesis of unnatural substrates for PikC oxidation studies: exploring PikC as a bio-catalyst for C-H activation

### Background

The enzyme PikC is a P450 monooxygenase from the organism *Streptomyces venezuelae*. [1] it catalyzes the last hydroxylation step in the bio-synthesis of natural product macrolide antibiotics methymycin/neomethymycin and pikromycin/neopikromycin pathway, (**Scheme 1**).[1] PikC stereo- and regioselectively oxidizes 12-membered endogenous substrate YC-17 at the allylic tertiary C-10 to give methymycin or it can oxidize the ethylene from C-12 to give neomethymycin.[1] Interestingly, PikC also oxidizes 14-membered endogenous substrate narbomycin at the allylic tertiary C-12 to give pikromycin or it can oxidize the exo-ethylene from C-14 to give neopikromycin. PikC shows greater substrate tolerance than most known P450's involved in macrolide hydroxylation because of its capability of oxidizing both 12- and 14-membered macrolides.




**Scheme 1.** Hydroxylated natural products of YC-17 and Narbomycin (Substrates for PikC). The numbers below the molecule refer to product ratio of the PikC oxidized metabolites.

Macrolides are a special class of polyketide natural products with antibiotic activity that are produced as secondary metabolites from Actinomycete bacteria.[3] Macrolide structures consist of a macrolactone, generally 12-, 14- or 16-membered rings, constructed by polyketide synthases (PKS), to which one or more deoxysugar residues may be attached (by the action of glycosyl transferases) and also contain additional hydroxyl or epoxide groups installed by cytochrome P450 enzymes.[3,4]

A promising approach for the development of useful C−H oxidation procedures involves employing cytochrome P450 monooxygenases as bio-catalysts given their capacity to perform difficult oxidations in a regio- and stereoselective manner. Due to its innate substrate flexibility and distinct substrate anchoring mode, through a salt-bridge interaction with the dimethylamino group from the desosamine sugar, PikC promised to be an excellent candidate for bio-catalysis.[4,5] However, to employ PikC as a bio-catalyst *in vitro* redox partners are needed to activate molecular oxygen by transferring reducing equivalents from NAD(P)H to PikC.[7] Since the endogenous redox partners of PikC are currently unknown, to replicate its activity *in vitro* spinach ferrodoxin reductase and spinach ferrodoxin, which are quite expensive, have been employed.[7] To

solve this problem the Sherman group developed and engineered self sufficient version PikC fused to and RhFRED reductase domain.[7] Previous results from the collaborative work from the Montgomery and Sherman groups reported the application of $PikC_{D50N}$-RhFRED for the moderately regioselective hydroxylation of several desosamine linked carbocyclic rings (referred to as "carbolides"), proving PikC to be capable of oxidizing unnatural substrates.[5] These encouraging results lead us to believe that PikC, represents a promising bio-catalyst for the selective oxidation of C-H bonds, which is a central challenge in organic chemistry.

