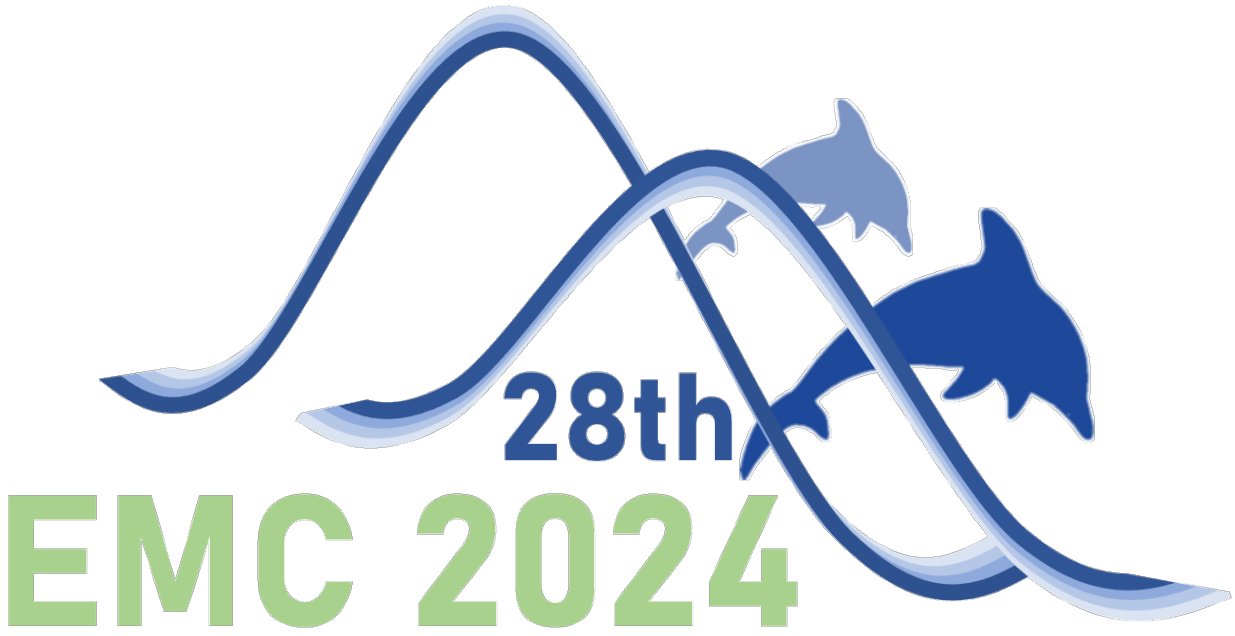
The 28th Enzyme Mechanisms Conference



January 3rd - 7th, 2024
Naples Grand Beach Resort
Naples, FL

Audrey Lamb and Graham Moran,
co-chairs

Vahe Bandarian, Karen Allen and Tom Meek,
Organizing Committee

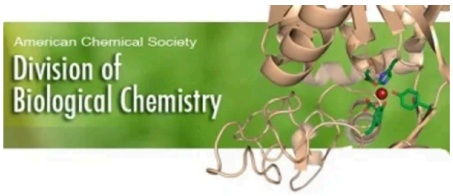
Contents

EMC Sponsors

John Kozarich & Marcia Durso

Anonymous Conferee

Anonymous Conferee



Anonymous Conferee

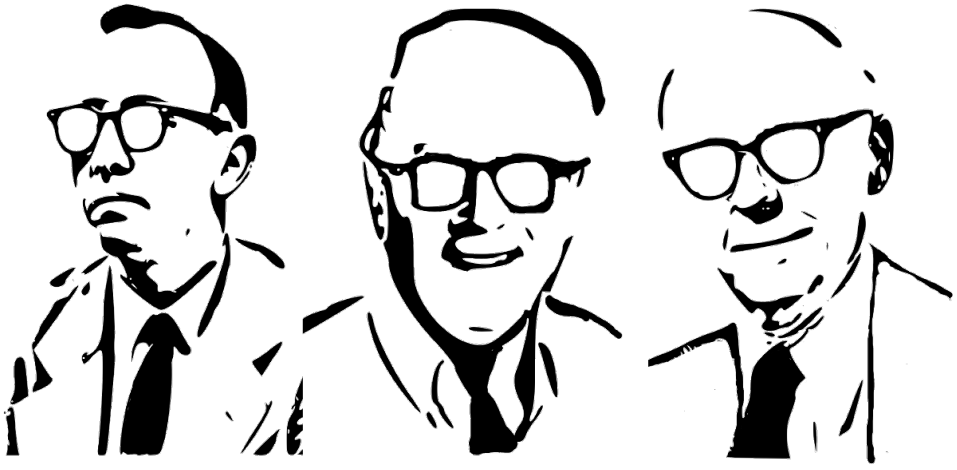
Christopher Whitman

Anonymous Conferee

Anonymous Conferee

The Enzyme Mechanisms Conferences

The Enzyme Mechanisms Conferences have brought together academic and industrial scientists to discuss new ideas at the forefront of mechanistic enzymology. The goal is to foster collegial interactions among chemists and biochemists who seek to understand the chemical basis for enzymatic catalysis and regulation of enzyme action and those who apply that knowledge for practical applications.



The conference has been held biennially since it was founded in 1969 by Tom Bruice, Bill Jencks, and Myron Bender. Over the past 55 years, the conference has provided an outstanding forum for the presentation and discussion of the most exciting advances in our understanding of the mechanisms of enzyme action and the application of this knowledge to synthetic chemistry, pharmaceutical design and agriculture.

The community of the Enzyme Mechanisms Conference includes people who are citizens of and reside in nations from around the world, are members of cultures and religions that represent all possibilities on Earth, are born to families both traditional and non-traditional, and have the full range of physical abilities, gender expression, sexual orientation and age. We use this diversity to provide a wide range of perspectives for the work of our common passion: determining how enzymes catalyze reactions and using that information for the betterment of life and the sustainability of our planet. We promote collegial academic debate in a welcoming environment and do not tolerate discrimination.

55 Years of EMCs

Year	Destination	Chair(s)
2024	Naples, FL	Audrey Lamb & Graham Moran
2022	Tucson, AZ	Wilfred van der Donk
2019	New Orleans, LA	Vahe Bandarian
2017	St. Pete Beach, FL	Richard Silverman
2015	Galveston, TX	Ken & JoAnn Johnson
2013	Coronado, CA	Thomas Meek
2011	St. Pete Beach, FL	John Richard & Tina Amyes
2009	Tucson, AZ	Karen Allen
2007	St. Pete Beach, FL	Chris Whitman
2005	Asilomar, CA	JoAnne Stubbe
2003	Galveston, TX	Frank Raushel
2001	Marco Island, FL	Vern Schramm
1999	Napa, CA	Richard Armstrong
1997	Naples, FL	John Kozarich
1995	Scottsdale, AZ	Dale Poulter
1993	Key Largo, FL	John Gerlt
1991	San Diego, CA	Joe Villafranca
1989	St. Petersburg, FL	Paul Bartlett
1987	Asilomar, CA	Tony Fink
1985	Tarpon Springs, FL	Gene Cordes
1983	Asilomar, CA	Judith Klinman
1981	Sanibel Island, FL	Perry Frey
1979	La Jolla, CA	George Kenyon
1977	Tucson, AZ	Joe Coleman
1975	San Juan, PR	Al Mildvan
1973	Los Angeles, CA	Paul Boyer
1971	Santa Barbara, CA	Tom Bruice
1969	New Orleans, LA	Bill Jencks

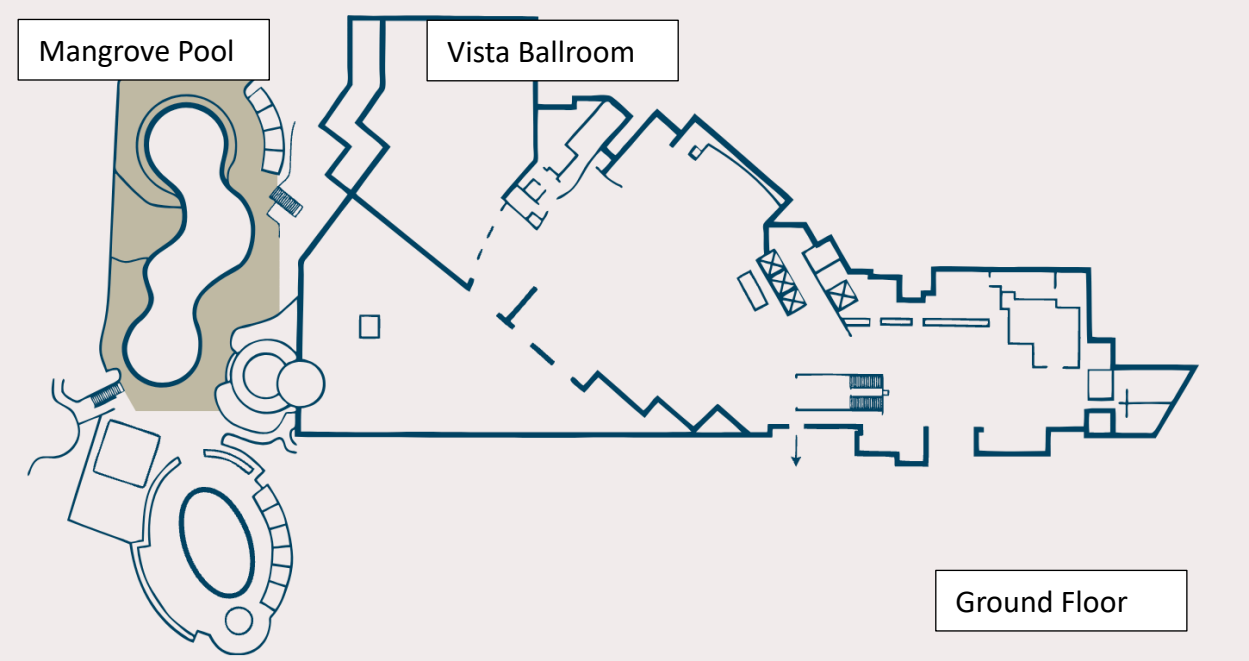
28th Enzyme Mechanisms Conference

Naples Grande Beach Resort, Naples Fl January 3-7, 2024

Registration and Conference Check-in: The hotel front desk is located in the area immediately in front of the main entrance of the lobby from the circle drive. The conference registration desk will be located at the top of the escalators to your left as you enter the lobby and will be open from 3-6 pm on the 3rd of January. If you are unable to obtain your registration materials during these times, please locate the conference chairs: Audrey or Graham (or email audrey.lamb@utsa.edu, gmoran3@luc.edu).

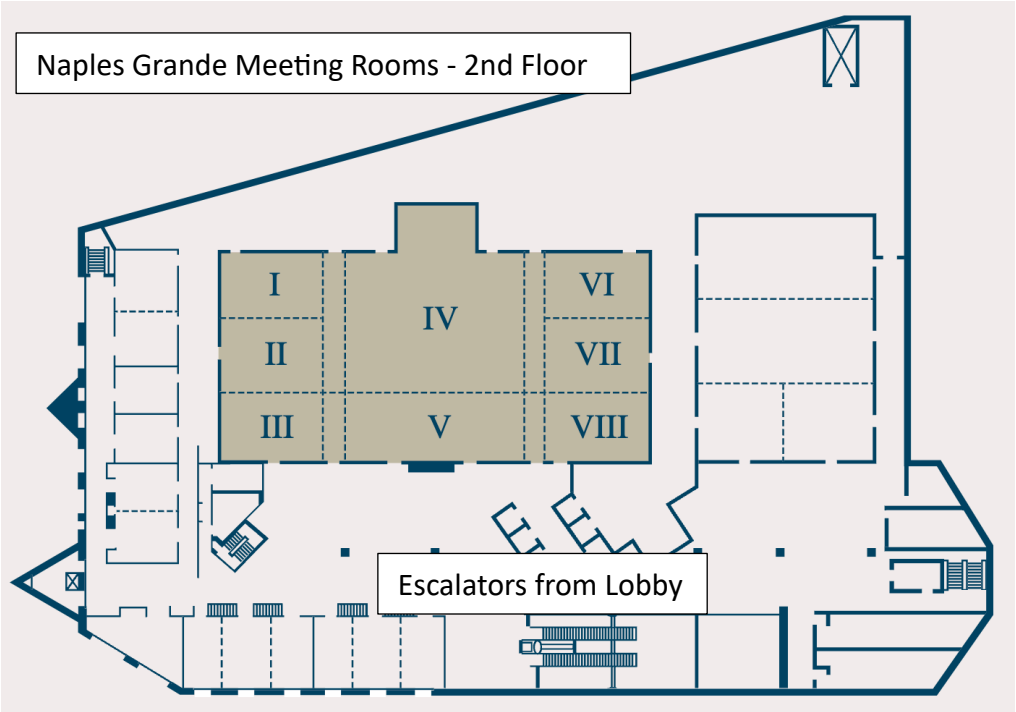
Name Tags: Conferees and registered guests are kindly asked to wear their name tag at all times while attending the scientific sessions, meals and social functions.

Opening Reception: The conference Welcome and Opening Reception will be held on Wednesday January 3rd from 6:00 pm to 8:00 pm at the Mangrove Pool (in the event of rain, the reception will be in the Vista Ballroom). Conferees and registered guests are welcome to attend.



Drink Tickets: At registration, all conference attendees will receive drink tickets good for soft drinks, water, wine, beer, or the specialty beverage of the day during evening cash bar events.

Lecture Sessions: The nine scientific sessions will be held in combined *Royal Palm IV-V*.



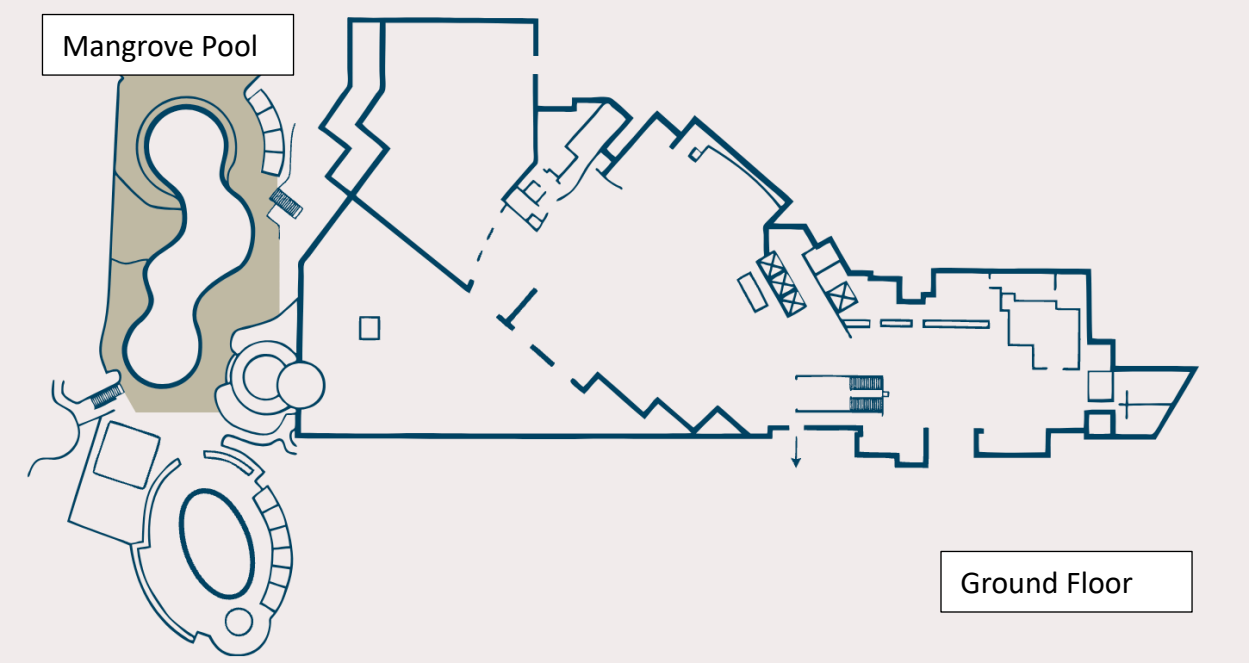
Poster sessions: Four poster sessions will be held in *Royal Palm I-III* spanning 10am-12pm and 8:50-10pm on both Thursday and Friday. Posters will be on display throughout the conference. A cash bar will be available. Posters may be mounted on Thursday morning and should be removed by Friday midnight. Even number poster presenters should be at their posters on Thursday, whereas odd number poster presenters will defend their posters on Friday.

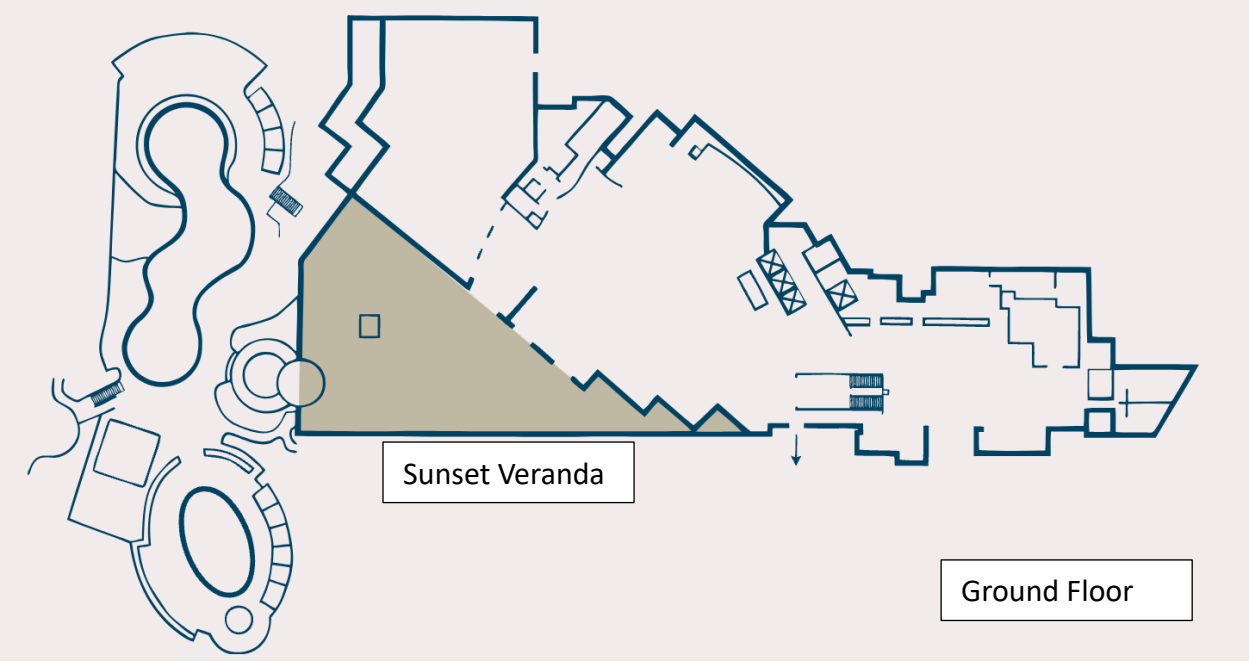
Breakfast: Breakfast will be available for conferees and registered guests *Royal Palm VI-VIII* beginning at 7:30 am on Thursday, Friday and Saturday. On Sunday, boxed breakfasts will be available for your travel home.

Lunch: Lunch will be available for conferees and registered guests *Royal Palm VI-VIII* from noon until 1pm on Thursday, Friday and Saturday.

Dinner: Dinner will be available for conferees and registered guests *Royal Palm VI-VIII* beginning at 5:30 pm on Friday.

Closing Banquet: The closing banquet will commence with a reception from 6-7 pm on the Sunset Veranda followed by the banquet from 7-9 pm surrounding the Mangrove Pool . Conferees and registered guests are welcome to attend.





Registered guests: Registered guests are invited to the opening reception, breakfasts, lunches, Friday dinner, coffee breaks, and the closing banquet.

Program

Wednesday, January 3

6:00-8:00 pm
Opening Reception - Mangrove Pool

Thursday, January 4

7:00-8:30 am: Breakfast – Royal Palm 6-8

Session 1 – **Spoonbill** – Royal Palm 4-5
Chair – **Marcus Hartmann** (Max Planck Institute for Biology)

8:00-8:10 am: Welcome

8:10-8:40 am: **Nozomi Ando** (Cornell U)
Conformational Ensembles of Flexible Multi-Domain Enzymes

8:40-9:10 am: **Dennis Murphy** (GSK)
Stereochemical discrimination of a covalent, irreversible werner helicase inhibitor series

9:10-9:40 am: **Alexey Silakov** (Penn State U)
Understanding why some [FeFe] hydrogenases tolerate the presence of oxygen

9:40-10:00 am: Three Three-minute Poster Pitches
1. **Anna Zmich** - Structural and mechanistic exploration of a thermostable Cystathionine γ-lyase reveals a new catalytic function
2. **Madison Smith** - Cellular redox homeostasis with a flavin-disulfide electron conduit
3. **Antonio Del Rio Flores** - De novo biosynthesis of azide by a promiscuous N-nitrosylase

Poster Session 1
10:00 am-12:00 pm
Coffee Break/Poster Session - Royal Palm 1-3

12:00-1:00 pm
Lunch - Royal Palm 6-8

Session 2 – **Alligator** – Royal Palm 4-5
Chair – **Dhara Shah** (Arizona State U)

1:00-1:30 pm: **Aimin Liu** (UT San Antonio) – Bringing amino acids together: unlocking new chemical potential

1:30-2:00 pm: **Adrian Keatinge-Clay** (UT Austin) - Engineering polyketide synthases using the correct module boundary

2:00-2:30 pm: **Suzanne Walker** (Harvard U) - How bacteria acylate their cell envelopes to protect themselves from antibiotics

3:00-7:00 pm
Free Time/Dinner (not provided)

4:00-5:00 Industry Career Workshop with **David Olsen** (Merck) - Pharmaceutical Industry Job Hunting: Distinguishing Yourself from the Crowd

Session 3 – **Mangrove**– Royal Palm 4-5
Chair – **Edwin Antony** (St Louis University)

7:00-7:30 pm: **Erika Taylor** (Wesleyan U) - Rethinking the Aminoglycosides Mechanism of Action: Evidence for Inhibition of Heptosyltransferase I from *Escherichia coli*

7:30-8:00 pm: **Frederick Stull** (Western Michigan U) - Exceptions to the oxidase paradigm of the flavoprotein amine oxidase superfamily

8:00-8:30 pm: **Reuben Peters** (Iowa State U) - TerDockin: Stuffing carbocations into terpene synthases to refine catalytic activity

8:30-8:50 pm: Three Three-minute Poster Pitches
1. **Zoe Hoffpauir** - The riboflavinator: A new hope
2. **Yu Yue** - Peptide extension after chain reversal during non-ribosomal peptide biosynthesis
3. **Mark Schmidt-Dannert** - Terpene synthases

Poster Session 2
8:50-10:00 pm
Drinks/Poster Session - Royal Palm 1-3, Royal Palm Foyer

Friday, January 5

7:00-8:30 am
Breakfast – Royal Palm 6-8

Session 4 – **Bottlenose** – Royal Palm 4-5
Chair – **Jeff McFarlane** (Fort Lewis College)

8:00-8:10 am: Welcome/Announcements

8:10-8:40 am: **Lana Saleh** (New England Biolabs) - 5-Methylpyrimidine dioxygenases (5mYOXs) and their partner proteins in base modification

8:40-9:10 am: **Robin Teufel** (U Basel) - Flavoenzyme-catalyzed redox tailoring of bacterial tropone natural products

9:10-9:40 am: **Oscar Juarez** (IIT) - The electron transfer and ion pumping mechanisms of the evolutionary divergent NQR family

9:40-10:00 am: Three Three-minute Poster Pitches
1. **Jake Lachowicz** - Structural and Mechanistic Determinants of ddhNTP Catalysis by the Ancient Antiviral Enzyme, Viperin
2. **Josseline Ramos - Figueroa** - Uncovering the biosynthetic pathway towards the production of Ammosamide
3. **Monica MacDonald** - Mutagenesis as a Strategy for Pyrrolobenzodiazepines Biochemical Synthesis

Poster Session 3
10:00 am-12:00 pm
Coffee Break/Poster Session - Royal Palm 1-3, Royal Palm Foyer

12:00-1:00pm
Lunch - Royal Palm 6-8

Session 5 – **Manatee** – Royal Palm 4-5
Chair – **Holly Ellis** (East Carolina U)

1:00-1:30 pm: **Andy Gulick** (U at Buffalo) - Structural and mechanistic studies of enzymes involved in siderophore biosynthesis