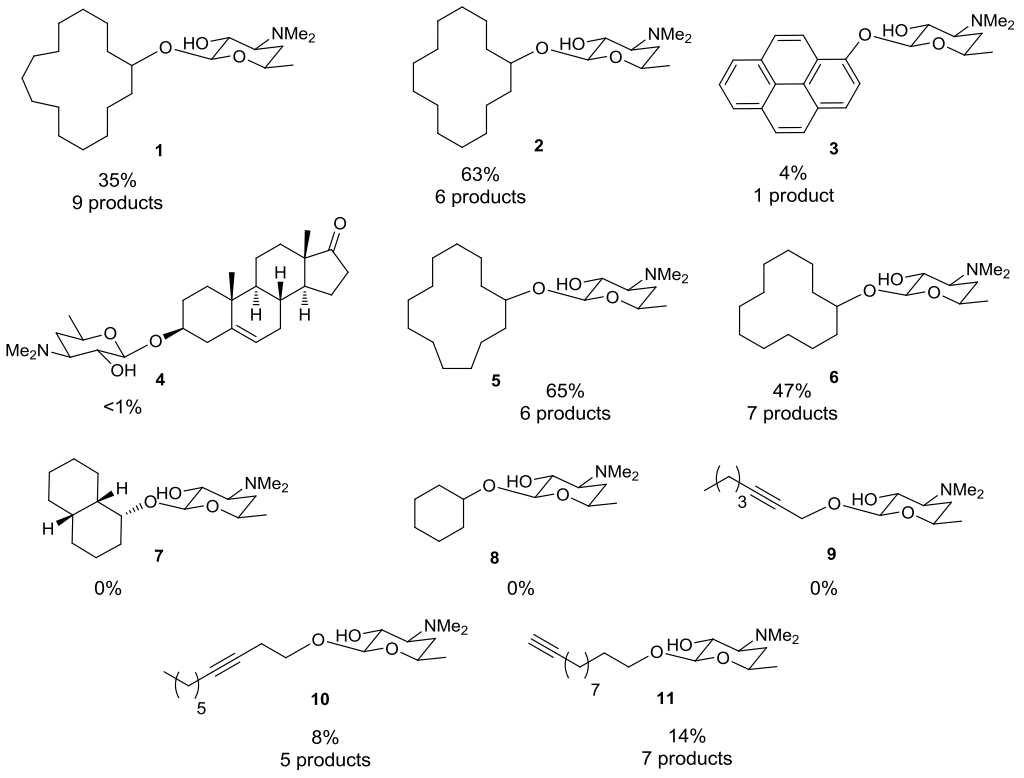
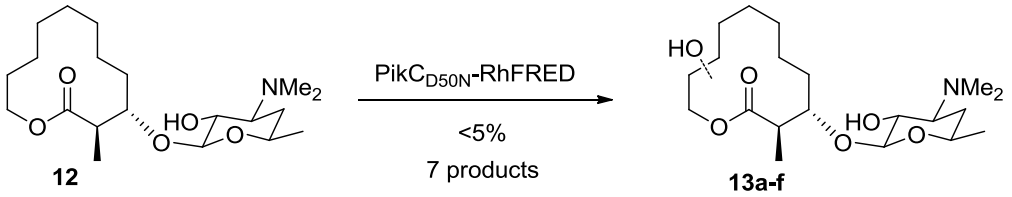



**Figure 1.** Substrates previously evaluated for $PikC_{D50N}$-RhFRED oxidation.[5]

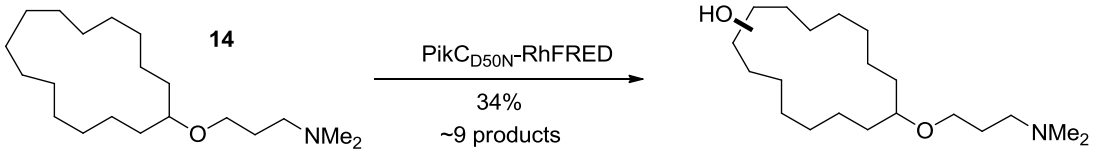
**Previous work in the Montgomery lab**

Achieving high levels of regio- and stereoselectivity for unnatural substrate hydroxylation has been challenging, most substrates yielded several products in the $PikC_{D50N}$-RhFRED oxidation. When I joined the Montgomery lab the first substrate I synthesized to address these issues was macrolide **12**, as it showed more resemblance to the endogenous substrate YC-17 than the previously evaluated 12-membered carbolide **6**. Improvement in the regioselectivity and stereoselectivity of the oxidation upon treatment with $PikC_{D50N}$-RhFRED, in comparison with carbolide **6**, was expected. However the results of the enzymatic reaction between substrate **12** and $PikC_{D50N}$ revealed a low conversion ratio and several products. The reason why the reaction was so low yielding and unselective could be because in macrolide **12**, there is no tertiary allylic or secondary exocyclic oxidation sites corresponding to the reactive C-H bonds in YC-17, therefore no highly favored pathway for oxidation. Macrolides **15** and **16** are currently being synthesized to address these issues and will be evaluated for $PikC_{D50N}$-RhFRED oxidation (*see figure 3*).




**Scheme 2.** PikC $_{D50N}$-RhFRED oxidation of macrolide **12.**

Substrates containing anchoring groups other than desosamine have also been evaluated for PikC$_{D50N}$-RhFRED oxidation. Compound **14** was synthesized and contains an *N,N*-dimethylaminopropanoxy moiety as the anchoring group (with Allison R. Knauff). This 15-membered carbocycle was bound and oxidized by PikC$_{D50N}$ in moderately high yields but with little selectivity. This result shows that the desosamine sugar moiety is not essential for PikC to be able to bind its substrates. In this case a linear chain linker still containing the a terminal *N,N*-dimethylamino group, which is necessary to form a salt-bridge interaction at the PikC$_{D50N}$ active site, was sufficient for oxidation. Besides the surprising result from the PikC$_{D50N}$ oxidation, **14** showed high antibacterial activity against the bacterial strains tested.




**Scheme 3.** PikC $_{D50N}$-RhFRED oxidation of compound **14.**

**Current work in the Montgomery lab and proposed sabbatical work in the Sherman lab**

Given the results obtained for compound **14**, substrates **17**-**21** are currently being synthesized to further explore PikC's capability to accept molecules containing other anchoring groups (*see figure 3*). With some of these compounds I plan to perform the PikC oxidation assays myself. This is one of the aspects of my project I have little familiarity with as I work exclusively on the synthesis of the unnatural substrates. A sabbatical in the Sherman lab enable me to become more involved with the protein biochemistry and enzymology aspects of my project. I also plan to become proficient in the mutagenesis and protein engineering techniques that increase PikC's efficiency and allow it to become a self-sufficient enzyme and be employed successfully as a C-H activation catalyst. I also expect to be able to test some of these compounds for antibacterial activity through the Center for Chemical Genomics (CCG) located in the Life Sciences Institute, as previous unnatural substrates have shown surprising bioactivity against antimicrobial targets including MRSA.[5]

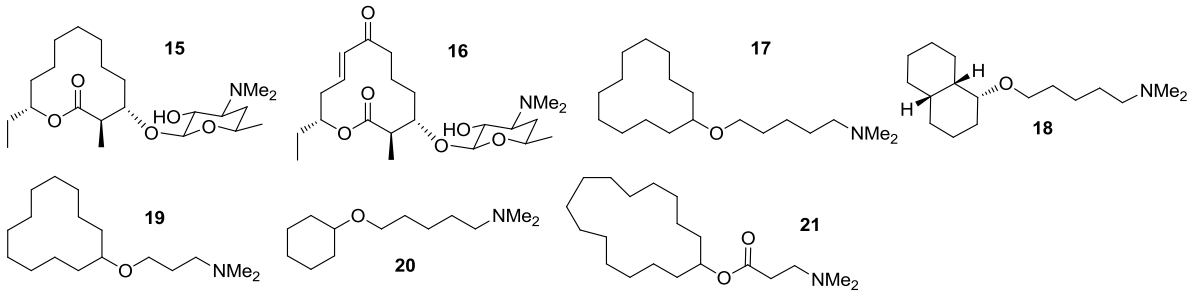


**Figure 2.** Proposed PikC substrates currently in preparation

The second objective I am hoping to accomplish through the sabbatical in Professor Sherman's laboratory is the isolation of the macrolide biosynthesis intermediates narbonolide and 10-deoxymethynolide which are generated after the chain elongation and cyclization steps catalyzed by the Pik-PKS and thioesterase respectively.[7,8] These intermediates contain the free hydroxyl group to which the desosamine sugar is attached in a subsequent glycosylation step. If these compounds were to be isolated prior to glycosylation they could serve as useful starting materials for further transformations such as attachment of different anchoring groups other than desosamine. The attachment of the *N,N*-dimethylaminopropanoxy linker (as in compound **14**) to narbonolide and 10-deoxymethynolide and evaluation of these compounds in the $PikC_{D50N}$-RhFRED oxidation assay could yield insights into the role of desosamine in the selectivity of the oxidation, as the C-C bonds present in the *N,N*-dimethylaminopropanoxy moiety are able to rotate freely, unlike those in desosamine which are constrained in a ring. Also, different sugars could be attached to these non-glycosylated intermediates. An example of such sugars would be D-mycinose, which is one of the sugars attached to macrolide natural product antibiotics Tylosin and Mycinamicin.[3] The YC-17 and Narbomycin analogs generated could be evaluated against the Cytochrome P450's involved in the biosynthesis of these two natural products (e.g. TylI/TylHI, MycG/MycI[9]) and to potentially generate novel and diverse macrolide antibiotics. Self-sufficient fusion proteins of these enzymes have been developed by the Sherman group and catalyze hydroxylation and/or epoxidation with high efficiency (unpublished). Results from the oxidation studies on these P450's could lead to their exploration as bio-catalysts for hydroxylation (through C-H activation) as well as epoxidation, which is one of the reactions catalyzed by the MycG P450's.[9]