1:30-2:00 pm: **Diane Retallack** (Primrose Bio) - Discovery and development of enzyme therapeutics using the advanced *Pseudomonas fluorescens*-based protein expression platform

2:00-2:30 pm: **Michelle Chang** (UC Berkeley) - Synthetic biology approaches to new chemistry

3:00-5:30 pm
Free Time

5:30-7:00 pm
Dinner – Royal Palm 6-8

Session 6 – **Heron** – Royal Palm 4-5
Chair – **Catherine Goodman** (ACS Journals)

7:00-7:30 pm: **Wilfred van der Donk** (U Illinois) - Genome Mining for New Enzymology

7:30-8:00 pm: **David Olsen** (Merck) - Malaria aspartyl protease inhibitors: From an unknown mechanism of action screening hit to dual-targeting of essential enzymes

8:00-8:30 pm: Founders Award Lecture
Leo Betancurt (Pasteur Institute/NEB) -Proofreading mechanism for family D DNA polymerases

8:30-8:50 pm: Three Three-minute Poster Pitches
1. **Elijah Kissman** - A dynamic metal coordination sphere controls chemoselectivity in radical amino acid halogenases
2. **Javeria Akram** - Characterization of oxidant specificity in bacterial flavoprotein amine "oxidases"
3. **Mercedes Fisk** - A rare NRPS-NIS hybrid for the biosynthesis of the siderophore Norcardichelin

Poster Session 4

8:50-10:00 pm

Drinks/Poster Session - Royal Palm 1-3, Royal Palm Foyer

Saturday, January 6

7:00-8:30 am
Breakfast – Royal Palm 6-8

Session 7 – **Otter** – Royal Palm 4-5
Chair – **Jeremy Lohman** (Michigan State U)

8:30-8:40 am: Welcome/Announcements

8:40-9:10 am: **Erik Ralph** (Pfizer) - Targeting Glycogen Synthase I for the treatment of glycogen storage diseases

9:10-9:40 am: **Katherine Davis** (Emory U) - Examining the structural basis for atypical heme enzyme reactivity

9:40-10:10 am: **Brian Miller** (Florida State U)- Evolutionary origins of vertebrate glucose homeostatic regulation

10:10-10:30 am
Coffee Break - Royal Palm Foyer

Session 8 – **Egret** – Royal Palm 4-5
Chair – **Mark Snider** (College of Wooster)

10:30-11:00 am: **Rafael Guimaraes da Silva** (U St. Andrews) - Structure and mechanism of a nucleotide sanitizing enzyme with a role in cancer

11:00-11:30 am: **Neil Marsh** (U Michigan) - Prenylated-flavin dependent decarboxylases

11:30 am-12:00 pm: **Dean Brown** (Jnana Pharmaceuticals) - Discovery and characterization of JNT-517, an inhibitor of SLC6A19 for the treatment of phenylketonuria

12:00-12:30 pm: **Leslie Poole** (Wake Forest U) - Structural and dynamic features of peroxiredoxins aligned with their roles in peroxide defense and signaling

12:30-2:00 pm
Lunch - Royal Palm 6-8

Session 9 – **Hibiscus** – Royal Palm 4-5
Chair – **Andrew Murkin** (University at Buffalo)

2:00-2:30 pm: **Samer Gozem** (Georgia State U) - Bringing together quantum mechanics, molecular dynamics, and experiments to study flavoproteins

2:30-3:00 pm: **Sharon Hammes-Schiffer** (Princeton U)- Proton-Coupled Electron Transfer in Enzymes

3:00-3:30 pm: **Jack Tanner** (U of Missouri) - Mechanism-based covalent inactivation of the flavoenzyme proline dehydrogenase

3:30-4:00 pm: **Joan Broderick** (Montana State U) - Delineating Mechanistic Steps in Radical SAM Reactions

4:00-4:10 pm: Award Ceremony

4:10-6:00
Free Time

Closing Banquet
6:00-7:00 pm
Reception with hors-d'oeuvres - Sunset Veranda

7:00 pm
Banquet – Mangrove Pool

Sunday, January 7

AM depart conference

Speaker Abstracts

Conformational Ensembles of Flexible Multi-Domain Enzymes

Nozomi Ando[§]

[§] *Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NA, USA*

Flexible, multi-domain enzymes catalyze remarkable chemical transformations by moving either their co-factor or substrate over large distances, from active site to active site. In this talk, I will describe recent work on two such enzymes: (1) the cobalamin-dependent methionine synthase (MetH),[1] which performs three different reactions by moving its cobalamin cofactor between three different active sites and (2) the first component of the non-ribosomal peptide synthetase (NRPS) pathway for gramicidin S biosynthesis[2]. I will discuss how to experimentally characterize conformational switching in flexible enzymes by combining small-angle X-ray scattering (SAXS) – which captures a wholistic view of conformational ensembles – with the single-particle or single-molecule perspectives provided by cryo-electron microscopy (cryo-EM) and single-molecule Forster resonance transfer (smFRET).

References

1. Watkins, M. B.; Wang, H.; Burnim, A.; Ando, N. *PNAS* **2023**, *120*, e2302531120.
2. Sun, X.; Alfermann, J.; Li, H.; Watkins, M. B.; Chen, Y.-T.; Morrell, T. E.; Mayerthaler, F.; Wang, C.-Y.; Komatsuzaki, T.; Chu, J.-W.; Ando, N.; Mootz, H. D.; Yang, H. *Nat. Chem.* **2023**. https://doi.org/10.1038/s41557-023-01361-4.

Stereochemical Discrimination of a Covalent, Irreversible Werner Helicase Inhibitor Series

Dennis Murphy, Ph.D.

Screening, Profiling, and Mechanistic Biology, GSK. Upper Providence, PA, USA

The Werner helicase is a synthetic lethality target for MicroSatellite Instability-High/Mis-Match Repair Deficient (MSI-H/dMMR) cancers, identified through genome-wide screening of genetic dependencies[1] and CRISPR rescue[2]. The enzyme is a ReQ3 helicase with both 3'- 5' exonuclease and ATPase dependent 3'-5' unwinding activities. Its primary function is binding at stalled replication forks and mediating resolution of a wide variety of DNA lesions. In-house screening identified three covalent inhibitors that alkyated cysteine-727. One compound containing a tricyclic core was optimized with a cyclic vinylsulfone warhead, yielding four compounds differing only in the configuration of the two stereogenic centers. Kinetic analysis utilized an internally quenched unwinding substrate and results were fit to the two-step, irreversible inhibition model. The bimolecular inactivation rate (k_{inact}/K_I) showed a nearly 500 fold potency range for the four compounds. The x-ray strucutre of the most potent analog was solved and showed a binding pocket for the tricycle and a covalent bond between cys-727 and the warhead. Modeling of the three weaker stereoisomers showed that a hydrogen bond with a nearby arginine and the proximity of the electronegative sulfone likely contributed to the variation in potency. While there were significant differences in the absolute binding and inactivation rates of the four analogs against the apo-enzyme versus the actively unwinding helicase, the rank order of the compounds remained the same.

References

1. E.M. Chan. . . F. Vazquez; A.J. Bass, *Nature* **2019**, *568*, 551.
2. F.M. Behan. . . K. Yusa; M.J. Garnett, *Nature* **2019**, *568*, 511.

Understanding why some [FeFe] hydrogenases tolerate the presence of oxygen

Kyle C. Jorgensen, Rinat Khundoker, Patrick S. Corrigan, Alexey Silakov

Department of Chemistry, Pennsylvania State University, University Park, PA, USA

[FeFe] hydrogenases catalyze reversible hydrogen evolution at rates as high as 10,000 turnovers per second. This exceptional catalytic ability is attractive for the use of hydrogenases in renewable energy applications and biohydrogen production. Unfortunately, most of these enzymes degrade irreversibly upon exposure to minute amounts of oxygen, presenting major roadblocks for study and implementation in practical or industrial applications. The recent finding of an [FeFe] hydrogenase from *Clostridium beijerinckii* (*Cb*HydA1) that does not degrade in the presence of O_2 is a long-awaited breakthrough in the field of enzymatic hydrogen catalysis because it presents an unprecedented opportunity to implement this very efficient enzyme into sustainable systems. We employed various spectroscopic methods, electrochemistry, bioinformatics, and theoretical modeling to investigate this unique enzymatic system.[1,2] The presented work provides crucial details necessary to understand the mechanism of O_2 tolerance and uncover the structural basis for this desirable phenotype. Our sequence similarity analysis also suggests that this enzyme represents a large group of yet-to-be-characterized [FeFe] hydrogenases, setting an exciting avenue for future studies of these enzymes. Furthermore, we illustrate the plausibility of engineering the O_2-tolerant [FeFe] hydrogenases for efficient coupling to cyanobacterial photosystem I. As such, results obtained in this work establish the basis for future photosynthetic H_2 production strategies.

References

1. P.S. Corrigan, J.L. Tirsch, A. Silakov, *J. Am. Chem. Soc.* **2020**, 142, 28, 12409-12419
2. P.S. Corrigan, S.H. Majer, A.Silakov, *J. Am. Chem. Soc.* **2023**, 145, 20, 11033-11044

Bringing amino acids together: unlocking new chemical potential

Aimin Liu

Department of Chemistry, University of Texas San Antonio

Abstract: The catalase-peroxidase enzyme KatG is a heme-dependent protein critical for the virulence of Mycobacterium tuberculosis (Mtb). Its catalase function is essential for the pathogen to mitigate oxidative stress from hydrogen peroxide in infected host cells. The catalase activity of KatG is distinct from other catalases due to the presence of a protein-derived cofactor, methionine-tyrosine-tryptophan (MYW) covalent triad, an auto-catalytical post-translationally modification. In the presence of the MYW cofactor, a 37,000:1 catalase:peroxidase ratio in competing peroxide consumption efficiency. In this presentation, the crosslinked cofactor in Mtb is naturally present in two distinct forms: Met-Tyr-Trp and Met-Tyr-Trp-OOH. The two forms show different spectroscopic signatures in UV-vis, EPR, resonance Raman and active site crystal structures. We have found the interconversion between the two forms, i.e., how the due to the N-linked hydroperoxyl group is installed and how it is removed. The impact of the indole-nitrogen hydroperoxyl adduct on the catalase activity and peroxidase activity of KatG is also determined. A novel cofactor-enabled catalase mechanism will be discussed. These studies take a new angle to obtain an in-depth molecular-level understanding of the factors governing catalase and peroxidase activities. The knowledge learned will establish a foundation for whether the catalase activity of KatG can be targeted to inhibit KatG Mtb.

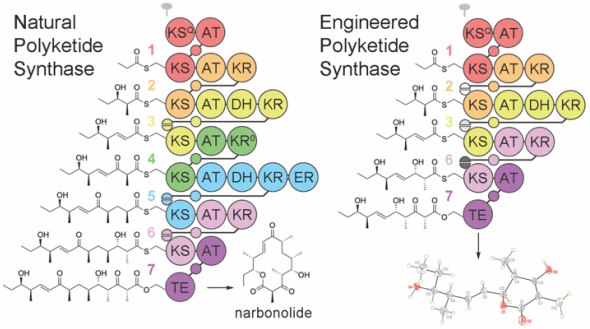
Engineering Polyketide Synthases Using The Correct Module Boundary

Miyazawa, T.,[1] Zhang, J.,[1] Hirsch, M.,[2] Ray, K.A.,[1] Lutgens, J.,[1] Bista, R.,[1] Desai, R.R.,[2] Fitzgerald, B.J.,[1] and Keatinge-Clay, A.T.[1]

[1]*Dept of Molecular Biosciences, The University of Texas at Austin, Texas, USA*

[2]*Department of Chemistry, The University of Texas at Austin, Austin, Texas, USA*

The modular nature of polyketide assembly lines and their ability to generate diverse, bioactive products make them attractive targets for combinatorial engineering. Our lab demonstrated that triketide synthases engineered utilizing the updated module boundary downstream of the ketosynthase (KS) domain, possess improved activities compared to synthases engineered utilizing the traditional boundary upstream of KS.[1-4] To access larger synthases and test their synthetic capabilities, we developed a BioBricks-like route that accelerates the construction of polyketide assembly line expression plasmids. This allowed us to rapidly engineer 5 tri-, 25 tetra-, and 125 pentaketide synthases from the updated modules of the pikromycin synthase (*Streptomyces venezuelae* ATCC 15439). Every combinatorial possibility of modules 2-6 inserted between modules 1 and 7 was assessed through LC/MS analysis of *E. coli* K207-3 culture extracts. The observed products and shunt products, confirmed by high-resolution mass spectrometry (as well as NMR and crystallography for some products), provide insights into the gatekeeping activities of the pikromycin KS domains. While all KSs strongly gatekeep for chemistries at the a- and b-carbons, the extent to which they gatekeep for chemistries downstream of the b-carbon vary.[5] These gatekeeping activities should be considered when engineering novel polyketide assembly lines.



Tri-, tetra-, and pentaketide synthases were constructed from modules of the pikromycin synthase. Products (crystal structure of Pik12367 product shown) provide insights into KS gatekeeping.

References

1. Miyazawa, T., Hirsch, M., Zhang, Z., Keatinge-Clay, A.T. (2020). An in vitro platform for engineering and harnessing modular polyketide synthases. *Nat. Commun.* 1:80.
2. Miyazawa, T., Fitzgerald, B.J., Keatinge-Clay, A.T. (2021). Preparative production of an enantiomeric pair by engineered polyketide synthases. *Chem. Commun.* 57:8762-8765.
3. Zhang, L. et. al. (2017). Characterization of giant modular PKSs provides insight into genetic mechanism for structural diversification of aminopolyol polyketides. *Angew. Chem. Int. Ed. Engl.* 56:1740-1745.
4. Keatinge-Clay, A.T. (2017). Polyketide synthase modules redefined. *Angew. Chem. Int. Ed. Engl.* 56:4658-4660.
5. Hirsch, M., Fitzgerald, B.J., Keatinge-Clay, A.T. (2021). How *cis*-acyltransferase assembly-line ketosynthases gatekeep for processed polyketide intermediates. *ACS Chem. Biol.* 16:2515-2526.

How Bacteria Acylate Their Cell Envelopes to Protect Themselves from Antibiotics

Suzanne Walker

Department of Microbiology, Blavatnik Institute, Harvard Medical School, Boston, MA, USA

Bacteria acylate extracellular polymers such as peptidoglycan and teichoic acid to resist innate immune factors and some antibiotics. How bacteria move acyl groups from the cytoplasm to these extracellular polymers has been a longstanding question. Many bacterial acylation pathways include a membrane-bound O-acyl transferase (MBOAT) protein and an extracellular SGHN hydrolase family protein. We have now elucidated the role of these proteins in bacterial polymer acylation pathways. We will talk about how acyl groups are transferred from a cytoplasmic acyl donor (either an acyl carrier protein or acetyl-CoA) through two covalent intermediates before reaching their final destination on an extracellular polymer.

Rethinking the Aminoglycosides Mechanism of Action: Evidence for Inhibition of Heptosyltransferase I from *Escherichia coli*

Jozafina Milicaj,[#] Bakar A. Hassan[#], Hazel M. Holden[ψ], Yuk Y. Sham[§], Erika A. Taylor[#]

[#]*Department of Chemistry, Wesleyan University, Middletown, CT, USA*

[§] *Bioinformatics & Computational Biology Program, University of Minnesota, Minneapolis, MN, USA*

[ψ] *Department of Biochemistry, University of Wisconsin, Madison, WI, USA*

A clinically relevant inhibitor for Heptosyltransferase I (HepI) has been sought for many years because of its critical role in the biosynthesis of lipopolysaccharides on bacterial cell surfaces. While many labs have discovered or designed novel small molecule inhibitors, these compounds lacked the bioavailability and potency necessary for therapeutic use. Extensive characterization of the HepI protein has provided valuable insight into the dynamic motions necessary for catalysis that could be targeted for inhibition. Structural inspection of Kdo_2-lipid A suggested aminoglycoside antibiotics as potential inhibitors for HepI. Multiple aminoglycosides have been experimentally validated to be first-in-class nanomolar inhibitors, with the best inhibitor demonstrating a Ki of 600 +/- 90 nM. Detailed kinetic analyses were performed to determine the mechanism of inhibition while circular dichroism spectroscopy, intrinsic tryptophan fluorescence, docking, and molecular dynamics simulations were used to corroborate kinetic experimental findings. While aminoglycosides have long been described as potent antibiotics targeting bacterial ribosomes' protein synthesis leading to disruption of the stability of bacterial cell membranes, more recently researchers have shown that they only modestly impact protein production. Our research suggests an alternative and novel mechanism of action of aminoglycosides in the inhibition of HepI, which directly leads to modification of LPS production in vivo. This finding could change our understanding of how aminoglycoside antibiotics function, with interruption of LPS biosynthesis being an additional and important mechanism of aminoglycoside action. Further research to discern the microbiological impact of aminoglycosides on cells is warranted, as inhibition of the ribosome may not be the sole and primary mechanism of action. The inhibition of HepI by aminoglycosides may dramatically alter strategies to modify the structure of aminoglycosides to improve the efficacy of HepI inhibition while minimizing undesired clinical side effects when administering these compounds to fighting bacterial infections.

References

Milicaj, J.; Hassan, B. A.; Cote, J. M.: Ramirez-Mondragon, C. A.; Jaunbocus, N.; Rafalowski, A.; Patel, K. R.; Castro, C. D.; Muthayala, R.; Sham, Y. Y.; Taylor, E. A., *Scientific Reports*, **2022**, *12*, 7302. (DOI: 10.1038/s41598-022-10776-x)

Exceptions to the Oxidase Paradigm of the Flavoprotein Amine Oxidase Superfamily

Frederick Stull

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Flavoprotein amine oxidases (FAOs) are a ubiquitous class of enzymes that use a flavin prosthetic group to oxidize amine-containing substrates in the reductive half-reaction of their catalytic cycles. The resulting enzyme-bound flavin hydroquinone must then be reoxidized in the oxidative half-reaction, and a longstanding assumption applied to FAOs is that these oxidases use dioxygen (O_2) to reoxidize their flavin hydroquinones. We have discovered several bacterial FAOs that react poorly with O_2. Instead of using O_2, they transfer the electrons from substrate oxidation to cytochrome c proteins, making them dehydrogenases. We have performed mechanistic studies on nicotine oxidoreductase, one of these FAO family dehydrogenases, to probe the structural features that enable this enzyme to use cytochrome c as an oxidant, and also those that suppress reactivity of its flavin hydroquinone with O_2.

TerDockin: Stuffing carbocations into terpene synthases to refine catalytic activity

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Terpene synthases catalyze reactions proceeding through often intricate carbocation cyclization and/or rearrangement cascades, yielding the frequently polycyclic cores from which terpenoids are further elaborated. The highly reactive nature of carbocations, which can be quenched (deprotonated) by even peptide backbone carbonyls, requires tight control for specific product outcomes. Thus, terpene synthases are hypothesized to utilize steric constraints as well as catalytic base positioning in otherwise inert active sites to regulate product outcome. We have helped develop and are utilizing a computational approach towards understanding terpene synthase activity termed TerDockin. We have validated TerDockin through retrospective analysis of a previously reported single residue switch in a class I diterpene synthase, specifically the *ent*-kaurene synthase from *Bradyrhizobium japonicum* (BjKS), and have applied the approach in combination with the Rosetta Modelling Suite's enzyme design functionality in a design-build-test cycle to prospectively engineer specific alternative product outcome from premature deprotonation of the initially formed pimarenyl carbocation intermediate at a particular position. Perhaps more interestingly, we are currently attempting to engineer an entirely distinct carbocation cascade involving rearrangement rather than further cyclization. In addition, we are applying this approach to the less studied class II diterpene cyclases, again beginning with validation by retrospective analysis of alternative single residue switches that lead to distinct product outcomes, which we will follow up with more rational design efforts. Together, our work is helping provide access to the vast array of potential terpene backbones, many of which are of significant biological and/or pharmaceutical interest.

Phage-encoded glycosyltransferases hypermodify DNA with diverse glycans

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Enzymatic modification of DNA nucleobases can coordinate gene expression, protection from nucleases, or mutagenesis. We recently discovered a new clade of phage-specific cytosine methyltransferase (MT) and 5-methylpyrimidine dioxygenase (5mYOX, *e.g.,* TET) enzymes that produce 5-hydroxymethylcytosine (5hmC) as a precursor for additional post-replicative enzymatic hypermodifications on viral genomes. Here, we identify phage MT- and 5mYOX-dependent glycosyltransferase (GT) enzymes that catalyze linkage of diverse glycans directly onto 5hmC reactive nucleobase substrates. Using targeted bioinformatic mining of the phage metavirome databases, we discovered thousands of new biosynthetic gene clusters (BGCs) containing enzymes with predicted roles in cytosine sugar hypermodification. We developed a pathway reassembly platform for high-throughput functional screening of GT-containing BGCs, relying on the endogenous *E. coli* metabolome as a substrate pool. We successfully reconstituted a subset of phage BGCs and isolated novel and highly diverse sugar modifications appended to 5hmC, including mono-, di-, or tri-saccharide moieties comprised of hexose, N-acetylhexosamine or heptose sugars. Structural predictions and sugar product analyses suggest that phage GTs are related to host lipopolysaccharide, teichoic acid, and other small molecule biosynthesis enzymes and have been repurposed for DNA substrates. An expanded metagenomic search revealed hypermodification BGCs within gene neighborhoods containing phage structural proteins and putative genome defense systems. These findings enrich our knowledge of secondary modifications on DNA and the origins of corresponding sugar writer enzymes. Post-replicative cytosine hypermodification by virus-encoded GTs is discussed in the context of genome defense, DNA partitioning and virion assembly, and host-pathogen co-evolution.

Biosynthesis of Bacterial Tropone Natural Products through Enzymatic Salvaging of Catabolic Shunt Products

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The structurally diverse bacterial tropone natural products often adopt crucial roles in mutualistic and antagonistic symbiotic interactions with eukaryotic hosts in marine and terrestrial environments. Their biosynthesis relies on an unusual intertwining of primary and secondary metabolism, in which the initial steps are shared for the various tropones and rely on enzymes from phenylacetic acid catabolism. In this pathway, a distinct reactive open-chain aldehyde intermediate is formed, which is either further degraded to central metabolites, or, in the case of tropone biosynthesis, cyclized to afford the characteristic tropone scaffold. The structural diversification of this shared natural product precursor is then mediated by tailoring enzymes that are specific to each type of tropone and producer strain. In my talk, I will highlight how structurally and functionally distinct flavoenzymes are employed for the functionalization of tropone scaffolds in the late-stage biosynthesis of various tropones such as the virulence factor tropolone, or the broad-spectrum antibiotics 3,7-dihydroxytropolone and tropodithietic acid.

The electron transfer pathway and sodium transport mechanism of the evolutionary divergent bacterial respiratory complex NQR

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NQR is a six subunit and six cofactor respiratory enzyme that catalyzes the transfer of electrons from NADH to ubiquinone, feeding the lower part of the electron transfer chain. NQR is the only known respiratory enzyme that couples the electron transfer reaction to the pumping of sodium across the plasma membrane, playing a critical role in hundreds of pathogenic bacteria. In these microorganisms, NQR is the main NADH dehydrogenase and the most important ion pump. The sodium gradient produced by NQR is used to sustain critical processes, including ATP synthesis, nutrient transport, pH regulation, ion homeostasis, as well as virulence factor secretion and drug efflux, which are particularly important in pathogenic and multidrug resistant bacteria.

The NQR family has evolved separately from other respiratory enzymes and ion transporters, and has unique structural and functional properties. For instance, this is the only known enzyme that can use riboflavin as a redox cofactor, instead of using the typical flavins, such as FMN or FAD. NQR contains two covalently-bound FMN molecules, which are incorporated by the only know flavin transferase, ApbE. Moreover, the ubiquinone binding sites found in the enzyme are also completely different compared to the sites found on human enzymes. Finally, the mechanism that couples the redox reactions within cofactors to the movement of sodium across the membrane is not found in any other enzyme described, and appear to follow a novel biochemical strategy.

Due to the essential role that this enzyme plays in pathogenic bacteria and to the lack of this enzyme in the human host, it has become a prime candidate for the development of novel antibiotics against drug-resistant microorganisms.