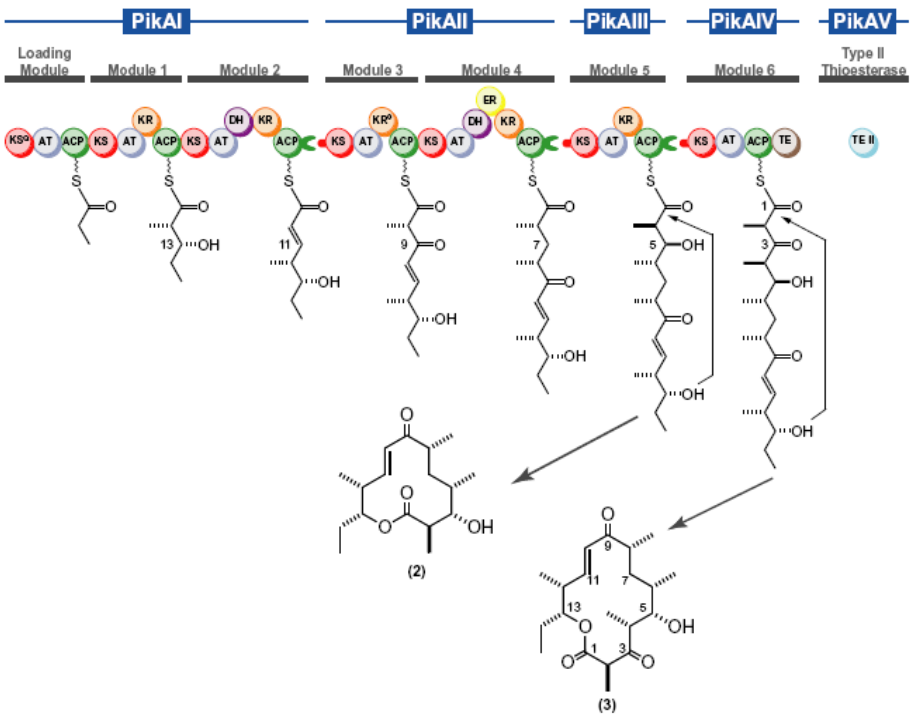



**Figure 3.** Biosynthesis of 10-deoxymethynolide and narbonolide by the Pik polyketide synthase.[7]

The intermediates narbonolide and 10-deoxymethynolide can be isolated from a *S. venezuelae* mutant (SC1022 or SC1015), in which genes DesVII and DesVII are deleted. These genes code for two glycosyl

transferases that in combination are responsible for attaching desosamine to these macrolactone intermediates.[6] This deletion renders the organism incapable of modifying narbonolide and 10-deoxymethynolide any further, and inhibiting the completion of the synthesis of the final products, therefore making it possible to isolate the desired aglycone intermediates upon culturing these strains of the organism.

My work in the Montgomery lab so far has focused solely on the organic synthesis aspect of the project. I synthesize the unnatural substrates but the biological evaluation (PikC oxidation studies and antibacterial assays) are performed entirely in the Sherman lab by graduate student Karoline Chiou. Although the project is very interdisciplinary, it combines aspects of synthetic organic chemistry, medicinal chemistry and enzymology, and thus, the multi-faceted nature of this project is fostered through the collaboration with Professor Sherman. For this collaboration I have been regularly attending group meetings at the Sherman lab and interact with other graduate students involved in the project but have not had the opportunity to obtain the molecular biology and protein biochemistry skills to perform the laboratory work there myself.