Structural and Mechanistic Studies of Enzymes Involved in NRPS-Independent Siderophore Biosynthesis

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Over the past four decades, nearly half of all FDA-approved drugs have been natural products, derivatives thereof, or molecules with a natural product pharmacophore.[1] A long-standing interest in our lab is to understand the fundamental structural basis of the enzymes involved in natural product biosynthesis, with a goal of aiding the discovery and characterization of biosynthetic gene clusters not yet fully defined. Siderophores are natural products that are produced by bacteria in low iron environments to aid in iron acquisition. One class of siderophores, commonly containing catechol or phenolate groups, is produced by the nonribosomal peptide synthetase (NRPS) enzymes. In contrast, other bacteria produce NRPS-independent siderophores (NISs) that frequently contain hydroxamate groups to coordinate iron.[2] Both NRPS and NIS pathways contain adenylate-forming enzymes that produce amide bonds through activation of a carboxylate, followed by attack of an amine upon an adenylate intermediate. Here, we will describe our studies to characterize several NIS synthetases. We have characterized IucA[3] and IucC,[4] two NIS synthetases that produce aerobactin, a critical virulence factor in a hypervirulent clinical strain of *Klebsiella pneumoniae*. In a series of mechanistic studies, we have determined that the IucA reaction proceeds through a quarternary complex in which all three substrates bind in an ordered fashion prior to both partial reactions.[5] Additionally, we present the structure of DesD, an NIS synthetase responsible for the production of desferrioxamine, bound to an inhibitor that mimics the adenylate.[6] Combined these studies explain the active site architecture and inform the discovery and characterization of newly discovered NIS pathways.

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Discovery and development of enzymes and enzyme therapeutics using an advanced Pseudomonas fluorescens-based protein expression platform

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The Pfenex Expression Technology® platform is a robust, cost-effective and scalable system for recombinant protein production supporting protein engineering discovery and protein product development for commercialization. An overview of how this *Pseudomonas fluorescens* based expression platform was specifically developed for recombinant protein production will be presented. Case studies demonstrate how the extensive toolbox of genetic elements and host strains, along with automated strain screening workflows, enable rapid screening of proteins variants to identify lead candidates for development. Addressing complex and challenging protein structures involves broad exploration of expression strategies.

The Pfenex platform has been successfully used to support hit-to-lead screening of antibody derivatives and is currently being applied to the screening of improved mRNA manufacturing enzymes. Primrose Bio has developed a collection of superior RNA polymerases (Prima RNApols™) and is working to further improve and develop polymerases with high mRNA yields and quality. Accelerating enzyme development via high throughput expression, enrichment and analysis of engineered hits is critical to streamlining the bridge from discovery to development of a robust and scalable manufacturing process for Prima RNAPols.

A comprehensive strain selection and an early process development program utilizing the Pfenex platform were key to establishing the foundation for late-stage success of Jazz Pharmaceuticals' Rylaze™, a recombinant *Erwinia chrysanthemi* asparaginase for the treatment of acute lymphoblastic leukemia (ALL) or lymphoblastic lymphoma (LBL) in patients who have developed hypersensitivity to *E. coli*-derived asparaginase.

Synthetic biology approaches to new chemistry

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Living systems have evolved the capacity to carry out many chemical transformations of interest to synthetic chemistry if they could be redesigned for targeted purposes. Our group is interested in using synthetic biology as a platform to study how enzymes function *in vivo* and to use this understanding to build new synthetic pathways for the production of pharmaceuticals, materials, fuels, and other chemicals using living cells.

Genome Mining for New Enzymology

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The genome sequencing efforts of the past 20 years have revealed that ribosomally synthesized and post-translationally modified peptides (RiPPs) constitute a large class of peptide natural products. These molecules are produced in all three domains of life, their biosynthetic genes are ubiquitous in the currently sequenced genomes, and their structural diversity is vast. Furthermore, they are increasingly recognized for their involvement in fighting or causing human disease. This presentation will discuss the use of genome mining and synthetic biology for the discovery of new RiPPs that has proven to be an excellent platform to discover unusual enzymology involved in their biosynthesis.[1-2]

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Malaria aspartyl protease inhibitors: From an unknown mechanism of action screening hit to dual-targeting of essential enzymes

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The malaria parasite is insidious. It is transmitted by a tiny mosquito, infecting over 200 million people a year resulting in a death rate of about one person every minute of the day. Arteminisin, a life saving Nobel Prize winning drug for the treament of malaria, is starting to exhibit its limitations as parasite resistance to the molecule is now established in SE Asia and Africa. Robust antimalarial drugs are needed to fill the gap to help save lives. To this end, a phenotypic screen of an aspartyl protease inhibitor library identified a very potent hit molecule – even more potent than chloroquine (+ control). However, the target of this potent screening hit was unknown. Resistance selection followed by whole cell genome sequencing pointed to plasmepsin X as the initial target enzyme. However, subsequent *in vitro* (biochemical and intracellular) profiling of more advanced leads suggested they functioned by inhibiting two different but related plasmepsin enzymes. This dual targeting yielded chemical matter with a very high barrier to the selection of resistance and *in vitro* and *in vivo* efficacy against all 3 stages (liver, blood and sexual/mosquito) of the parasite lifecycle. A structure-guided medicinal chemistry program optimized potency, pharmacokinetics and pharmacodynamics properties of the leads resulting in the identification of a clinical development compound, MK-7602.

Proofreading Mechanism for Family D DNA polymerases

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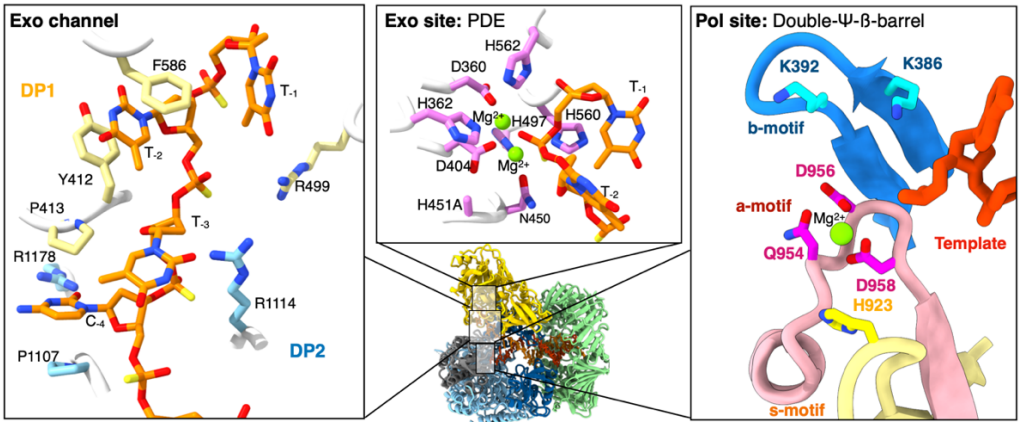
Replicative DNA polymerases copy entire genomes at high fidelity as they possess both polymerase and proofreading/exonuclease activities. These enzymes are present in all domains of life and are classified into four families: A, B, C, and D. PolD, a family D DNA polymerase found exclusively in Archaea, contains two subunits, a DP1 exonuclease subunit and a DP2 polymerase subunit. Interestingly, the DP1 exonuclease subunit of PolD contains an active site phosphodiesterase (PDE) motif that is not found in any other DNA polymerase family and the proofreading mechanism of this PDE motif is not well understood. To obtain a comprehensive understanding of the proofreading mechanism of PolD, we resolved the first Cryo-EM structure of PolD captured in its exonuclease proofreading mode. This structure reveals single-stranded DNA within the DP1 subunit with the terminal phosphodiester bond poised for cleavage within the PDE motif. Further, this structure reveals a unique channel that stabilizes the terminal 4 bases of the nascent DNA strand through multiple specific contacts between DP1 and DP2 amino acid residues and the DNA. Based off the structure, various PolD mutants were utilized to determine the contribution of these residues to exonuclease activity, kinetic rates, and mismatch bypass and observed that residues closer to the PDE motif have a higher impact as they stabilize the DNA substrate for catalysis. This work expands our understanding of how PolD achieves high fidelity DNA replication and extends the repertoire of protein domains known to be involved in DNA proofreading.



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Targeting Glycogen Synthase I for the treatment of glycogen storage diseases

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The central role of glycogen synthase in glycogen homeostasis makes it a promising therapeutic target to inhibit for treatment of glycogen storage diseases. The enzyme is allosterically regulated by G6P binding and is the paradigm for regulation via hierarchical multi-site phosphorylation. These aspects give rise to unique challenges in defining the “correct” form of the enzyme to utilize in screening funnels and in interpreting kinetic behavior by small molecule inhibitors.

Examining the structural basis for atypical heme enzyme reactivity

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It used to be thought that enzymes with the same structural scaffold catalyzed the same reaction, varying only by substrate. We have since discovered that members of many enzyme superfamilies share a common fold, and even employ a similar mechanistic strategy, yet produce dramatically different reaction outcomes. Heme-dependent enzymes are a prime example of this phenomenon. Those studied to date not only share a highly conserved fold, but also a mechanistic procedure that relies on the generation of a highly reactive Fe^{IV} =O (ferryl) species for the incorporation of molecular oxygen into their respective substrates. However, this enzyme class is replete with untapped potential as we continue to discover new chemical transformations beyond the archetypal hydroxylation reaction, ranging from ring expansion and epoxidation to nitration and aromatic coupling. The basis for this diversification is not well-understood but is often attributed to subtle changes in active site architecture that could alter substrate positioning, facilitate new proton/electron transfer pathways, or modulate the behavior of reactive intermediates. We present a series of structural studies aimed at elucidating strategies these fascinating enzymes employ to effect alternate reactivities, despite their highly conserved structural and mechanistic features.

Evolutionary Origins of Vertebrate Glucose Homeostatic Regulation

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Protein regulation is ubiquitous in biology. It plays an essential role in coordinating biochemical processes, and protein mis-regulation is a prominent cause of disease. Past experimental investigations of individual proteins have uncovered the molecular basis of many fundamental regulatory tactics, including allosteric control of enzyme activity and regulation of function via protein-protein interactions. Despite this fact, we know little about how evolutionary trajectories alter the intrinsic structural properties of proteins to facilitate the emergence of new regulatory strategies. Here, we investigate the evolutionary origins of glucose homeostatic maintenance, a hallmark of vertebrate physiology. At the heart of glucose homeostasis lies glucokinase (GCK), a key metabolic enzyme that functions as the body's glucose sensor. GCK activity is controlled by two distinct regulatory mechanisms. GCK is allosterically regulated by its substrate glucose, displaying a cooperative kinetic response with a midpoint value matching physiological blood glucose concentrations. GCK is also regulated by a heteromeric interaction with the glucokinase regulatory protein (GKRP), which acts as a competitive inhibitor of GCK and sequesters the enzyme within the hepatic nucleus. We resurrected contemporaneous ancestors along the GCK and GKRP phylogenies to establish the timing and molecular events leading to regulation in this system. Using a suite of biochemical and biophysical techniques, we demonstrate that GCK underwent conformational expansion during early vertebrate evolution (~600 Mya), resulting in an ability to sample a unique, super-open conformation. The GCK-GKRP regulatory interaction arose ~75 million years later, via *de novo* evolution of a binding loop in GKRP that co-opted a hydrophobic surface present only in GCK's super-open conformation. Our results show how biophysical features of proteins, including conformational space and pre-existing hydrophobic surfaces, are exploited by evolution to respond to, and mediate, changes in organismal physiology via protein regulation.

Structure and mechanism of a nucleotide sanitizing enzyme with a role in cancer

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The human enzyme 2′-deoxynucleoside 5′-phosphate *N*-hydrolase 1 (DNPH1) catalyses the *N*-ribosidic bond cleavage of 5-hydroxymethyl-2′-deoxyuridine 5′-monophosphate (5hmdUMP) to generate 2-deoxyribose 5-phosphate and the nucleobase 5-hydroxymethyluracil. Activity of DNPH1 leads to resistance to PARP inhibitors, a class of anticancer drugs used in the clinic to treat cancers with $BRCA^{-/-}$ genotype, such as many breast, prostate, and ovarian cancers. Conversely, inhibition of DNPH1 activity re-sensitizes tumours to PARP inhibition and potentiates the drug.[1] DNPH1 reaction proceeds by a double-displacement mechanism where Glu104 attacks the substrate C1′ to displace the nucleobase and form a covalent intermediate with the 5-phospho-2-deoxyribosyl group. Glu55 acts as the general base to activate a water molecule to hydrolyse the intermediate and complete the catalytic cycle.[2] Of the residues making up the conserved catalytic triad Tyr24, Asp80 and Glu104, only the role of Glu104 is established. We solved crystal structures of WT-DNPH1 in its unliganded form and bound to dUMP (a poor substrate),[3] and of D80N-DNPH1 bound to the physiological substrate 5hmdUMP and in its unliganded form. This work was complemented by functional analysis of the reaction catalysed by variants E104A-, E104D-, Y24F-, Y24H-, D80A-, D80N-, H56A-, and R30A-, using HPLC, pH-rate profiles, solvent deuterium isotope effects, ^{1}H-NMR, and rapid kinetics. A role for Tyr24 and Asp80 in catalysis is proposed that includes alternating H-bond patterns with Glu104 and modulating the pK_a of this residue for nucleophilic attack.

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Prenylated flavin-dependent decarboxylases

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Prenylated-FMN (prFMN) dependent decarboxylases (also known as UbiD-like enzymes) are the most recently discovered family of decarboxylases. The modified flavin facilitates the decarboxylation of unsaturated carboxylic acids through a novel mechanism involving 1,3-dipolar cyclo-addition chemistry. UbiD-like enzymes have attracted considerable interest for biocatalysis applications due to their ability to catalyse (de)carboxylation reactions on a broad range of aromatic substrates at otherwise unreactive carbon centres. The prenylated-FMN cofactor is synthesized from reduced FMN and dimethylallyl phosphate by a specialized prenyl transferase (UbiX). However the process by which reduced prFMN is oxidized to the active form remains poorly understood.

I will present recent studies from our lab aimed at understanding the mechanisms and scope of prFMN-dependent decarboxylases and the process by which the newly synthesized, reduced prFMN cofactor oxidatively matures into the catalytically active form.

Discovery and characterization of JNT-517, an inhibitor of SLC6A19 for the treatment of phenylketonuria

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Phenylketonuria (PKU) is caused by pathogenic variants in the phenylalanine hydroxylase (*PAH*) gene. PAH converts phenylalanine (Phe) to tyrosine and the absence of PAH leads to toxic accumulation of Phe in the blood and brain, resulting in significant neuropsychiatric symptoms and impaired quality of life. Solute carrier family 6 member 19 (SLC6A19) is the major transporter responsible for the (re)absorption of neutral amino acids, including Phe, in the intestine and kidney. In a well-established mouse model of PKU *(Pahenu2)*, previous reports have demonstrated that a genetic loss of *Slc6a19* causes aminoaciduria, including increased excretion of Phe as well as a 70% reduction in plasma Phe. We describe the discovery effort using our RAPID (Reactive Affinity Probe Interaction Discovery) chemoproteomics platform which led to small molecules that inhibited SLC6A19 and demonstrated in vivo activity in the *Pahenu2* model. This work led to the identification of JNT-517, a first-in-class clinical candidate for the treatment of PKU.

Structural and Dynamic Features of Peroxiredoxins Aligned with Their Roles In Peroxide Defense and Signaling

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While peroxiredoxins (Prxs) are highly active catalysts of peroxide reduction across biology, organisms somewhat paradoxically express them in high amounts, and express multiple distinct gene products that could be perceived as having redundant functions (3 types in E. coli, 6 types in mammals). However, selective knockouts of each, e.g. in mouse models, do not support such redundancy, which may in part stem from their non-defense roles in regulating or transmitting redox signals to other cellular proteins. Recent advances have enabled new insights, using both high resolution structural data and NMR-based analysis of site-specific dynamics information, into how individual Prxs can be "tuned" to align with their distinct biological roles. There is also building evidence that signal-induced posttranslational modifications of Prxs further expand the ability of these proteins to modulate their catalytic and regulatory properties depending on the needs of the cell, tissue and organism.

lavoproteins Through the Quantum Mechanical Looking Glass

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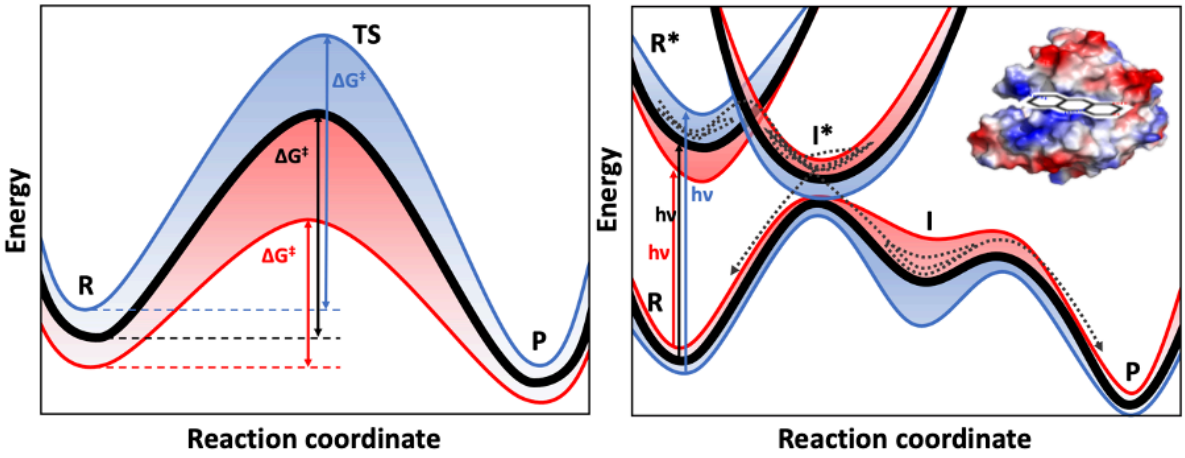
Flavin is famously a cofactor in various oxidoreductases, transferases, lyases, isomerases, and ligases. However, when it is excited by light, flavin is still a remarkably versatile cofactor that can undergo photoredox, proton-coupled electron transfer, fluorescence, intersystem crossing, and/or photochemical reactions. In fact, several families of flavoproteins (LOV, BLUF, and CRY) mediate light sensing and response in organisms. We develop and apply computational tools to study how flavoproteins tune the spectroscopy, photophysics, and photochemistry of their flavin cofactor. I will introduce two such tools: Electrostatic tuning maps [1-2] and average protein electrostatic configurations (APEC). The latter is a hybrid quantum mechanical / molecular mechanical (QM/MM) approach that performs multi-configurational quantum chemical computations in an ensemble of protein structures obtained from molecular dynamics simulations [3-4]. Just as QM/MM methods have been widely used to model transition state activation energies in enzymes (left panel), we use APEC to study the excited state energetics of protein-bound cofactors (right panel).



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Proton-Coupled Electron Transfer in Enzymes

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Proton-coupled electron transfer (PCET) reactions play a vital role in a wide range of biological processes. This talk will summarize the main concepts from our PCET theory and will focus mainly on the application to the enzyme ribonucleotide reductase (RNR), which is essential for DNA synthesis and repair. Our general theoretical formulation for PCET includes the quantum mechanical effects of the electrons and transferring protons, as well as the motions of the donor-acceptor modes and protein environment. This PCET theory enables the calculation of rate constants and kinetic isotope effects for comparison to experiment. Our application of this theory to PCET in soybean lipoxygenase provides an explanation for the unusually large kinetic isotope effects in terms of hydrogen tunnelling in a constrained enzyme environment. A more recent application explores the PCET pathway in RNR, which entails six PCET reactions spanning more than 32 Angstroms across an aqueous interface. Our quantum mechanical/molecular mechanical (QM/MM) free energy simulations provide insight into the roles of conformational motions, hydrogen bonding, water interactions, and proton relays.[1,2,3] Further analysis of RNR with our PCET theory elucidates the role of hydrogen tunneling and provides additional mechanistic insights. Moreover, kinetic modeling of the overall reverse radical transfer process in RNR illustrates the time evolution of radical transport following radical injection and identifies the key rate constants that may be tuned to alter the timescale and mechanism of this process.[4] This level of understanding will assist in guiding protein engineering efforts in this biochemically and pharmacologically significant enzyme.

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Mechanism-based covalent inactivation of the flavoenzyme proline dehydrogenase

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Proline dehydrogenase (PRODH) and Δ^1-pyrroline-5-carboxylate (P5C) reductase (PYCR) form a metabolic relationship known as the "proline cycle", a novel pathway that impacts cellular growth and death pathways. PRODH catalyzes the FAD-dependent oxidation of proline to pyrroline-5-carboxylate (P5C), while PYCR1 catalyzes the reverse transformation, the NAD(P)H-dependent reduction of P5C to proline. The proline cycle is central to the metabolic shift that enables tumorigenesis and supports the metastatic cascade of cancer cells. PRODH is upregulated in metastasizing breast cancer cells, and PYCR1 is one of the most consistently upregulated enzymes across multiple cancer cell types. We are developing chemical probes against PRODH and PYCR1 to aid the investigation of the cellular mechanisms of the proline cycle in cancer and to explore the tractability of these enzymes as therapeutic targets. As part of the effort, we screened libraries of target-focused fragments to develop structure-affinity relationships for PRODH and PYCR1. These studies serendipitously led to the discovery of S-heterocyclic compounds that irreversibly inactivate PRODH by covalently modifying the FAD N5 atom,[1] reminiscent of the first PRODH inactivator characterized over a decade ago, *N*-propargyliminoglycine.[2] In this presentation, I will discuss the structures and inactivation mechanisms of the three known classes of mechanism-based covalent inactivators of PRODH. Although all three result in the covalent modification of the N5 atom and lock the FAD into a reduced, inactive state, their mechanisms of inactivation are quite different, as are the structures of the modified enzyme. The prospects for leveraging this information in chemical probe discovery will also be discussed.

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Revealing Radical SAM Mechanisms Via Time-Resolved FQ-EPR

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Enzymes of the radical *S*-adenosyl-L-methionine (radical SAM, RS) superfamily, the largest in nature, use a [4Fe-4S] cluster and SAM to catalyze remarkably diverse radical reactions.[1] RS enzymes that act on protein or peptide substrates include the glycyl radical enzyme activating enzymes (GRE-AEs) important in anaerobic metabolism, and enzymes that catalyze epimerizations, splicing reactions, and cross-linking reactions during maturation of **ri**bosomally-encoded, **p**ost-translationally modified **p**eptides (RiPPs). We are using freeze-quench techniques coupled to electron paramagnetic resonance spectroscopy to gain insights into mechanistic steps and reaction intermediates in these reactions. We examine the reaction of the GRE-AE pyruvate formate-lyase activating enzyme (PFL-AE) with peptide substrate mimics such as RVSG$_{734}$YAV,[2] a model for the site of glycyl radical formation on the native substrate PFL, as well as RVS(Dha)YAV,[3] which has a dehydroalanine (Dha) in place of glycine and results in a change in reactivity from H-atom abstraction to adenosylation. Time-resolved freeze-quench electron paramagnetic resonance spectroscopy shows that at short mixing times for both substrates result in formation of the central organometallic intermediate, Ω, in which the adenosyl 5'C is covalently bound to the unique iron of the [4Fe-4S] cluster. Freeze-trapping the reactions at longer times reveals the formation of subsequent intermediate/product radicals dependent on identity of the substrate. Of central importance, freeze-quenching at intermediate times reveal the conversion of Ω to the nominally 'free' 5'-dAdo• radical, which can be observed to convert to a peptide-based radical upon cryo-annealing. These observations reveal the 5'-dAdo• radical to be a well-defined intermediate, caught in the act of initiating subsequent chemistry, providing new insights into the mechanistic steps of radical initiation by radical SAM enzymes.

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Posters Titles

Poster presenters should be at their posters at the following times:

Even numbered:
Thursday, Poster Session 1, 10:00 am-12:00 pm
Thursday, Poster Session 2, 8:50-10:00 pm

Odd numbered:
Friday, Poster Session 3, 10:00 am-12:00 pm
Friday, Poster Session 4, 8:50-10:00 pm

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4	Angerhofer, Alexander	Substrate Binding in Oxalate Decarboxylase as seen by ^{13}C-ENDOR
5	Antony, Edwin	CryoEM unveils the intricacies of long-range electron transfer in the nitrogenase-like DPOR complex
6	Bauer, Olivia	Thermodynamic Dependencies of Reaction Intermediate Formation of M379A Mutant Tyrosine Phenol-lyase
7	Bigley, Andrew	Characterization of PhoK-type Phosphatases Implicated in Organophosphate Flame-Retardant Degradation
8	Bilotti, Katharina	Mismatch discrimination and sequence bias during end-joining by DNA ligases
9	Bista, Ramesh	Combinatorial Biosynthesis of Large Polyketides Using Hybrid Engineered Polyketide Synthases
10	Blankenship, Sara	An Active-Site Cysteine to Serine Mutation in DOKDC Decreases Enzyme Activity through the Formation of PMP
11	Bourland, Ronnie	The Discovery of Enzymes Required for Biosynthesis of The HS1 Capsular Polysaccharide of Campylobacter Jejuni
12	Bruffy, Samantha	Mechanistic insight into aldolase activity with ketones
13	Cao, Mengtong	Discovery and Characterization of PRMT1-PRMT6 Interaction
14	Carberry, Anna	Mechanism of human DNPH1 reaction: implications for anticancer therapy
15	Cen, Yana	Activation of SIRT6 Deacetylation by DNA Strand Breaks
16	Chang, Yuxuan	Repurposing the 70 years old drug hydralazine for glioblastoma treatment
17	Chekan, Jonathan	Copper-Dependent Peptide Cyclases In Plant Peptide Biosynthesis

18	Chen, Jeff	Investigations into a multinuclear non-heme iron dependent oxidative enzyme that produces hydantoin-containing macrocyclic peptides
19	Conley, James	Computational and Structural Investigation of the Improved RNA-Clamping Activity of Amidino-Rocaglates on eIF4A1
20	Dangerfield, Tyler	Dynamics of Exonuclease Proofreading by T7 DNA Polymerase During DNA Replication
21	DelRioFlores, Antonio	De novo biosynthesis of azide by a promiscuous *N*-nitrosylase
22	Doleschal, Megan	Cell-Based Covalent Deubiquitinase-Capturing Assay for Inhibitor Discovery
23	Donu, Dickson	Nicotinamide Riboside Activates SIRT5 Deacetylation
24	Eastman, Karsten	Intermolecular electron transfer in radical SAM enzymes as a new paradigm for reductive activation
25	Elijah, Kissman	A dynamic metal coordination sphere controls chemoselectivity in radical amino acid halogenases
26	Ellis, Holly	Hydrogen Sulfide Oxidation Enzymes Critical in Maintaining Bacterial Virulence
27	Errickson, Max,	Biosynthesis of UDP-β-L-Arabinofuranoside for the Capsular Polysaccharides of *Campylobacter jejuni*
28	Fisk, Mercedes	Characterization of a Rare NRPS-NIS Hybrid System for the Biosynthesis of the Siderophore, Nocardichelin
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30	Gaynes, Matthew	Structure and Function of the Monoterpene Cyclase, Sabinene Synthase
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32	goodey, nina	The inverse solvent viscosity effect in Glu57Asp *M. tuberculosis* IGP synthase
33	Guo, Yisong	An S = 1 Iron(IV) Intermediate Revealed in a Non-Heme Iron Enzyme-Catalyzed Oxidative C-S Bond Formation
34	Hewitt, Patrick	Characterization of Intermediates formed in the Reactions of the Diheme Cytochrome-*c* Peroxidase from *Nitrosomonas europaea* with Hydrogen Peroxide
35	Higgins, Melanie	Biosynthesis of the aminocyclitol from Hygromycin A
36	Hoffman, Esther	Probing the Post-Translational Catalytic Cleavage Mechanism Required to Activate a New NylC Enzyme
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68	Ramos-Figueroa, Josseline	Uncovering the biosynthetic pathway towards the production of Ammosamide C
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96	Yu, Yue	Peptide extension after chain reversal during the biosynthesis of nonribosomal peptides
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100	Zhang, Yong	Mechanistic manifold in a hemoprotein-catalyzed cyclopropanation reaction with diazoketone
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Poster Abstracts

1. Structural and Kinetic Analysis of a Novel NRPS Adenylation Domain FbsH

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Non-ribosomal peptide synthetases (NRPSs) produce diverse natural products such as antibiotics and siderophores. Siderophores are important chelating agents that numerous pathogenic bacteria use to survive in low iron conditions. Engineering NRPSs to produce diverse siderophore analogs could lead to the generation of novel antibiotics, toxins, and other therapeutics that take advantage of this unique iron uptake system in bacteria. The highly pathogenic and antibiotic-resistant bacteria *Acinetobacter baumannii* produces the siderophore fimsbactin, which uses catechol and hydroxamate groups to bind iron. Previous studies have reported the full *in vitro* reconstitution of fimsbactin A biosynthesis and proposed a mechanism of NRPS branching to produce the final product[1]. However, the substrate promiscuity of the assembly line enzymes in this biosynthetic pathway has not yet been explored. Here we report two novel structures of the stand-alone aryl adenylation enzyme FbsH bound to its native substrate 2,3-dihydroxybenzoic acid (DHB) and a salicyl-AMS inhibitor. Structural analysis of the FbsH allowed us to engineer variants with an expanded binding pocket to incorporate bulky DHB analogs. Kinetic analysis showed that wildtype FbsH can accommodate analogs that alter the catechol group but is more selective for analogs at the C4 position and that expanding the binding pocket improves the tolerance for these bulky substrates. Finally, full *in vitro* reconstitution analysis showed that the altered substrates can progress down the fimsbactin assembly line to the downstream cyclization domains; however, selectivity for DHB analogs in catalytic domains that catalyze the final steps prevented the production of fimsbactin analogs.

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2. Characterization of oxidant specificity in bacterial flavoprotein amine "oxidases"

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The flavoprotein amine "oxidase" superfamily is involved in various redox reactions in biological systems. They catalyze C-N bond oxidation in the amine containing substrate while using flavin adenine dinucleotide (FAD) as a prosthetic group. Their reaction proceeds in a two-step mechanism involving hydride transfer from substrate to the bound FAD cofactor, and the reduced flavin is subsequently reoxidized by a physiological electron acceptor. For about a century, this enzyme superfamily has been assumed to all be oxidases i.e., donating electrons from substrate oxidation to dioxygen (O_2). However, our lab has demonstrated poor re-oxidation of two bacterial flavoprotein amine oxidases, nicotine oxidoreductase (NicA2)[1] and pseudooxynicotine amine oxidase (Pnao)[2], by O_2. Instead, experimental evidence shows that a cytochrome c protein closely encoded in the genome is the natural electron acceptor of these enzymes[2], demonstrating that these enzymes are in fact dehydrogenases. These findings led us to hypothesize that other enzymes in this family may also be cytochrome c using dehydrogenases despite the "oxidase" name applied to the superfamily. A genomic and phylogenetic analysis of 5000 flavoprotein amine oxidases indicated the presence of many potential dehydrogenases in this superfamily. We are currently testing the reactivity of representative flavoprotein amine oxidases from each clade towards dioxygen as well as the cytochrome c closely encoded to the flavoprotein gene in the genome of the organism. Kinetic analysis of the reactivity collected thus far suggests that the use of cytochrome c is more widespread in this enzyme superfamily than previously realized.

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3. Transient State Analysis of Bacterial Dihydropyrimidine Dehydrogenase

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Dihydropyrimidine dehydrogenase (DPD) is an oxidoreductase that catalyses the reduction of the 5,6-vinylic bond of uracil and thymine using electrons derived from NAD(P)H. Despite the relative simplicity of the chemistry catalysed, known DPDs have a complex architecture, for which a satisfactory explanation has not yet been offered. DPDs generally utilize six cofactors per subunit (FAD, $4Fe_4S_4$, and FMN) to couple the oxidation of NAD(P)H to the reduction of the pyrimidine substrate. Electrons from NAD(P)H are taken up at the FAD and traverse ~60 Å through the protein, via the Fe-S clusters, to the FMN, where they are delivered to the pyrimidine substrate. DPD from *Sus scrofa* (SsDPD) has been studied extensively and displays an unusual catalytic mechanism. Two electrons are taken up at the FAD, from NADPH, and are rapidly transmitted to the FMN, establishing the two electron reduced, FAD•4(Fe4S4)•FMNH2, resting-state of the enzyme. Only in the presence of additional NADPH, to reinstate this form of the enzyme, will turnover be completed via reduction of the pyrimidine, indicating that the two-electron reduced state is the active form of the enzyme. This has been termed reductive activation. Herein we present the first comprehensive characterization of DPD from *E. coli* (EcDPD). EcDPD displays a similar reductive activation process as well as half-of-sites behavior, similar to what has been observed with SsDPD. However, the mechanism of EcDPD is distinct from the mammalian homologue in that it shows diminished effector roles for its substrates. EcDPD will readily take up electrons from NADH in the absence of pyrimidine substrate and will complete turnover by reducing the pyrimidine substrate in the absence of excess NADH, albeit at a slower rate than in the presence of NADH.

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4. Substrate Binding in Oxalate Decarboxylase as seen by ^{13}C-ENDOR

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The Mn-dependent bicupin enzyme Oxalate Decarboxylase (OxDC, EC 4.1.1.2) from *Bacillus subtilis* catalyzes the redox-neutral disproportionation reaction of oxalate into formate and carbon dioxide at low pH. The quaternary structure of the enzyme is a hexamer composed of a dimer of trimers where the monomeric bicupin domains are linked together at the vertices of a triangle. The monomer unit has two cupin domains which both contain a high-spin Mn(II) ion coordinated by three histidines and a glutabmate.[1] Based on X-ray structures and site-directed mutagenesis experiments, the N-terminal cupin domain is considered to be the site of catalysis. A catalytically competent long-range electron transfer pathway exists across subunit boundaries via a π-stacked tryptophan pair at the vertices of the trimeric structure.[2] It allows for 1-electron oxidation of the N-terminal Mn(II) from an oxidized Mn(III) in the C-terminal domain after substrate binds in the active site.[2]

In this contribution we present pulsed EPR and ^{13}C-ENDOR (electron nuclear double resonance) studies of ^{13}C-labeled substrate bound to the N-terminal Mn(II) ion in the site-directed mutant enzyme W96F OxDC. W96F is used as a model for the active site since it shows structural fidelity to the WT enzyme but an order of magnitude slower catalysis, making it easy to trap the substrate-bound state.[2] Electron spin echo envelope modulation (ESEEM) water counting experiments on the N-terminal Mn(II) in W96F show the displacement of all water molecules bound to Mn upon addition of oxalate suggesting a side-ways bi-dentate binding mode of the substrate to the metal ion. This result was confirmed by X-Band ^{13}C-ENDOR data on both WT OxDC at pH 8.5 (in the inactive pH range) and W96F at pH 5.0 (in the active pH range) using ^{13}C-labeled oxalic acid. Density Functional Theory (DFT) calculations support a slightly asymmetric, yet side-ways bi-dentate binding mode of the substrate to Mn^{2+} in the active site. The ENDOR–derived hyperfine coupling constants were validated by DFT and showed the two ^{13}C in the labeled substrate at distances of 2.9 and 3.1 Å from the Mn(II) ion.

One of the implications of this result is that dioxygen is unable to bind to the active site Mn during turnover which prevents it from directly reacting with the substrate and directs the chemistry toward decarboxylation. In our current mechanistic proposal dioxygen may act as an initiator, possibly binding to and oxidizing the C-terminal Mn ion which can then oxidize the substrate-bound N-terminal Mn(II) to initiate catalysis.

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5. CryoEM unveils the intricacies of long-range electron transfer in the nitrogenase-like DPOR complex

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Dark-operative protochlorophyllide oxidoreductase (DPOR) catalyzes the 2-electron reduction of protochlorophyllide to chlorophyllide in chlorophyll biosynthesis. In the presence of ATP, electron donor and acceptor component proteins assemble and facilitate electron transfer (ET) across multiple metal clusters. Cryo-electron microscopy (CryoEM) imaging of DPOR reveals its symmetrical halves function asymmetrically: one half facilitates ET via aligned amino acid residues while the other obstructs it with misaligned residues. Our study uncovers a mechanism where the binding of the electron donor component and ATP hydrolysis in one half prompts long-range allosteric conformational changes in the other, orchestrating ET and substrate reduction. Notably, we uncover that a Cu cluster at the interface significantly impacts DPOR function. These findings reveal the structural blueprint for coordination of long-range electron transfer in nitrogenase-like enzymes.

6. Thermodynamic Dependencies of Reaction Intermediate Formation of M379A Mutant Tyrosine Phenol-lyase

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Tyrosine phenol-lyase (TPL) is a pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyzes the reversible β-elimination of L-tyrosine into phenol and ammonium pyruvate as well as the irreversible elimination other amino acids with good leaving groups on the β-carbon. Due to the versatility of the PLP cofactor in stabilizing carbanionic reaction intermediates and the intrinsic ability of TPL to catalyze an array of substrates with good leaving groups, TPL has been used to synthesize numerous L-tyrosine analogs that are biochemically and pharmaceutically relevant, like L-DOPA.[1] Thus, engineered TPL variants with wider substrate specificity and improved catalytic efficiency are of great interest. Our lab has recently shown the M379A TPL variant to be a robust catalyst with an increased range of substrates, specifically for 3-substituted tyrosine analogs which cannot be accommodated by native TPL.[2] However, the M379A mutation also alters the conformational dynamics that are coupled with TPL catalysis.[2,3] Here, we further characterize how the M379A mutation affects the protein dynamics that play a key role in TPL reaction mechanism. The rate of formation of the M379A aminoacrylate intermediate shows an obvious nonlinear temperature dependence and has significantly increased change in entropy ($\Delta S^{\ddagger}$) and change in enthalpy ($\Delta H^{\ddagger}$) when compared to the native TPL. Additionally, our data show that occupation of the void created by the M379A substitution results in a large change in heat capacity ($\Delta C_p^{\ddagger}$). These results indicate that the M379A substitution alters the vibrational capacity of the aminoacrylate intermediate and active site conformational dynamics. Additionally, investigation into the effect of hydrostatic pressure on the formation of the M379A TPL quinonoid intermediate shows the presence of multiple distinct equilibrium species, a unique aspect that is not seen in native TPL. Current work is focused on elucidating the pressure dependent processes of M379A TPL quinonoid intermediate and further investigating how the M379A mutation has affected transition state thermodynamic activation parameters.

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7. Characterization of PhoK-type Phosphatases Implicated in Organophosphate Flame-Retardant Degradation

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The PhoK-type phosphatases are of interest in bioremediation and biotechnology due their potential for the sequestration of heavy metals and their implication in the degradation of toxic organophosphate flame retardants.[1-2] The PhoK-type phosphatases are thought to have evolved from the nucleotide pyrophosphatase/phosphodiesterase (NPP) enzyme family. The NPP-family is part of the larger alkaline phosphatase superfamily, but unlike the majority of the alkaline phosphatase family the NPP-enzymes catalyze diesterase reactions rather than phosphatase reactions.[3] In the PhoK-type enzymes the enzymatic specificity has reverted back to a phosphatase. The limited structural information for the PhoK-type enzymes suggests that a key lysine substitution supports the dianionic transition state, but nothing is known about the substrate specificity of these enzymes.[4] Initial characterization of the PhoK enzyme implicated in the degradation organophosphate flame retardants in *Sphingobium* sp. TCM1 (*Sb*-PhoK) found that gene expression was regulated in response to organophosphate flame retardants, but no kinetic characterization has been carried out on organophosphate flame-retardant derived phosphoesters.[2] Bioinformatics analysis has now identified homologs of *Sb*-PhoK from numerous members of the *Sphingomonadaceae* bacterial family. Interestingly, homologs from known or suspected degraders of the organophosphate flame retardants appear to be more closely related to each other than homologs from more closely related species. A set of homologs of *Sb*-PhoK from species that are known to degrade organophosphate flame retardants as well as representatives from non-degrading species has been cloned and expressed in E. coli. Each homolog is characterized with a broad set of substrates including common metabolic compounds as well as phosphoesters derived from organophosphate flame retardants to determine the substrate specificity of each enzyme and to determine the extent to which the PhoK enzymes have evolved specifically toward the degradation of the organophosphate flame retardants.

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8. Mismatch discrimination and sequence bias during end-joining by DNA ligases

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DNA ligases, critical enzymes for in vivo genome maintenance and modern molecular biology, catalyze the joining of adjacent 3'-OH and 5'-phosphorylated ends in DNA. To determine whether DNA annealing equilibria or properties intrinsic to the DNA ligase enzyme impact end-joining ligation outcomes, we used a highly multiplexed, sequencing-based assay to profile mismatch discrimination and sequence bias for several ligases capable of efficient end-joining. Our data reveal a spectrum of fidelity and bias, influenced by both the strength of overhang annealing as well as sequence preferences and mismatch tolerances that vary both in degree and kind between ligases. For example, while T7 DNA ligase shows a strong preference for ligating high GC sequences, other ligases show little GC-dependent bias, with human DNA Ligase 3 showing almost none. Similarly, mismatch tolerance varies widely among ligases, and while all ligases tested were most permissive of G:T mismatches, some ligases also tolerated bulkier purine:purine mismatches. These comprehensive fidelity and bias profiles provide insight into the biology of end-joining reactions and highlight the importance of ligase choice in application design.

9. Combinatorial Biosynthesis of Large Polyketides Using Hybrid Engineered Polyketide Synthases

Ramesh Bista[1], Takeshi Miyazawa[1], Hayden Stegall[1], Kiran Thirukonda[1], Hubert Jaegy[1], Joshua Lutgens[1], Jie Zhang[1], Adrian T. Keatinge-Clay[1]

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Modular polyketide synthases (PKSs) are enzymatic assembly lines comprised of 3-30 modules that collaborate to generate complex polyketide products with diverse applications, ranging from agriculture to medicine. Leveraging their modularity through combinatorial engineering is a promising strategy in the pursuit of designer polyketide production, especially since the recent redefinition of the module[1]. Recently, our lab developed a BioBricks platform to engineer a comprehensive array of triketide, tetraketide, and pentaketide synthases through shuffling the updated modules of the Pikromycin synthase. Despite all combinations being explored, only a fraction of the synthases produced detectable quantities of their anticipated product. The results indicated that the ketosynthase (KS) domains impede the activities of these engineered synthases by acting as gatekeepers against non-native intermediates.

Analysis of the products and shunt products produced by these synthases provided valuable insights into the gatekeeping tendencies of KS domains. Most KS domains of the Pikromycin synthase possess gatekeeping residues in their substrate tunnels that reject non-cognate intermediates[2]. The study also unveiled the existence of KSs, such as those in the Rapamycin synthase, that display greater substrate promiscuity. These KS domains lack commonly observed gatekeeping residues in their substrate tunnels and were observed to nonspecifically accept non-native polyketide intermediates so they can be relayed down the assembly line. Thus, we developed a 2-plasmid, BioBricks-like strategy to design larger PKSs, such as hexaketide and heptaketide synthases, that capitalize on the relaxed gatekeeping properties exhibited by the Rapamycin KSs.

A large proportion of these PKSs, engineered from the updated modules of the Pikromycin and Rapamycin synthases, yielded their anticipated products. These polyketides possess functionality that has not been observed in the macrolide antibiotics, and many have the potential to be converted into derivatives of the antibacterial agents Pikromycin and Methymycin through the transfer of a desosamine sugar. This research highlights the potential of utilizing updated modules containing promiscuous KS domains in the pursuit of engineered PKSs that synthesize designer polyketides.

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10. An Active-Site Cysteine to Serine Mutation in DOKDC Decreases Enzyme Activity through the Formation of PMP

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D-Ornithine/D-lysine decarboxylase (DOKDC) from *Salmonella enterica* serovar typhimurium is structurally like human and *E. coli* L-ornithine and L-lysine decarboxylases, despite low sequence homology. It is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that functions as a dimer with PLP bound at the dimer interface. Unlike other similar enzymes, DOKDC decarboxylates using an S_E2 mechanism, with inversion of stereochemistry, and is stereospecific for D-amino acids. Previous research has shown that Tyr-430, which is a phenylalanine in the active site in L-specific enzymes, plays a role in DOKDC's stereospecificity. A proton donor is necessary for the decarboxylation, but one has not yet been identified. Cys-387 was identified as a possible proton donor, based on crystal structure data, as it is approximately 4 angstroms from the Cα of substrate in the active site.

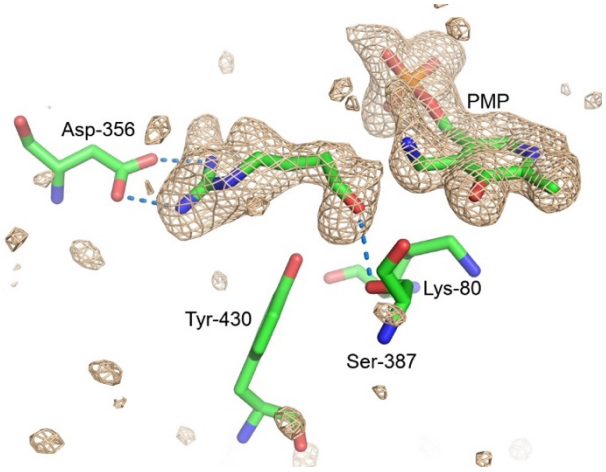

Figure 1. Decarboxylation-dependent transamination of PLP to PMP with the inhibitor D-Arg. The carbonyl of the aldehyde product is hydrogen bonded to Ser-387.

Kinetic studies on C387S DOKDC showed decreased activity compared to wild-type. The wild-type k_{cat} for the substrate D-Lys was 4.4 s^{-1} and D-Orn was 1.8 s^{-1} while the k_{cat} for D-Lys in C387S was 0.97 s^{-1} using the phenol red assay. No catalytic activity was observed for D-Orn. Activity was decreased, but not abolished with this mutation, which is consistent with data from eukaryotic L-ornithine decarboxylase (ODC), although the decrease in activity for ODC was much larger (1000x for ODC vs. approximately 10x for DOKDC). Stopped-flow experiments, mass spectrometry, and X-ray crystallography also showed that C387S forms pyridoxamine 5-phosphate (PMP) through decarboxylation-dependent transamination, which is a side reaction that inactivates the enzyme and is consistent with the equivalent mutation in ODC.

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11. The Discovery of Enzymes Required for Biosynthesis of The HS1 Capsular Polysaccharide of Campylobacter Jejuni

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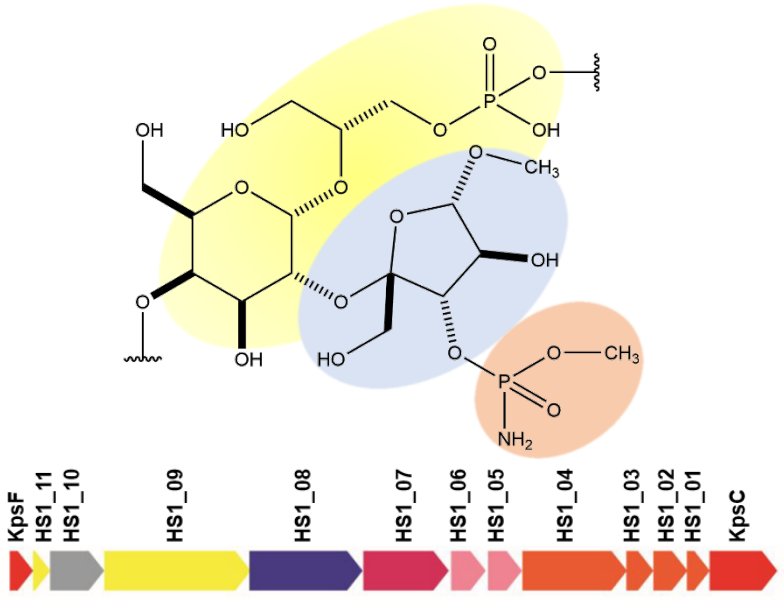
Campylobacter jejuni is a gram-negative pathogenic bacterium commonly found in poultry and is the leading cause of gastrointestinal infections in the United States. Similar to other gram-negative bacteria, *C. jejuni* possesses extracellular carbohydrate-based capsular polysaccharides composed of repeating units of monosaccharides bound via glycosidic linkages that contribute to bacterial colonization and pathogenicity. Serotype 1 (HS1) contains 13 different genes required for the production and presentation of the CPS. HS1_01 – HS1_07 are involved in the biosynthesis and transfer of methyl phosphoramidate. Each repeat unit within the HS1 CPS structure contains a backbone of glycerol phosphate with the C2 of glycerol linked to the anomeric carbon of galactose that is decorated with a fructose molecule with a methylated phosphoramidate moiety bound at C3 of fructose. The full-length HS1_11 protein was expressed, purified, and characterized. The kinetic parameters for HS1_11 was determined through HPLC and NMR experiments with a turnover rate of 13 s^{-1} when saturating both CTP and L-glycerol-3-phosphate. HS1_09 is a multidomain protein with two predicted catalytic functions and required truncations to separately characterize each domain. The HS1_09 truncation from amino acids 286-703 catalyzed the formation of the disaccharide, 4-((R)-glycerol-1-phosphate)-1-O-methyl-α-D-galactopyranose, from CDP-glycerol and 1-O-methyl-α-D-galactopyranose. The biosynthesis of the disaccharide was monitored through HPLC experiments and confirmed with NMR and mass spectrometry. The HPLC derived turnover rate for 1-O-methyl-α-D-galactopyranose was 7.8 h^{-1}.