The proposed sabbatical work in the Sherman lab will allow me to gain experience in techniques of biochemistry and molecular biology, skills that complement the exclusively synthetic organic chemistry training I am acquiring as a graduate student in the Montgomery lab. It will also give me the experience to become more immersed in the cross-disciplinary aspects from my project, beyond the synthetic chemistry of the substrate molecules. I will be able to be involved in both the synthesis and biological evaluation of my own compounds, which would not have been possible if I did not have the opportunity to do the CBI student sabbatical in the Sherman lab.

**References**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February 18, 2011

**RE: Negretti CBI sabbatical request**

Dear Anna,

I would like to express my support for the sabbatical plan submitted by Solymar Negretti. Her work in my lab involves organic synthesis, specifically the preparation of novel structures to be examined in the labs of David Sherman. The structures that she has prepared are designed to probe the generality of oxidations mediated by engineered cytochrome P450 (pikC) enzymes. Modifications in the oxidizable portion of the structures as well as the anchoring segment are being examined. Currently, she is passing along material to students in the Sherman lab, where the P450 oxidations are conducted, and she has no hands-on involvement in the biochemical aspects of the project. As she proposes in her attached document, Solymar hopes to carry out her sabbatical in the Sherman lab, where she will conduct experiments involving pikC mutagenesis and protein engineering, pikC oxidations of the compounds that she has previously prepared, isolation of the macrolides naturally derived from the pikC system, and hands-on evaluation of the derivatives that she has prepared in the CCG. These techniques are all completely new to her, and she will be unable to gain meaningful experience in these areas without the time commitment that the sabbatical arrangement allows. I believe that her work in this sabbatical plan will provide her with outstanding training in areas new to her, and that the work will facilitate new directions in this collaborative project.

Sincerely,

John Montgomery

John Montgomery
Professor of Chemistry

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life sciences institute

MARCH 2, 2011

Professor Anna Mapp
Director, NIGMS Chemistry/Biology Interface Training Grant
Department of Chemistry
University of Michigan
Ann Arbor, MI 48109

Dear Anna:

I am writing in support of Ms. Solymar Negretti's research sabbatical application that is an important training component of her CBI fellowship. Solymar has participated in several combined group meetings between my group and the Montgomery group over the past couple of years as we pursue collaborative work on C-H bond activation using biosynthetic P450 enzymes. Since John began his sabbatical in January, Solymar has been routinely attending P450 subgroup meetings that include Karoline Chiou, Dr. Shengying Li, and Rafay Shareef from my laboratory. This is providing Solymar with deeper insights into the molecular biology, protein biochemistry and enzymology aspects of the project that she plans to pursue during her proposed sabbatical.

Following detailed discussions with John and Solymar, we plan is to have her work directly with Karoline and Shengying on the analysis of her synthetic substrates for C-H bond activation involving PikC P450 and an additional series of biosynthetic macrolide monooxygenases that accommodate large ring macrolactones. Based on our collaboration with the Montgomery lab, we are gaining increasing knowledge of the anchoring group requirements, and the tolerance of the enzyme toward non-traditional appendages that provide considerable additional synthetic flexibility for substrate preparation. Solymar has already made significant contributions to this effort, and the next natural step in her training as a Ph.D. student and CBI fellow is to work directly on the biochemical analysis and biological evaluation of the products formed from these remarkably versatile catalysts. New products obtained from her P450 biocatalysis work will be directly analyzed using a series of whole cell microbial targets that are available in my laboratory and the Center for Chemical Genomics. These include VRE, MRSA, and a series of engineered *E. coli* and fungal strains that Solymar can work with under BSL2 conditions. This sabbatical period will enable Solymar to gain important additional training in areas that she has shown significant interest, but would be otherwise very difficult to do. I am confident that her participation will fulfill both the letter and spirit of the CBI training grant mandate for this sabbatical experience.

With best wishes.

Sincerely,

David H. Sherman

David H. Sherman, Ph.D.
Hans W. Vahlteich Professor of Medicinal Chemistry (College of Pharmacy)
Professor of Microbiology & Immunology (Medical School)
Professor of Chemistry (College of Literature, Science & Arts)
Director, Center for Chemical Genomics