12. Mechanistic insight into aldolase activity with ketones

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Aldolases are prodigious C–C bond forming enzymes, but their reactivity has only been extended past activated carbonyl electrophiles in special cases.[1] To probe the mechanistic origins of this limitation, we compared the reactivity of a pair of pyridoxal-phosphate (PLP)-dependent aldolases. Our results reveal how aldolases are limited by proton transfer with solvent, which undermines aldol addition into ketones. We show, however, that transaldolases lack the sophisticated H-bonding network required for a viable proton transfer pathway, thereby enabling efficient addition into unactivated ketones. The resulting products are non-canonical amino acids with side chains that contain chiral tertiary alcohols. This study reveals the principles for extending aldolase catalysis beyond its previous limits and enables convergent, enantioselective C-C bond formation from simple starting materials.

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13. Discovery and Characterization of PRMT1-PRMT6 Interaction

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Abstract: Numerous studies have found that individual members of protein arginine methyltransferases (PRMTs) interact with other cellular proteins to regulate crucial biological processes from gene transcription, DNA repair, RNA splicing, to signal transduction. Although PRMT enzymes are well known to exist as homodimeric complexes, the understanding of heteromeric interactions between different PRMT members remains very limited. In this study, we revealed and characterized a heteromeric interaction between two members of the PRMT family, PRMT1 and PRMT6. Intriguingly, PRMT6 undergoes methylation by PRMT1, which makes it a 'modified modifier'. Through LC-MS/MS analysis and site-directed mutagenesis analyses, we determined that R106 is a major methylation site induced by PRMT1. Furthermore, the steady state kinetics, biochemical tests, and cellular assays showed that PRMT1-mediated methylation suppresses the activity of PRMT6 on Histone H3 methylation. These results point out the intricate cross-talking relationship within the PRMT family, where one member can modulate the functions of another. Together, this work illustrates the dynamic interplay between PRMT1 and PRMT6 and provides a new understanding of the regulatory mechanisms underlying protein arginine methylation[1].

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14. Mechanism of human DNPH1 reaction: implications for anticancer therapy

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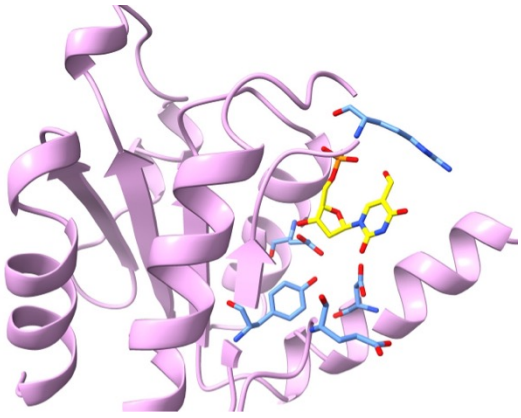
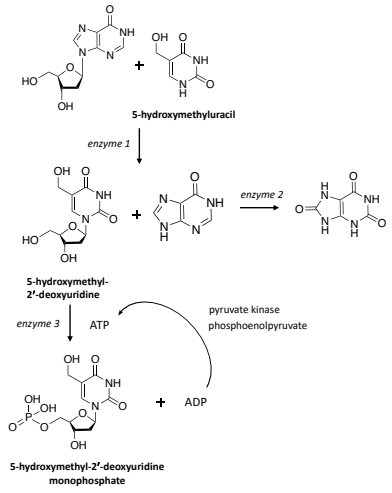
The enzyme 2'-deoxynucleoside 5'-monophosphate *N*-hydrolase 1 (DNPH1) catalyses the *N*-ribosidic bond cleavage of 5-hydroxymethyl-2'-deoxyuridine 5'-monophosphate (5hmdUMP), a cytotoxic nucleotide whose erroneous incorporation into DNA restores and enhances sensitivity of *BRCA*-deficient cancers to anticancer poly(ADP-ribose) polymerase inhibitors (PARPi). DNPH1 upregulation is linked to PARPi resistance in *BRCA*$^{-/-}$ cancer cells, thus the enzyme is a promising inhibition target both to potentiate PARPi action and to resensitise cancer cells resistant to PARPi therapy as a result of 5hmdUMP depletion.

This work reports the first one-pot, room temperature protocol for biocatalytic synthesis of 5hmdUMP from 5-hydroxymethyl uracil nucleobase, and use of this physiological substrate for both steady-state and pre-steady state kinetic analysis of the *Hs*DNPH1 reaction mechanism. Isolation of the first chemical step of catalysis shows glycosidic bond cleavage by a conserved glutamate nucleophile is not rate-limiting and points to an anionic nucleobase intermediate along the reaction profile. Mutation of catalytic triad residues Tyr24 to Phe or Asp80 to Ala reduces k_{cat} 600- or 300- fold respectively relative to wild-type enzyme. A bell-shaped pH rate profile is flattened upon mutation of conserved Asp80 or His56 to Ala, indicating that acid-base catalysis is operational in wild-type enzyme and a complex network of residues function to influence the pKa's of important catalytic groups for reaction. Kinetic analysis is supported by crystallographic data and highlights the importance of the 5-hydroxymethyl group for substrate affinity. These findings lay the foundation for inhibitor design against *Hs*DNPH1.[1]





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15. Activation of SIRT6 Deacetylation by DNA Strand Breaks

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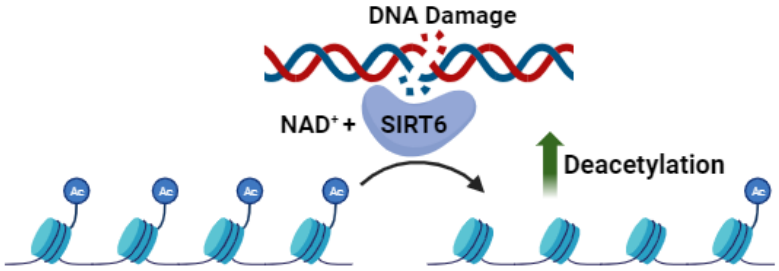
SIRT6 is an emerging regulator of longevity. Overexpression of SIRT6 extends the lifespan of mice.[1] Conversely, SIRT6 knockout mice demonstrate severe metabolic defects and shortened lifespan.[2] The discrepancy between SIRT6's weak in vitro activity and robust in vivo activity has led to the hypothesis that this enzyme can be activated in response to DNA damage in cells. Here, we demonstrate that the deacetylase activity of SIRT6 can be stimulated by DNA strand breaks for synthetic peptide and histone substrates.[3] The mechanism of activation is further explored using an integrative chemical biology approach. SIRT6 can be preferentially activated by DNA lesions harboring a 5'-phosphate. The N- and C-termini of SIRT6 are strictly required for DNA break-induced activation. Additionally, the defatty-acylase activity of SIRT6 is also sensitive to DNA breaks, although the physiological significance needs further investigation. Collectively, our study sheds important light on the cellular regulation of diverse SIRT6 activities, and suggests possible strategies for effective SIRT6 activation.



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16. Repurposing the 70 years old drug hydralazine for glioblastoma treatment

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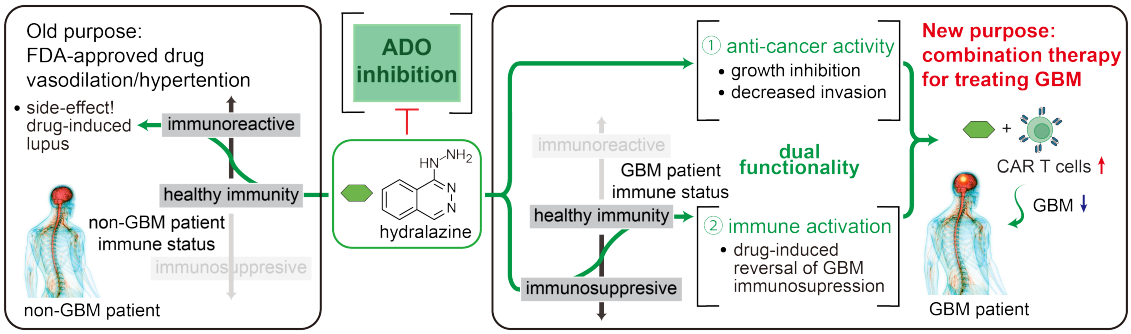

Overview: A small-molecule drug to be repurposed on the basis of its known cellular effects to enhance CAR T therapies for GBM.

Hydralazine, a drug with a hydrazine (–NHNH2) pharmacophore, has unknown mechanism(s) of action for treatment of hypertension and solid tumors.[1,2] Nevertheless, hydralazine is known to vasodilate by reducing intracellular Ca^{2+} release in smooth muscle cells[3] and overactivate T cells by inhibiting DNA methylation.[4] To map these mechanisms of actions, our group pioneered a unique chemoproteomics platform called reverse-polarity activity-based protein profiling (RP-ABPP).[5] Using RP-ABPP, we discovered that only a single enzyme called 2-aminoethanethiol dioxygenase (ADO), was targeted by hydralazine. ADO is an iron(II)- and oxygen-dependent dioxygenase that oxidizes cysteine-derived thiol groups from both small molecule metabolite substrates as well as important protein substrates. ADO has been well established as a therapeutic target as ADO is found to be correlated with the growth, invasion, and malignancy of glioblastoma (GBM), however no inhibitors have been developed to date.[6] Taken together, we envision that hydralazine will be the holy grail for GBM therapy both as a direct anti-cancer drug and as an immune activator of CAR T cell therapy.

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17. Copper-Dependent Peptide Cyclases In Plant Peptide Biosynthesis

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Cyclopeptide alkaloids are an abundant class of plant cyclopeptides with over 200 analogs described and bioactivities ranging from analgesic to antiviral. Structurally, cyclopeptide alkaloids are 4-5 amino acid macrocycles linked by an ether bridge between the phenolic oxygen of tyrosine and the β-carbon of a nearby amino acid. While these natural products have been known for decades, their biosynthetic basis remained unclear. Using a transcriptome-mining approach, we link the cyclopeptide alkaloids from Ceanothus americanus to dedicated ribosomally synthesized and post-translationally modified peptide (RiPP) precursor peptides. A bioinformatic search of these precursor peptides uncovered widely distributed gene clusters across eudicots containing a new type of copper-dependent BURP peptide cyclase. Guided by our predictions, we identify and isolate new cyclopeptides from Coffea arabica, which we named arabipeptins. We reconstituted enzymatic activity for the BURP peptide cyclase found in the biosynthesis of arabipeptin A (ArbB2), validating that it is responsible for installing the ether cross-link. Guided by modeling studies, we demonstrate multiple turnover catalytic activity for ArbB2 on a minimal peptide an explore its substrate scope. These results expand our understanding of the biosynthetic pathways responsible for diverse cyclic plant peptides and give molecular insights into a newly discovered copper-dependent peptide cyclase.

18. Investigations into a multinuclear non-heme iron dependent oxidative enzyme that produces hydantoin-containing macrocyclic peptides

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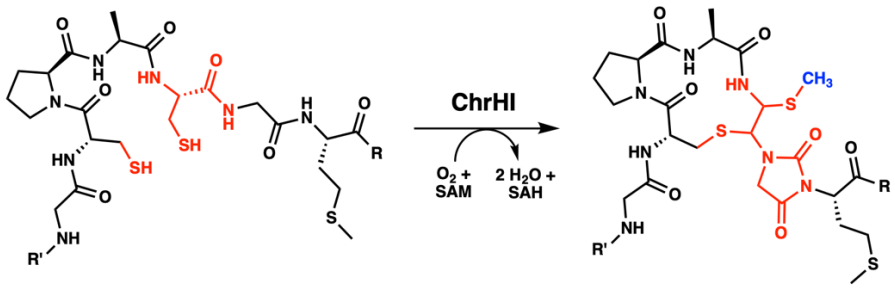
Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a class of diverse natural products. In RiPP biosynthesis, peptides are post-translationally modified by different enzymes to produce the mature natural product. An emerging class of modifying enzymes called *multinuclear non-heme iron dependent oxidative enzymes* (MNIOs, formerly DUF692) have been shown to perform widely different reactions on their peptide substrates. In the *Chryseobacterium* genus, an MNIO (termed ChrH) performs a remarkable rearrangement to install several functional groups (a macrocycle, a thiomethyl group, and a hydantoin heterocycle) onto a peptide precursor[1]. To investigate this reaction further, we characterized the substrate specificity of the enzyme towards different precursor peptides. We then identified the minimal substrate in order to probe the mode of substrate recognition by ChrH, and provide a synthetically accessible peptide for mechanistic studies. Mutating iron-binding ligands in the primary coordination sphere identified key residues for the unusual reactivity of ChrH. Finally, using comparative genomics, we identified and expressed homologs of ChrH. Interestingly, despite being highly similar, these enzymes appear to install different post-translational modifications. These findings on the broad substrate promiscuity of ChrH shed light on how the MNIO family of iron-dependent enzymes has evolved to perform such diverse reactivity.



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19. Computational and Structural Investigation of the Improved RNA-Clamping Activity of Amidino-Rocaglates on eIF4A1

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Eukaryotic Initiation Factor 4A-1 (eIF4A1) is an ATP-dependent RNA helicase that unwinds 5'-UTR mRNA secondary structure to facilitate cap-dependent translation initiation. Since initiation is the rate-limiting step of protein production, modulation of eIF4A1 activity is a key means by which cells regulate the translation of mRNA into protein. Overexpression or overactivity of eIF4A1 is common in cancer and some viruses, notably coronaviruses, utilize the unwinding activity of eIF4A1 to promote translation of viral RNAs. Rocaglates, named for the natural product Rocaglamide A (RocA), are a class of eIF4A inhibitors first identified for their cytotoxic activity against tumor cells. Additionally, rocaglates have been validated as antiviral agents and have promise as anti-parasite agents.[1,2] Rocaglates inhibit eIF4A1 activity by "clamping" the helicase onto polypurine RNA, preventing it from efficiently unwinding RNA and inhibiting translation initiation.[3,4] A new class of rocaglate derivatives, amidino-rocaglates (ADRs), containing a fourth heterocyclic ring fused to the cyclopenta[*b*]benzofuran core, has enhanced potency compared to RocA.[5] Here, I present the first structure of an ADR (CMLD012824) in complex with eIF4A1, a nonhydrolyzable ATP ground-state mimic AMPPNP, and a poly r$(AG)_5$ refined to 1.7 Å resolution. The binding pose of the ADR overlays well with that of RocA, revealing no new interactions, prompting investigation of the basis for the enhanced potency. Density functional theory (DFT) studies of RocA and CMLD012824 suggest that the ADR scaffold is inherently preorganized in eIF4A1-RNA-binding-competent conformation, whereas RocA must undergo an energetically costly conformational change in order to bind. This study illustrates how the rigidifying effect of the ADR ring fusion on the rocaglate scaffold can be used to improve potency for development as potential antineoplastic, antiviral, or antiparasitic drugs.

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20. Dynamics of Exonuclease Proofreading by T7 DNA Polymerase During DNA Replication

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In this study we explore the interplay of polymerase and exonuclease active sites of T7 DNA polymerase in selective error correction during high fidelity DNA replication. First, we characterize the substrate specificity of the proofreading exonuclease domain in a high-fidelity DNA polymerase[1], showing efficient proofreading of terminal mismatches and surprisingly, even greater efficiency for mismatches buried by correct bases suggesting that the polymerase may correct some mistakes by first extending them with the correct base. We then developed a homology model for the DNA primer strand in the exonuclease active site showing that the DNA must backtrack and 3 bases melt for the primer to partition to the exonuclease active site for hydrolysis. Based on these results we designed a DNA substrate containing the fluorescent cytosine analog tC^{o} at the n-2 position to measure kinetics of proofreading on physiologically relevant substrates[2]. Combining pre-steady state stopped flow fluorescence measurements of strand separation with single turnover rapid quench experiments allowed us to obtain a global fit to elucidate the rate constants and kinetic mechanism for high fidelity error correction. These studies revealed a unique intermediate state and stimulation of transfer to the exo site by nucleoside triphosphates. Hydrolysis-resistant oligonucleotides track DNA transfer without hydrolysis. Our proposed model explains faster removal of buried mismatches than single 3'-terminal mismatches, providing an additional avenue for error correction. This comprehensive model demonstrates how DNA transfer, coupled with base excision, facilitates efficient selective mismatch removal during DNA replication, enhancing fidelity by over 1000-fold.

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21. De novo biosynthesis of azide by a promiscuous *N*-nitrosylase

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Azides are energy-rich compounds endowed with diverse representation in broad scientific disciplines, including material science, synthetic chemistry, pharmaceutical science, and chemical biology. Despite ubiquitous usage of the azido group, the underlying biosynthetic pathways for its formation remain largely unknown. Here we report the characterization of what is to our knowledge the first biosynthetic pathway for de novo azide construction. We demonstrate that Tri17, a promiscuous ATP and nitrite-dependent enzyme, catalyzes organic azide synthesis through sequential *N*-nitrosation and dehydration of aryl hydrazines. Through biochemical, structural, and computational analyses, we further propose a plausible molecular mechanism for azide biosynthesis that sets the stage for future biocatalytic applications and biosynthetic pathway engineering.

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22. Cell-Based Covalent Deubiquitinase-Capturing Assay for Inhibitor Discovery

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Ubiquitin is known to play a key role in cell signaling and various eukaryotic cellular processes. Ubiquitination is a reversible process regulated by a large class of enzymes called deubiquitinases (DUBs). USP15, a DUB in the ubiquitin-specific protease family, plays an important role in mitophagy and Parkinson's disease. USP15 is known to deubiquitinate mitochondrial outer mitochondrial membrane (OMM) proteins in a Parkin E3 ligase-dependent manner, suggesting its antagonistic relationship with Parkin's ubiquitin ligase activity. We developed a cell-based DUB assay that utilizes a cell-permeable Biotin-cR_{10}-Ub-PA probe to covalently capture DUBs in their native cellular environment. AlphaLISA is used to quantitatively assess the covalent capturing of the target DUB by the Biotin-cR_{10}-Ub-PA probe upon cell lysis. We showed that this novel cell-based DUB assay is robust and adaptable to a high-throughput screening format. This new DUB assay can be employed in inhibitor discovery against many other human DUBs to identify potent and cell-permeable DUB inhibitors, thereby fast-tracking lead identification and inhibitor optimization.

23. Nicotinamide Riboside Activates SIRT5 Deacetylation

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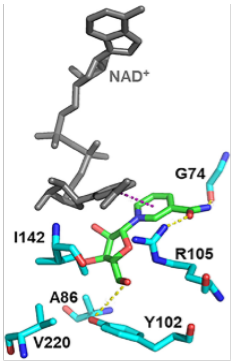
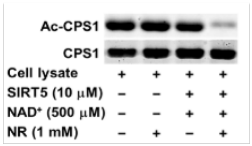
Human sirtuins play important roles in various cellular events including DNA repair, gene silencing, mitochondrial biogenesis, insulin secretion, and apoptosis. They regulate a wide array of protein and enzyme targets through their NAD^+-dependent deacetylase activities.[1] Sirtuins are also thought to mediate the beneficial effects of low-calorie intake to extend longevity in diverse organisms from yeast to mammals.[2] Small molecules mimicking calorie restriction to stimulate sirtuin activity are attractive therapeutics against age-related disorders such as cardiovascular diseases, diabetes, and neurodegeneration.[3] Little is known about one of the mitochondrial sirtuins, SIRT5. SIRT5 has emerged as a critical player in maintaining cardiac health and neuronal viability upon stress and functions as a tumour suppressor in a context-specific manner. Much has been debated about whether SIRT5 has evolved away from being a deacetylase because of its weak catalytic activity, especially in the *in vitro* testing.[4] We have, for the first time, identified a SIRT5-selective allosteric activator, nicotinamide riboside (NR). It can increase SIRT5 catalytic efficiency with different synthetic peptide substrates. The mechanism of action was further explored using a combination of molecular biology and biochemical strategies. Based on the existing structural biology information, the NR binding site was also mapped out. The knowledge gained in this study can be used to guide the design and synthesis of more potent, isotype-selective SIRT5 activators and to develop them into therapeutics for metabolic disorders and age-related diseases.





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24. Intermolecular electron transfer in radical SAM enzymes as a new paradigm for reductive activation

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Radical S-adenosyl-L-methionine (rSAM) enzymes are remarkable for their ability to bind multiple Fe-S clusters,[1-2] enabling them to catalyze a wide range of transformations leading to the synthesis of complex and diverse natural products. Central to their function is a 4Fe-4S cluster, which plays a critical role in cleaving SAM to produce a 5'-deoxyadenosyl radical.[3] This radical is pivotal in initiating the catalytic cycle through hydrogen atom transfer from the substrate. However, the function of additional auxiliary Fe-S clusters (ACs) in these enzymes has remained largely unexplored. Our study focuses on the rSAM enzyme PapB, known for catalyzing thioether cross-link formation between the β-carbon of an aspartate and a cysteine thiolate in the PapA peptide.[4] Intriguingly, one of the two ACs in PapB is observed to bind to the substrate thiolate, facilitating the formation of a thioether bond and transferring a reducing equivalent back to the protein. This study reveals that for PapB to initiate another catalytic cycle, it must undergo an electronic state isomerization, transferring the electron back to the SAM-binding cluster. Our investigation, utilizing a series of iron–sulfur cluster deletion mutants, supports a model where this isomerization has an essential intermolecular electron transfer step, which can be mediated by either redox-active proteins or small molecules, likely through the second AC in PapB. Remarkably, we found that a combination of FMN and NADPH can facilitate both the reductive and isomerization steps. These discoveries propose a novel paradigm in rSAM enzyme activation, highlighting the significance of intermolecular electron transfer in enzymes requiring multiple iron–sulfur clusters for efficient turnover. We will discuss the broader implications of these findings on our understanding of the biological activation of rSAM enzymes and their potential applications in biotechnology and medicine.

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25. A dynamic metal coordination sphere controls chemoselectivity in radical amino acid halogenases

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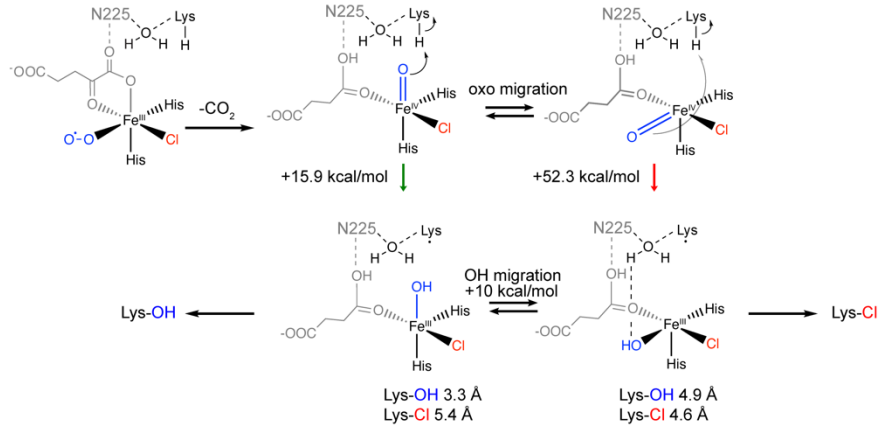
The enzymatic halogenation of unactivated C(sp3)-H bonds has the potential to provide a powerful biocatalytic platform for chemical synthesis. However, engineering efforts to expand the substrate scope or chemoselectivity (halogenation vs hydroxylation) of known halogenases and their phylogenetically related hydroxylases have been met with limited success, revealing our incomplete molecular-level understanding of their mechanism. In this work, we employed a combined mechanistic and protein engineering approach to uncover the molecular origins of chemoselectivity in the Fe^{II}/αKG-dependent lysine halogenase, HalA. Crystallographic and spectroscopic (XAS, EPR, Mössbauer) investigation of the V^{IV}-oxo-substituted intermediate in HalA combined with DFT calculations revealed that ligand isomerization at the metal coordination site is required for achieving both efficient C-H bond activation and the subsequent chemoselective halogenation via radical-rebound. Further investigations revealed the conserved second-sphere residues that constitute an extended H-bonding network with the metal center and control chemoselectivity in HalA. Site-directed mutagenesis at only three of these second-sphere positions in a lysine hydroxylase resulted in its successful engineering into a halogenase exhibiting activity and chemoselectivity that matches that of the related native halogenase, HalA.



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26. Hydrogen Sulfide Oxidation Enzymes Critical in Maintaining Bacterial Virulence

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Pseudomonas aeruginosa is a primary health concern for individuals with compromised immune systems and cystic fibrosis. Unfortunately, multidrug resistant *P. aeruginosa* is the predominant nosocomial infection present in healthcare settings. The multidrug resistance of *P. aeruginosa* can be attributed to numerous factors that involve evading the host immune response, and these responses differ among free-living cells and biofilm-forming communities.[1] Recent studies have proposed multiple roles for H_2S as a virulence factor in antibiotic resistance and alleviating the oxidative stress response in bacteria. In *P. aeruginosa,* H_2S is formed by both cystathionine β-synthase and cystathionine γ-lyase.[2] The production of H_2S must be managed in the cell to prevent damage to cellular processes and disruption of the cellular redox balance.[2]

Oxidation and assimilation of H_2S in bacteria is often catalyzed by a persulfide dioxygenase and sulfurtransferase (ST) in tandem. While enzymes responsible for H_2S production have been identified in *P. aeruginosa*, comparable enzymes for the oxidation of H_2S have not been clearly defined. The characterized mercaptopropionate dioxygenase (MDO) in *P. aeruginosa* has been proposed to catalyze the oxidation of 3-mercaptopropionate. However, kinetic investigations of MDO suggested the enzyme had a broader substrate specificity than previously identified and was unable to use 3-mercaptopropionate as a sulfur source in growth assays. The physiological role of MDO is questionable given the expression of a ST enzyme on the same operon as the thiol dioxygenase. In addition, the *mdo* operon was upregulated in proteomic analyses of *P. aeruginosa* under sulfur limiting conditions, suggesting a potential role for this operon in H_2S oxidation and subsequent assimilation. Studies were performed to identify the catalytic and physiological role of the ST enzyme. The ST enzyme contains four tandem rhodanese domains. Only two of the domains contain putative catalytic cysteine residues (Cys191 and Cys435) that could form a persulfide intermediate. Cys191 was not accessible in thiol quantification assays and was unable to form a Cys persulfide. Conversely, Cys435 was solvent accessible and the persulfide intermediate was formed with thiosulfate as the sulfur donor. HDX MS analyses confirmed the accessibility of Cys435 and flexible loop regions of the ST enzyme that would promote catalysis. The results obtained from these investigations provide overall insight to the catalytic mechanism and physiological role of the *mdo* operon in sulfide oxidation and assimilation.

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27. Biosynthesis of UDP-β-L-Arabinofuranoside for the Capsular Polysaccharides of *Campylobacter jejuni*

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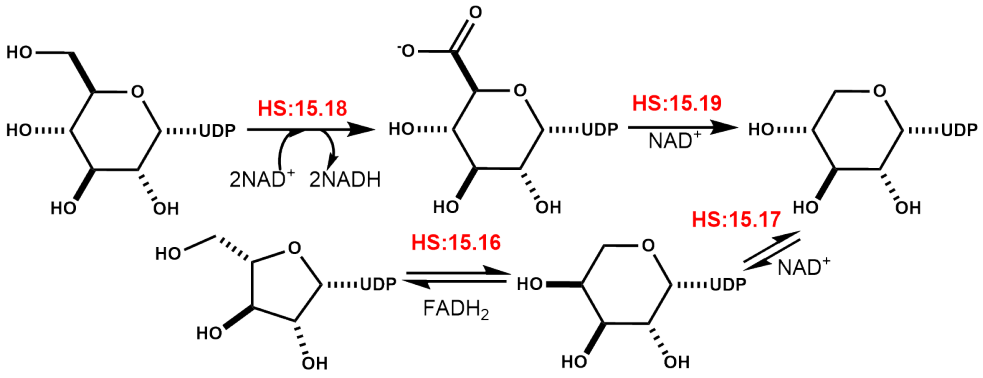
Campylobacter jejuni is the leading cause of food poisoning in North America and Europe. The exterior surface of this bacterium is coated with a capsular polysaccharide (CPS) which enables adherence to the host epithelial cells and evasion of the host immune system. Many strains of *C. jejuni* can be differentiated from one another by changes in the sequence of the carbohydrates found within the capsular polysaccharide. The CPS structures of serotypes HS:15 and HS:41 of *C. jejuni* have been chemically characterized and found to contain an L-arabinofuranoside moiety in the repeating CPS sequence. Sequence similarity and genome neighborhood networks were used to identify the putative gene cluster within the HS:15 serotype for the biosynthesis of the L-arabinofuranoside fragment. The first enzyme (HS:15.18) in the pathway was found to catalyze the NAD^+-dependent oxidation of UDP-α-D-glucose to UDP-α-D-glucuronate, while the second enzyme (HS:15.19) catalyzes the NAD^+-dependent decarboxylation of this product to form UDP-α-D-xylose. The UDP-α-D-xylose is then epimerized at C4 by the third enzyme (HS:15.17) to produce UDP-β-L-arabinopyranoside. In the last step HS:15.16 catalyzes the $FADH_2$-dependent conversion of UDP-β-L-arabinopyranoside to UDP-β-L-arabinofuranoside. The UDP-β-L-arabinopyranoside mutase catalyzed reaction was further interrogated by measurement of a positional isotope exchange (PIX) reaction within [^{18}O]-UDP-β-L-arabinopyranoside.

Scheme 1: Biosynthetic Pathway



28. Characterization of a Rare NRPS-NIS Hybrid System for the Biosynthesis of the Siderophore, Nocardichelin.

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Siderophores are synthesized by microbes for the sequestering and acquisition of iron from low ferric environments and can be produced through the Non-Ribosomal Peptide Synthetases (NRPSs), hybrid NRPS/polyketide synthases, as well as NRPS independent siderophore synthetases (NISs). In the biomedical field, these molecules have been explored for their role in maintenance of a healthy balance of iron content within the body, as novel antibiotics, anticancer treatments, vaccine developments, and iron content diagnostic testing. We identify here a novel biosynthetic gene cluster for Nocardichelin biosynthesized by a modular NRPS/NIS from *Nocardia carnea*. Of the identified cluster, we have examined three proteins which we call NcdE-ATx, NcdF, and NcdH. NcdE-ATx is an unusual acyltransferase appended to the C-terminus of the modular NRPS (NcdE) that is putatively responsible for the integration of a fatty acyl tail. NcdF is an NIS synthetase presumed to combine the products of NcdE-ATx to create a hydroxamate moiety with a fatty acyl tail. NcdH is likely an NRPS stand-alone condensation domain capable of connecting the salicyloxazolone moiety derived from the NRPS module NcdE to the product of NcdF at the hydroxamate moiety. To investigate this novel pathway and its possible promiscuities/limitations, a multi-aspect approach is taken which includes cluster confirmation, structural representation, and biochemical analysis. We present structures of NcdF and the acyltransferase domain of NcdE that allow the exploration of the current biosynthetic pathway hypothesis and its alternatives as well as provide insights for the design of novel siderophores utilizing this hybrid pathway.

29. Enzymatic Synthesis of Aryl CoA Surrogates for Cell Free Pathways

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Thioesters represent an important class of substrate that can be used for the synthesis carbon-carbon and carbon-nitrogen bonds in cell-free engineered enzyme pathways. The thermodynamically uphill energetics of Coenzyme A acylation are overcome by mechanistically coupling thioester synthesis to substrate decarboxylation in the case of the multicomponent α-keto acid dehydrogenases or ATP hydrolysis in the case of aromatic acid coenzyme A ligases, fatty acid synthases, polyketide synthases, and non-ribosomal peptide synthases. The relative structural and mechanistic simplicity of acylating aldehyde dehydrogenses (AAD's), which directly couple aldehyde oxidation to the synthesis of coenzyme A thioesters identifies them as particularly attractive targets for integration into engineered biochemical pathways. Coenzyme A is quite unstable and costly and for these reasons alternative thiol substrates that can serve as surrogates of coenzyme A in engineered enzyme pathways are sought.

In the present work we have targeted the expansion of the aldehyde and thiol substrate specificity of a propionaldehyde specific AAD which has been expressed as a stable, soluble enzyme subcomponent of bacterial microcompartments natively used for energy recovery from the 1,2-propane diol product of rhamnose and fucose metabolism of lignocellulosic bacteria[1]. We have further targeted the N-acetylcysteamine (SNAC) thioesters that can serve as acyl and aryl-coenzyme A surrogates. We have used transform restrained Rosetta[2] (trRosetta) models to evaluate the impact of AAD mutations designed to increase the volume of the hydrophobic aldehyde-binding active site. In silico models found to increase aldehyde active site volume without significantly disrupting pyridine nucleotide and coenzyme A-binding domains were used to evaluate potential increases in aldehyde substrate range based on the results of in silico docking studies[3].

Based on the results of in silico work, five mutants that substitute alanine for bulkier hydrophobic amino acid side chains were selected for production: A1 (F263A), A1a (I421A), A2 (I421A and F423A), A3 (Y418A, I421A, and F423A), and A4 (F263A, Y418A, I421A and F423A)). Three of these (A1a, A2, and A3) show increased aldehyde substrate range and the ability to use SNAC and other coenzyme A surrogates as co-substrates. As proof of concept, we have joined A2, the AAD variant found to have the highest substrate specificity for phenylacetaldehyde, with an engineered peroxisomal 6-aminopenicillanic acid N-acyl transferase (IAT)[4] in the one pot synthesis of penicillin G.

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30. Structure and Function of the Monoterpene Cyclase, Sabinene Synthase

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The C_{10} monoterpenes derived from the cyclization of geranyl diphosphate comprise a pool of volatile, polycyclic, and often strained-ring natural products.[1] The application of these compounds is broad, spanning uses from industry to medicine. Several monoterpene cyclases have been structurally characterized to date, including bornyl diphosphate synthase, limonene synthase, and cineole synthase.[2,3,4] A robust definition of the structural biology and mechanistic strategies of terpene biosynthesis can facilitate access to useful compounds and enable the prediction of enzyme structure with a targeted function.[5] To advance our understanding of structure-function relationships in monoterpene cyclases, we now report the X-ray crystal structure of (+)-sabinene synthase from the western red cedar, *Thuja plicata* (TpSS). Sabinene is a monoterpene product that can be used as a component in next generation biofuels or as a fragrance in perfumes and cosmetics; sabinene also exhibits anti-inflammatory and anti-fungal properties.[6,7] In addition to structure-function details we also report that TpSS has maximal activity with Co^{2+} as a cofactor, which is unusual since Mg^{2+} or Mn^{2+} are canonical metal cofactors for terpene cyclases. Further, relating structural similarities between TpSS and that of a sesquisabinene synthase from *Santalum album* also reveals common features in active site templates required for the generation of strained [3.1.0] bicyclic ring systems.[8]

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31. Evidence for the Cyclic Intermediate in ProTide Prodrug Activation

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Abstract: Phosphoramidate (ProTide) prodrug compounds were engineered by Christopher McGuigan and his group at Cardiff University[1]. Originally developed to improve treatments for HIV, ProTides have become a leading therapeutic method for treatment of viral infections. The chemical structure of ProTides generally consists of a phosphoramidate (P-N) core with a nucleoside analog, a phenolic leaving group, and an alanyl carboxyester. The proposed mechanism of activation (Figure 1) for ProTide prodrugs begins with the hydrolysis of the carboxyester by a carboxylesterase. The next step is hypothesized to be a spontaneous attack of the phosphorus by the newly formed carboxylate, resulting in the release of the phenol group and formation of a 5-membered ring intermediate. Another hypothesized spontaneous hydrolysis reaction opens the ring of the cyclic intermediate. In this proposed mechanism, the product from the hydrolysis of the ring has two possible paths to its formation. A nucleophilic attack at the phosphorus or at the carbonyl carbon by water will result in the formation of the same product. Using ^{18}O-labeled water, we show that the site of hydrolysis of the 5-membered ring intermediate occurs at the phosphorus atom. A ProTide analog compound and ^{31}P NMR was used to investigate the formation and subsequent breakdown of the 5-membered cyclic intermediate. These studies also describe the pH dependence on the rate of formation and breakdown of the cyclic intermediate.

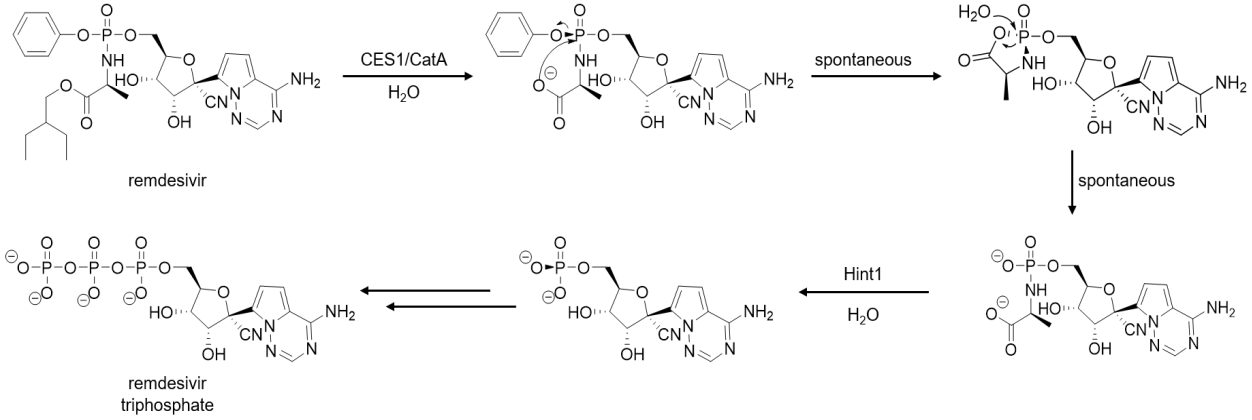

Figure 1: Proposed mechanism of activation for ProTide prodrugs using Remdesivir as an example.

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32. The inverse solvent viscosity effect in Glu57Asp *M. tuberculosis* IGP synthase

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M. tuberculosis indole-3-glycerol phosphate synthase (*Mt*IGPS) catalyses the fourth step in *M. tuberculosis* tryptophan biosynthesis pathway and is a potential drug target in TB treatment.[1] To understand the enzyme's mechanism and ligand binding, we tested the effects of several active site amino acid replacements.[2] Solvent deuterium kinetic isotope effect measurements indicated that a proton transfer step is at least partially rate-limiting for the wild type enzyme. We observed changes in the rate-liming step for several active site mutants, as indicated by the absence of a solvent deuterium kinetic isotope effects. While the wild type enzyme did not exhibit a solvent viscosity effect, a significant inverse solvent viscosity effect was observed for the Glu57Asp mutant, which exhibited a 50-fold decreased catalytic activity. We observed a bell-shaped rate vs. pH curve for *Mt*IGPS with maximum activity at pH = 7.5 with a pKa1 of 6.3 ± 0.1 and a pKa2 of 9.0 ± 0.1 Mutations at residue Glu57 resulted in significantly lower pKa2 values, suggesting a potential role for the negatively charged Glu57 stabilizing the positively charged, protonated form of the proposed catalytic acid Lys119. Additionally, through computational docking and molecular dynamics simulations of substrate analogs and proposed intermediates with *Mt*IGPS, we predicted interactions that take place in the active site at different stages of the catalytic mechanism. The results suggest that residue N189 plays an important role in orienting the intermediates during the catalytic mechanism, providing a possible explanation for the large decreases in catalytic activity observed for Asn189Ser, Asn189Gln, and Asn189Leu mutants. The findings implicate a highly coordinated active site structure with multiple critical interactions required for normal catalytic function.

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33. An S = 1 Iron(IV) Intermediate Revealed in a Non-Heme Iron Enzyme-Catalyzed Oxidative C-S Bond Formation

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Abstract: Ergothioneine (ESH) and ovothiol A (OSHA) are two natural thiol-histidine derivatives. ESH has been implicated as a longevity vitamin and OSHA inhibits the proliferation of hepatocarcinoma. The key biosynthetic step of ESH and OSHA in the aerobic pathways is the O_2-dependent C−S bond formation catalyzed by non-heme iron enzymes (e.g., OvoA in ovothiol biosynthesis), but due to the lack of identification of key reactive intermediate the mechanism of this novel reaction is unresolved. In this study, we report the identification and characterization of a kinetically competent *S*=1 iron(IV) intermediate supported by a four-histidine ligand environment (three from the protein residues and one from the substrate) in enabling C−S bond formation in OvoA from *Methyloversatilis thermotoleran*, which represents the first experimentally observed intermediate spin iron(IV) species in non-heme iron enzymes. Results reported in this study thus set the stage to further dissect the mechanism of enzymatic oxidative C−S bond formation in the OSHA biosynthesis pathway. They also afford new opportunities to study the structure-function relationship of high-valent iron intermediates supported by a histidine rich ligand environment.

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34. Characterization of Intermediates formed in the Reactions of the Diheme Cytochrome-*c* Peroxidase from *Nitrosomonas europaea* with Hydrogen Peroxide

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Bacterial Cytochrome *c* Peroxidases (BCcPs) are a family of diheme peroxidases with cryptic cellular function. Most characterized BCcPs are purported to function as defense against cytotoxic hydrogen peroxide,[1] while others have demonstrated the ability to catalyze cofactor maturation of partner proteins.[2,3] Conventional BCcPs contain two hemes in close proximity: one serving as the active site of peroxide reduction, and the other serving an electron transfer conduit to the active site heme. These hemes possess widely spaced reduction potentials that can vary by up to 600mV, and as such the peroxidatic heme is commonly referred to as the low potential (LP) heme and the electron transfer heme is referred to as the high potential (HP) heme. In the majority of conventional BCcPs, the HP heme must be reduced to trigger a conformational change which makes the LP heme accessible to peroxide binding and reactivity. The BCcP of *Nitrosomonas europaea* (*Ne*CcP) is one of few exceptions to this requirement among conventional BCcPs and is able to perform peroxidatic chemistry without pre-reduction.[4] The ability of *Ne*CcP to react with peroxide in either a "diferric" state (with both hemes oxidized) or a "semi-reduced" state (with the HP heme reduced) provides an opportunity to study how the initial oxidation state of the HP heme influences the peroxidatic reaction cycle of BCcPs. Here we present the use of UV-Visible spectroscopy in hand mixing and pre-steady-state modes to characterize the kinetics of the reactions of the diferric and semi-reduced *Ne*CcP with H_2O_2; and the use of UV-Visible and EPR spectroscopy to characterize species formed in the peroxidatic reaction cycle.

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35. Biosynthesis of the aminocyclitol from Hygromycin A

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Hygromycin A (HygA) is a broad-spectrum antibiotic produced by *Streptomyces hygroscopicus* that inhibits ribosomal peptidyl transferase.[1] HygA consists of three distinctive functional groups: furanose, cinnamic acid, and aminocyclitol. Although the aminocyclitol is critical for ribosome inhibition,[2] the unique methylenedioxy group found on the aminocyclitol is only vital for *in vivo* antimicrobial activity but not *in vitro* ribosome inhibition, suggesting its function is independent of ribosome binding.[3] Through isotope-labeling and mutagenesis experiments, a pathway has been proposed for the aminocyclitol biosynthesis.[3,4] This proposed pathway originates from glucose-6-phosphate, which is converted to *myo*-inositol-1-phosphate by Hyg18 and then dephosphorylated by Hyg25 to give *myo*-inositol. The C-5 is then oxidized by Hyg17 to form *neo*-inosose, followed by a transamination to *neo*-inosamine-2 by Hyg8. Hyg6 then installs a methyl group onto C-2 which sets up cyclization of the methylenedioxy group by Hyg7. This pathway presents interesting enzyme reactions which have yet to be validated or characterized. These include the oxidation of the C-5 of *myo*-inositol by Hyg17, which is unlike known *myo*-inositol dehydrogenases that oxidize the C-2 position. Furthermore, the methylenedioxy group is thought to be formed by Hyg7, which is annotated as a metallo-dependent hydrolase. More commonly, methylenedioxy groups are generated by cytochrome p450 or α-ketoglutarate nonheme iron-dependent enzymes, suggesting a possible new enzyme mechanism for methylenedioxy formation. Here, we will be presenting bioinformatics and biochemical analysis for Hyg17 and discussing the role of Hyg7 in methylenedioxy formation.

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36. Probing the Post-Translational Catalytic Cleavage Mechanism Required to Activate a New NylC Enzyme

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Plastics are ubiquitous in society, being widely used in packaging, textiles, industrial machinery, and construction materials. Nevertheless, 79% of plastic waste is dispersed into landfills and the broader environment due to current costly waste management strategies.[1] Subsequently, significant effort is being directed towards developing more sustainable methods of recycling plastic such as equipping enzymes that will enable the economically feasible biocatalytic degradation of plastics.[2,3] One ill-studied class of enzymes, dubbed nylC nylonases, are thought to hydrolyze nylon after undergoing a post-translational autocatalytic-cleavage reminiscent of the N-terminal nucleophile (Ntn) hydrolases.[4] However, the mechanism behind the cleavage of the nylC precursor is not well-known, making it difficult to characterize and engineer these enzymes for scaling to industrial standards. We have identified a new thermostable nylonase from Thermovenabulum gondwanense (TvgC) that undergoes a heat-induced catalytic-cleavage. Using SDS-PAGE gel densitometry, we can kinetically characterize the cleavage as well as conduct an in silico inspired site-directed mutagenic study to probe the critical residues involved in the mechanism. Thus far, a proof-of-concept experiment involving preliminary kinetic studies of wildtype and mutated TvgC have been successfully completed. Ultimately, this study will provide an in-depth analysis of residues required to activate TvgC for nylon-degradation, leading to a proposed mechanism of cleavage, and finally providing an essential foundation for upscaling nylC nylonases for industrial purposes.

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37. The Riboflavinator: A New Hope

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Riboflavin (vitamin B2) is essential to all organisms as it is the universal precursor to cofactors FAD and FMN. While animals lack riboflavin biosynthetic enzymes and obtain riboflavin from dietary sources of riboflavin, many pathogenic bacteria rely on endogenously synthesized riboflavin as they lack efficient riboflavin uptake machinery[1]. As such, the enzymes responsible for riboflavin biosynthesis are promising targets for novel antibiotics[2]. The individual enzymes responsible for riboflavin biosynthesis are slow with kinetic parameters insufficient to account for the amount of riboflavin being produced *in vivo*[3]. In order to justify the disconnect between the rates of riboflavin formation *in vivo* and *in vitro,* we hypothesize that these enzymes assemble into a macromolecular machine with enhanced catalytic efficiency called the Riboflavinator. The riboflavin biosynthetic pathway is composed of two distinct branches that converge at the penultimate step of riboflavin formation. The enzymes of this pathway have been studied for decades across multiple organisms, but an essential phosphatase has remained largely elusive outside of a few reported bacteria [4]. In order to understand the pathway in its entirety, we seek to identify the missing phosphatase of *Aquifex aeolicus* and detail protein-protein interactions that expedite riboflavin formation. This investigation combines kinetics, NMR, X-ray crystallography, and cryogenic electron microscopy to determine the mechanisms of the Riboflavinator's component parts, the individual enzymes, and generate a 3D blueprint of a macromolecular machine. The riboflavin biosynthetic pathway has been touted for decades as an ideal target for novel antibiotics, but decades of intense study has not yielded new antibiotics. For this reason, the Riboflavinator hypothesis represents A New Hope that understanding this pathway in its entirety will guide drug design to combat pathogenic bacteria.

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38. Exploring uncharted territory in RiPP biosynthesis: studies on curacozole, a cyanobactin from *Streptomyces curacoi*

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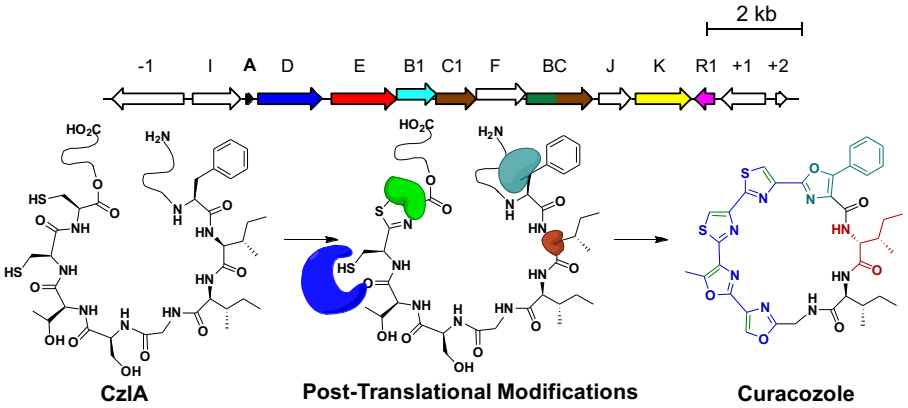

The majority of antibiotic, antiviral, and anticancer drugs used in modern medicine are natural products and their derivatives. Ribosomally synthesized and post translationally modified peptides (RiPPs) form a major class of bioactive natural products, comprising over 20 structurally distinct families. To unlock the full potential of RiPPs and opportunities for pathway engineering, we must develop a deeper understanding of their biosynthetic principles.[1,2] The cyanobactins are a class of RiPP first discovered in cyanobacteria. They are small cyclic peptides that are highly modified through side chain cyclization, epimerization, prenylation, and oxidation.[3] In recent years, similar compounds have been discovered in *Streptomyces*. This project aims to characterize the biosynthetic pathway of curacozole, a cyanobactin produced by the soil bacterium *Streptomyces curacoi.* A putative gene cluster has been proposed, but the functions of the many encoded enzymes are unknown as they share little similarity to the known biosynthetic gene clusters of cyanobactins, such as the patellamides and trunkamide.[4,5] *In vitro* assays with purified enzymes and the precursor peptide CzlA were performed and analyzed using LC-MS/MS analysis. Recent work on enzymes involved in heterocyclization, hydroxylation, and epimerization will be presented.

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39. Chemoproteomic profiling of metabolic drivers in acute myeloid leukemia

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Accumulation of oncometabolites generated from disordered metabolic enzymes are considered as novel pathognomonic hallmarks in cancer diseases including acute myeloid leukemia (AML).[1] As a genetically heterogeneous hematologic malignancy, around 20% of AML has an oncogenic mutation of isocitrate dehydrogenase (IDH), which transforms 2-oxoglutarate (2OG) into 2-hydroxyglutarate (2HG), which can competitively inhibit the Fe(II)/2OG-dependent dioxygenases[2], subsequently promoting leukemogenesis through a block in normal myeloid differentiation.[3] Genetically dependent tools have been widely applied to detect disordered enzyme expression but provided limited view on enzyme activity alteration, which is regulated by small molecule cofactors and directly regulates oncometabolite generation that induces disease development. Therefore, proteomic tools that are genetically independent are required to investigate system biology by connecting dysregulated protein activities with leukemogenesis. Reverse-polarity activity-based protein profiling (RP-ABPP) is such a powerful tool to capture catalytically essential electrophilic post-translational modifications[4] or oxidative cofactors in enzyme active sites[5] by using a nucleophilic hydrazine warhead. By using a suite of broadly reactive hydrazine probes that can be tuned to be highly selective and specific for a single protein, this method is hgly tunable towards diverse classes of therapeutic enzyme targets, enabling both global profiling of enzymatic activity and the discovery of potent and selective inhibitors[6] against a specific protein target that would potentially be a metabolic enzyme driver in AML development.

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40. Inhibition of Carboxyspermidine Decarboxylase by Difluoromethylornithine

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Carboxysper
midine decarboxylase (CASDC) is a pyridoxal 5'-phosphate (PLP) dependent enzyme that converts carboxyspermidine into the polyamine spermidine. Spermidine is an important signaling molecule and substrate with roles in gene expression that affect cell growth and proliferation. CASDC acts on carboxyspermidine produced by carboxyspermidine dehydrogenase. These enzymes form an alternative pathway to spermidine and are found in 75% of the most prevalent human gut microbes. CASDC is a potential drug target as spermidine production is necessary for viability in many bacterial species and spermidine production by gut microbes has been associated with human colon cancer in some studies. To better understand CASDC, we examined a similar PLP-dependent enzyme known as ornithine decarboxylase (ODC) found in eukaryotes. ODC uses ornithine to produce putrescine, a precursor in the eukaryotic biosynthesis of spermidine. Difluoromethylornithine (DFMO) is an FDA-approved inhibitor of ODC. CASDC and ODC have similar active site structures and we hypothesize that DFMO will act as a suicide inhibitor of CASDC by forming a covalent bond with Cys-306, analogous to the established ODC mechanism of inhibition. To demonstrate inhibition of CASDC we are using X-ray crystallography as well as kinetic assays. We have solved a 1.41 Å X-ray crystal structure of *Clostridium leptum* carboxyspermidine decarboxylase (ClCASDC) with the internal aldimine form of PLP bound in the active site. We have developed a coupled kinetic assay using phosphoenolpyruvate carboxykinase and malate dehydrogenase to measure CO_2 production by CASDC. We have also used a TgK rapid mixing device to measure single turnover reactions of CASDC by following PLP absorbance at 420 nm on a Cary 60 spectrometer. Future goals include solving separate X-ray crystal structures with spermidine and DFMO bound, assaying inhibition by DFMO and characterizing the CASDC mechanism using stopped-flow spectrometry.

41. The role of unique second-sphere amino acids in class Id RNRs

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Class I ribonucleotide reductases (RNRs) use either a metal-centered oxidant or a tyrosine-derived radical to initiate catalysis.[1,2] Class Id RNRs are the second example of an RNR subclass employing a metal-centered oxidant in their use of an oxidized dimanganese cluster as reaction initiator.[3,4] Assembly of the active $Mn_2^{III/IV}$ cluster in class Id RNR requires capture of superoxide by the $Mn_2^{II/II}$ form of the enzyme, enabled by a second sphere Lys side chain and a solvent exposed metal-binding site (Fig 1). In class Id RNRs, the radical-harboring Tyr of the Tyr•-dependent subclasses is conserved but it is not oxidized. Here we investigate the functional roles of the cofactor proximal Lys and the second-sphere Tyr via mutagenesis and characterization of sequence-divergent homologs. We also report an x-ray crystal structure of an activated class Id RNR. These results reveal how a high-valent metal-centered oxidant might be stabilized in a solvent-exposed cavity via cofactor shielding by the second-sphere Lys, which changes conformation to partially close the metal-binding site. Finally, we report characterization of a class Id RNR homolog that forms a small amount of inactive $Mn_2^{III/III}$-Tyr•. In its wild-type β subunit, the Tyr• does not destabilize the oxidized manganese cluster. But mutation of a class Id-conserved H-bonding partner to the Tyr increases the quantity of Tyr• formed, leading to loss of the active cofactor and enzyme activity. Discovery of a class I RNR that can form both an active metal-centered oxidant and an inactive Tyr-derived radical suggests how radical cofactors might evolve from their metal-centered counterparts.

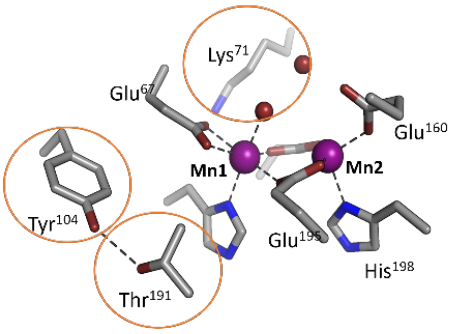

Figure 1. Class Id β RNRs crystal structure with second-sphere amino acids.[3]

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42. Structural basis of S-adenosylmethionine-dependent allosteric regulation in methylenetetrahydrofolate reductase

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Methylenetetrahydrofolate reductase (MTHFR) is a flavoprotein that serves as a metabolic nexus linking the folate and methionine methyl cycles, catalyzing the reduction of methylenetetrahydrofolate to methyltetrahydrofolate; this reaction commits a methyl group from folate to methionine metabolism. Human MTHFR (hMTHFR) employs an elaborate allosteric mechanism. While extensive functional characterization of human MTHFR has demonstrated an interplay between protein phosphorylation status and AdoMet-dependent inhibition, other factors, such as relative subunit orientations or FAD/cofactor status, have remained understudied due to the lack of functional structural models, specifically as they relate to the allosteric transitions influenced by the S-adenosylmethionine. Here, we report crystal structures of MTHFR from Chaetomium thermophilum (cMTHFR) in both its active R and its inhibited T-state. In the crystal structure of cMTHFR captured in the T state, FAD is occluded on its si-face by Tyr361, preventing it from binding to/engaging with substrate. Remarkably, the inhibited form of cMTHFR allows for the binding of two AdoMet molecules per subunit. Based on the structural framework provided by our cMTHFR model as captured in different states, we propose a possible mechanism to explain the allosteric structural transition of MTHFR, including the role of phosphorylation on the AdoMet-dependent inhibition of hMTHFR.

43. Discovery of Mechanism based Peptidomimetics Dual-Target Inhibitors of Cysteine Proteases SARS-CoV-2 3CL-Protease (M^{pro}) and Human Cathepsin L Inhibitors.

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Human cathepsin L (hCatL) and SARS-CoV-2 main protease (M^{Pro}) are cysteine proteases (CPs) involved in endosomal viral entry and viral replication of SARS-CoV-2 respectively. SARS-CoV-2 infection elevates the expression of hCatL, and overexpressed hCatL in turn accelerates the viral infection by forming a vicious cycle.[1] These properties of both CPs make them promising therapeutic targets. Our lab showed that clinical-stage hCatL inhibitor K777 reduced the viral infection in human cells at nanomolar concentrations even though it was found to be inactive against M^{Pro} [2]. The efficacy of the current FDA-approved drug nirmatrelvir is reduced with the arrival of new mutations within its molecular target M^{Pro} especially the mutations at the Gln recognizing S1 subsite which make SARS-CoV-2 highly resistant to nirmatrelvir.[3] In this project, we developed peptidomimetics with five new Gln mimics that inhibit both CPs (M^{pro} and hCatL). Peptidomimetics with our new 2-Pyridonyl-Ala (structural chimera of Phe and Gln) inhibits both CPs at nanomolar concentrations. It retains all crucial H-bonding with S1 subsite of M^{pro} and close mimic of Phe that retains inhibition constants for hCatL. In addition, these substrate-based peptidomimetics are engineered with new mild electrophilic centers that require high residence time in the active site and closer proximity to cysteine to form covalent adduct. Peptidomimetics δ-lactols and cyclic imino-ketones are nanomolar inhibitors of hCatL and displayed at least 100 fold selectivity over hCatB which has high structural similarity to hCatL. In fact, cyclic imino ketone formed reversible michael adduct with only hCatL over hCatB and blocked SARS-CoV-2 in Vero-E6 and A549/ACE2 cells at nanomolar concentrations.

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44. Structural and Mechanistic Determinants of ddhNTP Catalysis by the Ancient Antiviral Enzyme, Viperin

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Viperin is an interferon-inducible member of the radical S-adenosylmethionine (SAM) enzyme superfamily that inhibits and/or is involved in the replication of a remarkable range of DNA and RNA viruses through various mechanisms. It was recently discovered that human viperin catalyzes the formation of the novel molecule, 3'-deoxy-3',4'-didehydrocytidine triphosphate (ddhCTP), from CTP, which inhibits viral polymerases during viral genome replication.[1] Interestingly, homologues of viperin exist in bacterial, fungi, and archaea and possess divergent substrate specificity, producing alternative ddh-nucleotide triphosphate (ddhNTP) products (i.e. ddhUTP, ddhGTP).[2] The mechanism by which viperin and viperin-like enzymes (viperins) catalyze this reaction and the structural basis for their divergent nucleotide specificity is unknown. An understanding of viperin's mechanism and the structural regions governing substrate preference is needed for the development of tool compounds, elucidating the antiviral effects of different ddhNTP-catalyzing viperins, and defining a novel mechanism of dehydration catalyzed by the radical SAM superfamily. Mutagenesis of conserved active site residues in *Mus musculus* viperin demonstrates that arginine and tyrosine residues play a critical role in catalysis. In addition, by utilizing multiple sequence alignment, homology-based engineering, and X-ray crystallography, we find that viperin substrate specificity is mediated through the β-8 loop and can be engineered to prefer alternate NTP substrates.

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45. First characterized pyridoxal-L-phosphate independent epimerase with double active site serines

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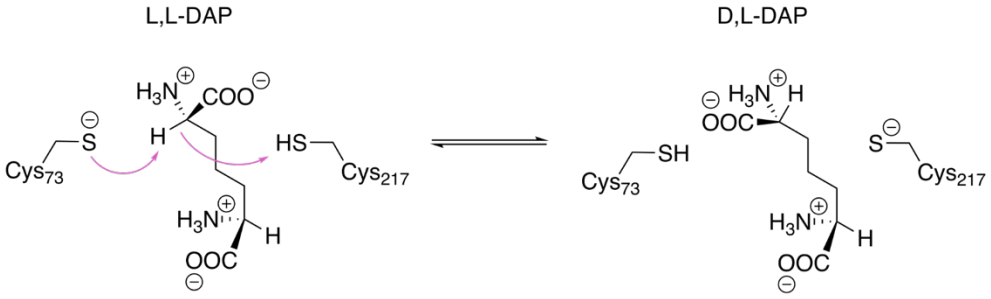
Pyridoxal-L-phosphate (PLP) independent epimerases and racemases are highly specific enzymes that reversibly invert the stereochemistry of amino acids and other metabolites.[1] These enzymes have been characterized for a wide variety of substrates including diaminopimelic acid (DAP), alanine, proline, glutamate, isoleucine, and O-ureidoserine, amongst others. These enzymes are entirely cofactor-independent, and operate via a very unusual two-base mechanism (Figure 1).[2] The active site typically contains two catalytic cysteine residues that form a thiol/thiolate pair. The thiolate acts as a general base and removes a proton from the substrate to form a planar, anionic intermediate, which then accepts a proton from the thiol general acid on the opposite face of the substrate, thereby inverting the stereochemistry of the chiral center.



One PLP-independent enzyme of importance is DAP epimerase (Figure 1). This enzyme is found in photosynthetic organisms and bacteria and catalyzes the conversion of LL-DAP to DL-DAP. DAP epimerase is a major antibiotic target as its reaction is part of two essential biosynthetic pathways: 1) biosynthesis of the essential amino acid lysine, and 2) DL-DAP biosynthesis for inclusion in the pentapeptide of Gram negative bacterial cell walls.[3] Despite a long-standing interest in production of inhibitors of DAP epimerase as antibiotics, the understood high specificity of this enzyme has made inhibitor synthesis challenging.

In this work, we report the characterization of an unusual DAP epimerase from cyanobacteria. This enzyme has double active site serine residues rather than the typical cysteines, yet still maintains catalytic activity. This is remarkable as mutation of cysteine to serine in other DAP epimerases has previously been shown to greatly reduce catalytic activity.[4] This poster presentation will discuss the characterization of this enzyme, its substrate scope, and comparisons to other DAP epimerases from diverse organisms.

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46. Cryo-EM Structure of the *E. coli* Acetyl-CoA Carboxylase Complex, a Tube-like Filament and Reaction Chamber

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Acetyl-CoA carboxylase (ACC) catalyzes the first committed step in fatty acid biosynthesis; conversion of acetyl-CoA to malonyl-CoA using ATP.[1] ACC catalysis is accomplished with three functional units, two enzymatic domains and a carrier protein that shuttles intermediates between active sites. The *Escherichia coli* ACC represents the vast majority of prokaryotic heteromeric ACCs and is made of four proteins. Crystal structures of the *E. coli* ACC subunits are known, but complex assembly and allosteric regulation remains a mystery.[2-3] We determined the structure of a complete *E. coli* ACC complex using single particle cryo-EM and find it assembles into polymorphic tubular filaments. These structures reveal the protein termini form protein:protein interactions (PPI) that promote complex assembly. Deleting the interacting termini abrogates tube formation and catalytic activity, suggesting the PPI interfaces are new druggable sites. Density for Mg·ADP and acetyl-CoA revealed the location of the active sites, which are on the tube interior and oriented toward each other. The tube-like structure facilitates shuttling of the carrier protein bound intermediates during catalysis. Our cryo-EM structures serve as a basis for understanding the structure-function relationships underpinning the complex allosteric regulation of heteromeric ACC enzymes from diverse organisms ranging from human commensal or pathogenic bacteria to cyanobacteria and plants.

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47. Mechanistic Studies of Class II Lanthipeptide Synthetases

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Lanthipeptides are a class of **ri**bosomally synthesized and **p**ost-translationally modified **p**eptides, RiPPs, that contain intramolecular thioether linkages. In class II lanthipeptides, a single, bifunctional enzyme, LanM, dehydrates select serine and threonine residues in the core peptide, yielding dehydroalanine and dehydrobutyrine intermediates, respectively. The dehydroamino acids subsequently undergo a Michael-type addition via a cysteine thiol to form (methyl)lanthionine rings. Recent insights from the study of a human LanC-like enzyme, LanCL1, provided a deeper understanding of analogous processes in eukarya[1]. The crystal structure of LanCL1 complexed to the product of a thia-Michael addition of glutathione to a dehydrobutyrine-containing peptide was solved. This revealed a histidine in the enzyme active site that may stabilize the enolate intermediate of the addition reaction[1]. When compared to cyclase homologs, this residue is not conserved. Here, we mutate the residue at the conserved position in LanM and assess how the mutation affects the cyclization reaction.

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48. The mechanism and bioengineering of lanthionine synthetase C-like (LanCL) enzyme

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In peptide biosynthesis, the Thia-Michael addition, involving dehydroalanine (Dha) and dehydrobutyrine (Dhb), is crucial. Our study contrasts the mechanisms of bacterial LanC and eukaryotic LanCL enzymes. LanC enzymes catalyze intramolecular cysteine-mediated additions, producing bioactive peptides, whereas LanCL enzymes in eukaryotes facilitate glutathione-mediated elimination of these residues. This research elucidates these enzymatic processes and aims to engineer control over product stereoselectivity.

Our investigation into LanC enzymes involved analyzing co-crystal structures of human LanCL1 with GSH and sERK peptide. We discovered an uncharacterized residue, His277, stabilizing the enolate intermediate's carbonyl oxygen. Binding affinity studies with LanCL1 variants and Fluorescein-Dha-Erk demonstrated that His277, while not essential for reactivity, plays a crucial role in carbonyl recognition and stabilization. Thus, this residue might be critical for directing the stereochemistry of enzymatic reactions.

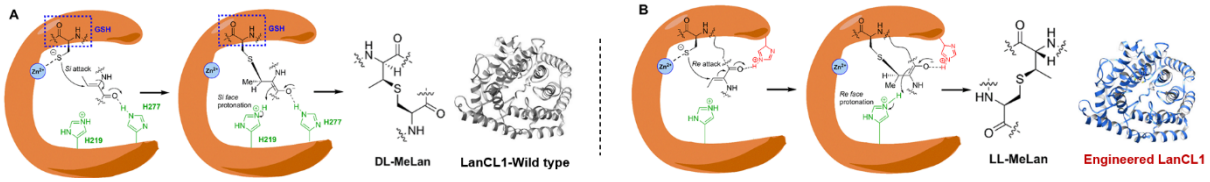

Fig. 1 The binding of Dhb-containing peptides differs in the formation of DL-MeLan and LL-MeLan, both of which are formed by the anti-addition of thiols across the double bond.

The mechanistic study of LanCL highlights bioengineering potential in stereochemistry control. In LanCL1, the reaction involves His277-mediated enolate stabilization, thia-attack on the *Si*-face of (Z)-Dhb, and protonation on the α-carbon (**Fig. 1**). Targeted mutations altering substrate geometry enable thia-attack on the Re-face of Dhb, leading to LL-product formation. This research not only clarifies LanCL enzyme mechanisms but also suggests new pathways for peptide synthesis through bioengineering.

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49. Mutagenesis as a Strategy for Pyrrolobenzodiazepine Biochemical Synthesis

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Pyrrolobenzodiazepines (PBDs) are potent cytotoxic compounds that form a covalent adduct with guanine bases in the minor groove of DNA, inhibiting transcription and replication in mammalian, and in some cases bacterial and fungal cells. The PBDs, tilimycin and tilivalline, are produced by gut bacterium *Klebsiella oxytoca* and cause antibiotic associated hemorrhagic colitis (AAHC) in humans. Tilimycin and tilivalline are synthesized through a non-traditional peptide synthesis machinery known as a non-ribosomal peptide synthetase (NRPS) pathway. This NRPS pathway consists of three proteins, NpsA, ThdA, and NpsB, which are involved in recognizing and loading the substrates into the pathway, forming an amide bond between the substrates, then releasing the final aldehyde product through a reduction reaction for final product cyclization[1]. NpsA and NpsB recognize and incorporate the substrates 3-hydroxyanthranillic acid (3HA) and proline respectively. Previous studies have shown novel PBDs that are structurally similar to tilivalline are produced in homologous pathways through the introduction of analogs of 3HA. Previously, PBD variants were biosynthesynthesized with wild-type enzymes and 3HA analogs[2,3]. We extend these studies through use of mutant enzymes as well as analogous substrates for the adenylation domain of NpsB. In this study, structure guided mutagenesis is used as a tool for incorporation of analogs in NpsA and for NpsB, using homologous PBD pathways as rationale for specific mutation sites. By engineering the pocket of NpsA and NpsB, analogs can not only be incorporated in the final PBD synthesis, but at high enough rates for downstream antibiotic/antitumorigenic use.

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50. Novel insights into the mechanism of the bacterial arsenite efflux pump ATPase, ArsA

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Arsenite-stimulated ATPase, ArsA is the cytosolic component of the arsenite efflux pump, ArsAB that confers arsenite resistance in many bacteria by coupling ATP hydrolysis to transport of arsenite via the membrane transporter, ArsB. Elucidating the molecular mechanism of the ArsAB pump holds the potential to inspire engineering of arsenic bioremediation strategies. ArsA belongs to the 'Intradimeric Walker A' (IWA) subset of P-loop NTPase superfamily,[1] along with enzymes that have diverse biological functions ranging from bacterial cell division regulation to nitrogen fixation. Unlike other IWA ATPases that are functional dimers, ArsA is a pseudodimer with two homologous nucleotide binding domains connected by a linker sequence.[2] The arsenite binding site is located at the pseudodimer interface. Nucleotide-dependent dramatic conformational rearrangements, notably at the dimer interface, is a key mechanistic feature of IWA ATPases. Such a conformational landscape is not structurally well-characterized for ArsA, precluding the elucidation of its catalytic mechanism. Here, we report the X-ray crystal structure of a novel post-hydrolysis conformation of ArsA from a thermotolerant bacteria *Leptospirillum ferriphilum*, and discuss implications for the catalytic mechanism of ArsA.

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51. Breaking the Chain: Inhibition of the SARS-CoV-2 Polymerase

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The ongoing challenge posed by SARS-CoV-2 underscores the critical need for a deeper understanding of its RNA replication mechanisms, particularly the role of the RNA-dependent RNA polymerase (RdRp) complex. This research delves into the intricate process of RNA replication facilitated by the nsp12 polymerase and its essential cofactors, nsp7 and nsp8[1]. By employing advanced biochemical and biophysical methods, we have delineated the intricate interactions within this complex, shedding light on the mechanism of SARS-CoV-2 RNA synthesis. A significant focus of our study is the examination of SARS CoV-2 nsp12 inhibition by antiviral agents Remdesivir and Bemnifosbuvir (AT-9010). Due to its essential function in the viral life cycle, the RdRp core enzyme, nsp12, is a prime target for antiviral drug development. Remdesivir, which received emergency approval during the pandemic, functions by stalling the polymerase complex [1,2]. In contrast, Bemnifosbuvir, still in clinical trials, promotes chain termination[3]. Here, we elucidate the mechanism of Remdesivir and Bemnifosbuvir incorporation by nsp12 and offer insights into their therapeutic potential and limitations. Our findings enhance the understanding of SARS-CoV-2 RNA replication and provide a critical evaluation of RdRp inhibitors. This research paves the way for developing more effective therapeutic strategies against SARS-CoV-2, contributing to the global effort in managing and eventually overcoming the pandemic. The exploration of combined therapy treatments, harnessing the unique properties of these inhibitors, represents a promising avenue for more effectively curtailing viral replication.

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52. Resolving redox state-dependent kinetics of substrate binding in a cytochrome *c* nitrite reductase: a voltammetric study

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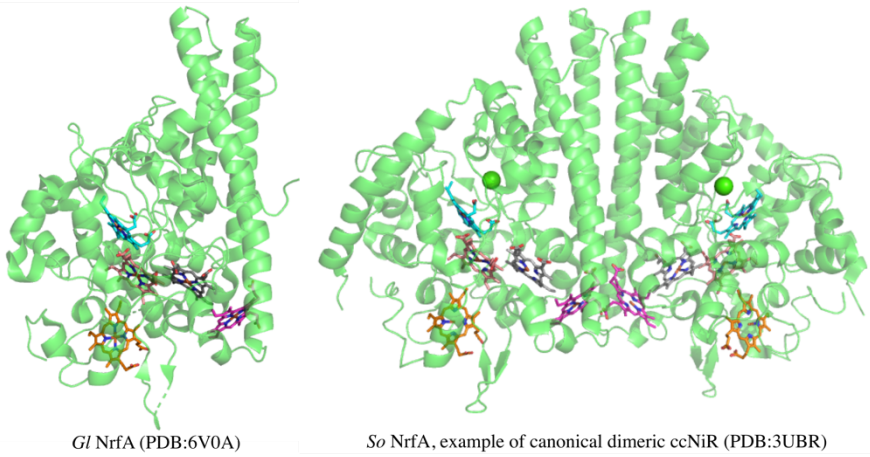
Redox enzymes are evolved by nature to couple long-range electron transfer with catalysis, and support high electron fluxes and turnover rates. NrfA, a widespread pentaheme cytochrome *c* nitrite reductase (ccNiR), converts the environmental pollutant nitrite into ammonia (a six-electron modification) with 100% product selectivity[1] and high turnover rate (1209 sec^{-1}). The NrfA from *Geobacter lovleyi* (*Gl*), a widespread microbe important in the biogeochemical nitrogen cycle, has been identified to be non-Ca^{2+} dependent and non-dimer-forming, unlike the canonical members of the ccNiR class.[2] We have applied protein film voltammetry (PFV) to study the mechanism of substrate binding, catalysis, and electron transfer in *Gl* NrfA. We also compare this bacterial ccNiR with other known ccNiR enzymes in terms of kinetic and mechanistic properties, which have previously been studied by direct electrochemistry for the *Escherichia coli* and *Shewanella oneidensis* ccNiR enzymes.[3] Results from catalytic and non-turnover electrochemistry show that irreversible steps are important in the NrfA catalytic cycle, and support our model: substrate binding is not at equilibrium during turnover, and substrate binding to the active site favors intramolecular electron transfer. As a result, the substrate-bound active site is easier to reduce by more than 100 mV compared to the active site empty of substrate. We derive a steady-state current equation to quantitatively fit the PFV data in the biologically relevant range of substrate concentrations, allowing for extracting the values of kinetic and thermodynamic constants. The proposed mechanism implies that the kinetics of substrate binding and electron transfer are tuned by evolution to optimize for fast turnover rates and energy conservation in the class of ccNiR enzymes.



Gl NrfA (PDB:6V0A) *So* NrfA, example of canonical dimeric ccNiR (PDB:3UBR)

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53. Carboxyspermidine dehydrogenase of microbial polyamine biosynthesis

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Polyamines are positively charged alkylamines found in almost all cells. Humans obtain them in three ways; dietary intake, metabolic production, or the uptake of polyamines made by microbes within the gut. Polyamines have wide-ranging functional roles in cell growth and proliferation and the dysregulation of microbe-produced polyamines has been implicated in conditions such as colon cancer, diabetes and obesity. The pathway most commonly used by gut microbes to produce polyamines differs from the human pathway. This alternative pathway uses carboxyspermidine dehydrogenase (CASDH), an enzyme without prior structural or functional characterization, to produce carboxyspermidine as a precursor to spermidine biosynthesis. We solved a 1.94 Å X-ray crystal structure of *Bacteroides fragilis* CASDH. This structure demonstrates the dimeric nature of BfCASDH, reveals the active site residues involved in catalysis, and leads us to propose updated functional annotations for two existing PDB entries. Steady-state kinetics were used to determine substrate specificity, k_{cat} and k_{cat}/K_m parameters for BfCASDH and *Clostridium leptum* CASDH. Putrescine and diaminopropane substrates have similar k_{cat}/K_m values suggesting that carboxynorspermidine or carboxyspermidine are potential products. NADPH is the coenzyme as no catalysis was observed with NADH. Binding titrations performed while observing tryptophan fluorescence suggest enhanced putrescine binding in the presence of aspartate semi-aldehyde. NADP+ and carboxyspermidine were observed to bind and do cause competitive inhibition of the forward reaction, but no turnover with these substrates was observed for the reverse reaction. These data provide the first steady-state kinetic and structural characterization of CASDH – a key enzyme in the production of polyamines by human gut microbes.

54. Structural and functional dynamics of site-specific vanadium-dependent haloperoxidases during a catalytic cycle

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Site-specific vanadium-dependent haloperoxidases (VHPOs) catalyze the two-electron oxidation and selective installation of aqueous halide ions onto small organic molecule substrates.[1] This enzymology has broad utility in the biosynthetic assembly of therapeutically useful bioactive compounds and represents a desirable biocatalytic alternative to conventional chemical halogenation reagents. Recent X-ray crystallographic studies have advanced our structural understanding of actinobacterial VHPOs involved in the biosyntheses of antimicrobial and anticancer meroterpenoid natural products;[2] however, insight into how these enzymes interact with their substrates remains elusive and has restricted their application. While fungal VHPOs have been identified that possess one large catalytic binding pocket,[3] we and others hypothesize that site-specific bacterial VHPOs segregate halide oxidation and organic substrate binding into two separate pockets. Moreover, we propose that a series of protein conformational changes occur during catalysis to ensure a distinct order of events. We have recently discovered and characterized a novel VHPO from the myxobacteria *Enhygromyxa salina* (esVHPO), which plays a role in the regioselective bromination of alkyl quinolone (AQ) molecules important for *Pseudomonas aeruginosa* quorum sensing and biofilm production.[4] By adapting pioneering experiments performed on flavin-dependent halogenase RebH,[5] we have purified a stable esVHPO intermediate after incubation with oxidative cofactors and co-substrates capable of single-turnover bromination following AQ substrate addition. Site-directed mutagenesis of non-vanadate coordinating residues in the halide oxidation pocket and putative substrate binding residues have given information into the mechanism of hypohalite transfer and distal AQ binding. In tandem, single particle cryo-EM analyses of esVHPO at various stages of the catalytic cycle shows distinct conformational changes, providing insight into the structural dynamics at play. An improved understanding of how Nature utilizes vanadate to perform controlled halogenation chemistry on substrates will further aid in the biocatalytic application of this enzymology.

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55. ABSTRACT WITHDRAWN

56. “Stuffed” Epimerase Domains in Pyochelin-like Natural Product Biosynthesis may be Defunct Methyltransferases and not Catalytically Required for Activity

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Nonribosomal peptide synthetases (NRPSs) are used by bacteria, fungi, and plants to generate secondary metabolites called nonribosomal peptides (NRPs). These bioactive NRPs also have medicinal applications as antibiotics, anticancer drugs, and immunosuppressants. A common feature of NRPs is the need for further tailoring for bioactivity such as epimerization, methylation, reduction, hydroxylation, heterocylization, etc. The inclusion of amino acids with D-stereocenters, which have been found to be important for the chemical functionality of the peptide, and as a way to evade protease degradation typically arise by the action of individual epimerase tailoring domains that have been well studied. Interestingly, 2-hydroxyphenylthiazoline natural products do not use common epimerase tailoring domains but have been hypothesized to employ noncanonical epimerase domains that are embedded, or ‘stuffed’, within the NRPS adenylation domain. Herein, we examine the adenylation-epimerase didomain of PchE from pyochelin biosynthesis, a 2-hydroxyphenylthiazoline siderophore from *Pseudomonas aeruginosa*. Two recombinant variants of PchE were isolated and shown to have adenylation activity by steady-state kinetics. Substrate and product analogs were synthesized to probe the epimerase chemistry of PchE. While the variants performed adenylation chemistry, epimerase activity was not enzymatically catalyzed. Indeed, racemization was spontaneous for the 2-hydroxyphenylthiazoline ethyl ester analogues. Based on similar findings for other analogous natural products, we propose that noncanonical stuffed epimerase domains of 2-hydroxyphenylthiazoline natural products are catalytically defunct methyltransferases and possibly an evolutionary remnant.

57. Transition State Analysis Reveals a Novel Mechanism for Enzyme-Catalyzed Aldose–Ketose Isomerization

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The enzyme-catalyzed interconversion of aldoses and ketoses generally involves one of two canonical mechanisms: most isomerases (e.g., triosephosphate isomerase) utilize Brønsted acid–base catalysis via an enediol(ate) intermediate while others (e.g., xylose isomerase) use a metal-assisted 1,2-hydride shift. Both mechanisms promote α-hydrogen transfer through the presence of a carbonyl group in the open-chain form of the substrate. The conversion of 5-methylthio-D-ribose 1-phosphate (MTR1P) to 5-methylthio-D-ribulose 1-phosphate (MTRu1P) catalyzed by MTR1P isomerase (MtnA) challenges this dogma because the substrate's phosphate ester prevents equilibration with an open-chain form. ^{2}H and ^{13}C kinetic isotope effects (KIEs) were measured to evaluate the nature of the rate-limiting transition state. The primary 2-^{2}H KIE and the 1- and 2-^{13}C KIEs probed the concertedness of furanose ring opening and hydrogen transfer. In addition, an inverse solvent KIE was observed, consistent with the involvement of an active-site cysteine residue as the base responsible for proton transfer. QM/MM hybrid calculations revealed excellent agreement between the experimental primary KIEs and those predicted for a concerted E2 elimination and were inconsistent with a stepwise proton- or hydride-transfer from an oxocarbenium-ion intermediate. We conclude that MtnA aligns the substrate for anti elimination of the protonated ring oxygen instead of phosphate to generate an enediol intermediate that undergoes tautomerization with return of the proton to C-1.

58. Studies on reductive activation of QueE – a radical SAM enzyme

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All members of the radical S-adenosyl-L-methionine (rSAM) enzyme superfamily reductively cleave the cofactor, SAM, to generate a 5'-deoxyadenosyl radical. The reduction of SAM is catalyzed by a [4Fe-4S] site differentiated cluster, which binds the amino and carboxylate moieties of SAM. The reduction of the cluster from the resting +2 to the catalytic +1 state requires a source of a reducing electron. Traditionally, the provision of necessary reducing equivalents for this reaction has been accomplished *in vitro* through inorganic reducing agents such as sodium dithionite or titanium citrate. Alternatively, "physiological" reducing systems like flavodoxin/flavodoxin reductase/NADPH have also been utilized. An intriguing observation made by numerous researchers studying rSAM enzymes is that sometimes *in vitro* reaction with dithionite fails to yield products, a catalytic reaction is evident upon switching to the flavodoxin/flavodoxin reductase/NADPH system. Under many conditions, particularly with dithionite, rSAM enzymes produce varying amounts of 5'-deoxyadenosine, whose provenance is generally presumed to be unproductive and abortive cleavage of SAM. This poster will show our progress towards understanding the role of multiple reducing systems on the rSAM enzyme, QueE, with a primary emphasis on exploring the limitations associated with utilizing non-physiologically relevant reducing agents such as dithionite. Our findings offer insights into the underlying reasons for the differing activation patterns observed between dithionite and other less potent reducing systems.

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59. Using AI to understand enzyme function and effects of cancer-associated mutations

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Our Artificial Intelligence (AI) methodology, Partial Order Optimum Likelihood (POOL)[1, 2], is used to predict biochemically active amino acids in the 3D structures of proteins. Computed electrostatic and chemical properties of individual amino acids serve as input features. Our most recent applications of POOL are described. POOL analysis uncovers the types of interactions that enable protein structures to transform the weakly acidic or basic side chains of amino acids into strong acids, strong bases, or nucleophiles in the active site. Examples are shown to illustrate how interactions with neighboring amino acids give the catalytic amino acids their strength of acidity / basicity and expanded buffer ranges. This analysis can also predict whether a mutation is likely to alter biochemical function. The μ4 value, a metric obtained from POOL and THEMATICS[3] that serves as a measure of the degree of coupling between one ionizable amino acid and its neighbors, is used to identify which protein mutations are likely to have significant impact on the biochemical activity. For examples of cancer-associated variants of human DNA polymerase kappa (pol kappa), THEMATICS and POOL are used to predict which ones have altered biochemical activity. Kinetic assays on undamaged and damaged DNA confirm predictions of reduced activity for the pol kappa variants R48I, H105Y, G147D, G154E, V177L, R298C, E362V, and R470C relative to wild type. The pol kappa variants T102A, H142Y, R175Q, E210K, Y221C, N330D, N338S, K353T, and L383F were predicted to have similar catalytic efficiency to WT pol kappa; these predictions were confirmed by experiment in vitro. This raises intriguing questions about the mechanisms for disease association in vivo.

This work is supported by NSF MCB-2147498.

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60. Phage-encoded 5-methylpyrimidine dioxygenases (5mYOXs)- activity, regulation, and their roles in DNA base modification

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5-methylpyrimidine dioxygenases (5mYOXs) are Fe(II) and 2-oxoglutarate- dependent dioxygenases that catalyze the oxidation of 5-methylpyrimidines (5mY) on nucleic acid polymers. Members of this superfamily are present across all domains of life and are essential for cellular processes such as active demethylation in gene regulation mechanisms in mammals, catalyzed by ten-eleven translocation (TET) dioxygenase. Characterized eukaryotic TETs are capable of catalyzing three sequential oxidations of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC). Base J biosynthesis (trypanosomes) occurs via hydroxylation of T to 5-hydroxymethyluracil (5hmU) by J-base binding protein (JBP) dioxygenase followed by addition of glucose by a glucosyltransferase. Recent work published by our group identified biosynthetic gene clusters from metaviriome databases (bacteriophage) that encode a cytosine C5-methyltransferase (C5-MT), a 5mYOX, and other DNA modification genes (ie. epimerase, glycosyltransferase). Both C5-MT and 5mYOXs from this study are GpC specific, divergent from eukaryotic homologs (CpG preference).[1] It is possible that the GpC specificity of phage-encoded DNA modifying enzymes may play a role in gene regulation.

We conducted metaviriome mining, identifying thousands of 5mYOX containing DNA modification pathways from bacteriophage. One phage homolog, "5mYOX43", a 5mC dioxygenase, has been characterized *in vitro* and displays divergent biochemical properties compared to the eukaryotic TETs. In addition to differing sequence context, 5mYOX43 is specific to dsDNA and stalls at 5hmC production. We show kinetically that the stalling mechanism is due to chemistry rather than binding affinity. We speculate that structural properties and active site residue positioning with regards to the 5xC substrate are determinants of the rate-limiting 5hmC to 5fC conversion.

We also describe the first study of phage-encoded T-hydroxylases from the 5mYOX superfamily. To probe the activity of 5mYOX subclasses and other DNA modifying enzymes, we developed a Golden-Gate based gene(ome) re-assembly platform for expression and functional screening of 5mYOX bacteriophage homologs and their partner proteins, using *E. coli* as a metabolite source. This demonstrated two subclasses of phage T-hydroxylases: (1) dependent on a regulatory protein, ParB, for activity and (2) ParB-independent. ParB-independent T-hydroxylases can oxidize T to 5hmU in the absence of a partner protein, in contrast to ParB-dependent T-hydroxylases which only exhibit oxidation of T when co-expressed with ParB. Future work will aim to elucidate the evolutionary divergence of T-hydroxylases and to understand the molecular mechanism of regulation of T-dioxygenase activity by ParB.

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61. Tipping the balance between DNA rehybridization and RNA:DNA duplex maintenance enables Cas9 to catalyze multiple turnovers

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The RNA-guided Cas9 endonuclease from Staphylococcus aureus (SauCas9) can catalyze multiple turnover reactions whereas Cas9 from Streptococcus pyogenes (SpyCas9) is a single-turnover enzyme. Here we dissect the mechanism of multipleturnover catalysis by SauCas9 and elucidate its molecular basis. We show that the multiple-turnover catalysis does not require more than stoichiometric RNA guides to Cas9 nuclease. Rather, the RNAguide loaded ribonucleoprotein (RNP) is the reactive unity that is slowly released from product and recycled in the subsequent reaction. The mechanism that RNP is recycled formultiple-turnover reaction entails the unwinding of the RNA:DNA duplex in the R-loop. We argue that DNA rehybridization is required for RNP release by supplementing the energy cost in the process. Indeed, turnover is arrested when DNA rehybridization is suppressed. Further, under higher salt conditions, both SauCas9 and SpyCas9 showed increased turnover, and engineered SpyCas9 nucleases that form fewer direct or hydrogen bonding interactions with target DNA became multiple-turnover enzymes. Thus, these results indicate that for both SpyCas9 and SauCas9, turnover is determined by the energetic balance of the post-chemistry RNP-DNA interaction. Due to the conserved protein core folds, the mechanism underpinning turnover we establish here is likely operant in all Cas9 nucleases.

62. Phytosiderophore biosynthetic enzymes from graminaceous plants: key tools for future food security

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Two billion people are grappling with iron-deficient diets[1]. This global health concern prompted the World Health Assembly to take action in May 2023, adopting a resolution for micronutrient food fortification, particularly emphasizing iron. Although abundant in the environment, iron often oxidizes from the readily accessible ferrous iron (Fe^{2+}) to sparingly soluble ferric iron (Fe^{3+}). Graminaceous crop plants, such as wheat, rice, corn, and barley, have evolved a mechanism to scavenge iron from the rhizosphere by biosynthesizing and secreting phytosiderophores derived from nicotianamine. These phytosiderophores form complexes with Fe^{3+} and are taken up by specialized importers[2]. Many researchers are actively engaged in engineering transgenic crop plants with enhanced phytosiderophore biosynthetic enzymes[3]. However, these endeavors are being pursued without the fundamental enzymology required to develop new plant varieties leading to more efficient iron uptake and higher iron content in food products. In this study, we present a biochemical characterization of nicotianamine synthase enzymes (NAS) from Barley (Hv) and Corn (Zm). While both isoforms readily aggregate into oligomers that are compromised structurally, a small fraction of monomer can be obtained using the size exclusion chromatography. The monomeric isoforms exhibit Fe^{3+} binding, as revealed by ICP-MS analysis, leading to a consequential decrease in enzymatic activity. These monomeric configurations predominantly adopt α-helical structures, and ZmNAS consistently demonstrates a reliable melting temperature of 85-90 °C. In the presence of Fe^{3+}, ZmNAS experiences a distinct reduction in melting temperature, reaching 70-75 °C. In contrast, the aggregated species exhibited no distinct transition in the melting curve, with no discernible melting temperature, ultimately precipitating from solution upon the addition of Fe^{3+}. We have predicted a cysteine residue in the C-terminal region that may play a regulatory role and will present kinetic and structural data testing the hypothesis that Fe^{3+} serves as a direct regulator of enzyme function[4]. In the future, we will provide structural and functional characterizations of the downstream enzymes in the biosynthesis of phytosiderophores: nicotianamine amino transferases (NAAT), and deoxymugineic acid synthases (DMAS). The outcomes of this research are poised to make a significant contribution to the development of superior enzymes for iron acquisition in grains, a critical step in ensuring future food security.

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63. Structural insights into substrate selectivity and iterative biosynthesis of the siderophore desferrioxamine by DesD

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Siderophores are high-affinity iron chelating compounds secreted by microbes for iron assimilation. Two systems responsible for siderophore biosynthesis are Non-ribosomal peptide synthetase (NRPS) and NRPS-independent synthetase (NIS). A lack of substrate-bound structures has impeded the mechanistic understanding of siderophore biosynthesis, substrate selection and enzymatic reactions of NIS family of proteins. The biosynthesis of desferrioxamine siderophores is catalyzed by DesD proteins that utilize an enigmatic iterative mechanism. In order to decipher the iterative mechanism and substrate selectivity in DesD, we designed an acyl-adenylate mimic inhibitor. Herein, we describe the first acyl-adenylate mimic-bound structure of DesD. With the help of five structures capturing liganded and unliganded states of DesD with cofactors and inhibitor, we delineated the substrate selectivity in the NIS family of proteins. Furthermore, we performed docking with the trimeric substrate guided by the solved acyl-adenylate mimic-bound DesD structure. Overall, this structural analysis and molecular docking helped us to propose a mechanism for the iterative biosynthesis desferrioxamine siderophores catalyzed by DesD.

64. Enzymatic Formation of Designed Multi-Crosslinked Peptide Architectures

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PapB is a promiscuous radical S-adenosyl-L-methionine (rSAM) enzyme, distinguished by its ability to introduce thio(seleno)ether crosslinks into a wide array of substrates.[1-5] Despite its versatility, the substrate range of PapB has not been fully explored, with previous research primarily focusing on its native Cys-X_3-Asp thioether motif. The inherent redox stability of thioether crosslinks positions PapB as a promising tool for biotechnological applications. This poster will discuss PapB's capability to process both nested and in-line crosslinks across varying distances between each crosslinking site. Additionally, we reveal that PapB can successfully process non-peptidic substrates, efficiently installing thioether crosslinks at predictable and consistent locations. These findings not only expand our understanding of the versatility of PapB, but also highlight its potential as a reliable biotechnological tool.

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65. Crystal Structures of *Proteus vulgaris* Tryptophan Indole-lyase Complexed with Alanine, Ethionine, and 7-Azatryptophan Show Dynamics and Ground State Strain

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Tryptophan indole-lyase (TIL; E.C. 4.1.99.1) is a pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyzes the reversible β-elimination of indole from L-tryptophan. The mechanism of elimination of indole from L-tryptophan starts with formation of an external aldimine of the substrate and PLP, followed by deprotonation of the α-CH of the substrate, forming a resonance stabilized quinonoid intermediate. Proton transfer and carbon-carbon bond cleavage of the quinonoid intermediate gives indole and an aminoacrylate intermediate, releasing pyruvate. L-Ethionine and L-alanine are competitive inhibitors that form equilibrating mixtures of external aldimine and quinonoid intermediates, with λ_{max}=508 nm[1]. 7-Aza-L-tryptophan is a very slow substrate for TIL, with k_{cat}~0.5% that of L-tryptophan[2]. We have now determined the X-ray crystal structures of the TIL complexes with L-ethionine, L-alanine, and 7-aza-L-tryptophan. These structures show mixtures of external aldimine and quinonoid intermediates, in both open and closed active site conformations. Furthermore, the structure of the 7-azatryptophan complex shows that the azaindole ring is bent about 20° in the closed quinonoid intermediate. This strain is due to steric clashes of the azaindole ring with Phe-37 and Phe-449. New hydrogen bonds are only formed, with OD2 of Asp-133 bridging N1 of the azatryptophan and NE2 of His-448, in the strained conformation (Figure 1).

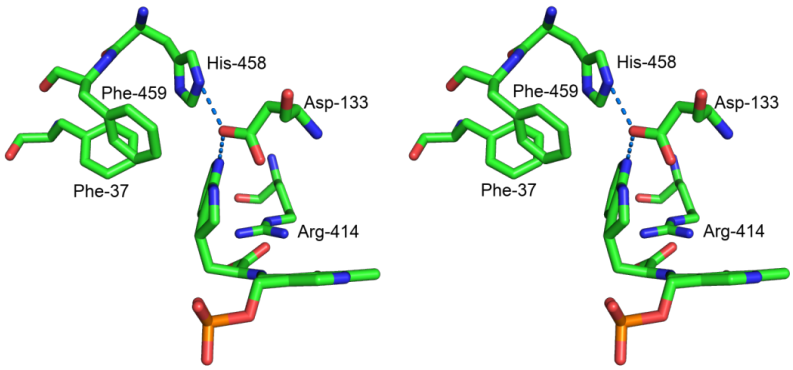

Figure 1. Complex of 7-aza-L-tryptophan with TIL.

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66. Mechanistic Studies of Mono- and Binuclear Hydrolytic Metalloenzymes

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The selective hydrolysis of the extremely stable phosphoester, peptide and ester bonds of molecules is required in a wide range of biological, biotechnological and industrial applications such as protein engineering, therapeutics, genomics, DNA repair and remediation of pesticides, nerve agents and plastics. In nature, these bonds are hydrolyzed by highly specialized mono- and binuclear metal center containing enzymes, which depending on the nature of the scissile bond are categorized as proteases/peptidases, esterases and phosphatases/nucleases and in general known as metallohydrolases. In their catalytic mechanisms, a multitude of chemical factors such as the nature of the metal center, metal ions, ligand environment, catalyst–substrate complexation, nucleophile and non-covalent interactions play key roles. These distinct factors are productively utilized by these catalysts for their efficient functioning.

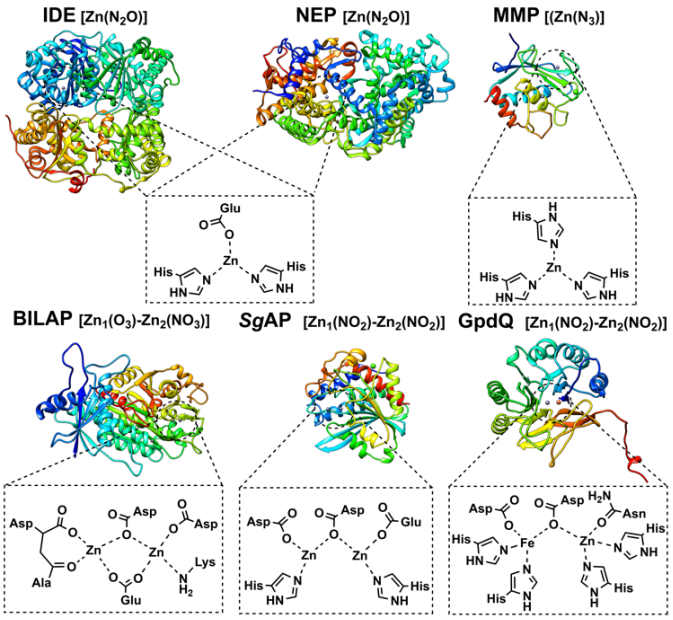

Figure 2: Metal center(s) of mono- and binuclear metallohydrolases

We have investigated mechanisms of distinct mono- [insulin degrading enzyme (**IDE**), neprilysin (**NEP**) and matrix metalloproteinase (**MMP**)] and binuclear [bovine lens leucine aminopeptidase (**BILAP**), *Streptomyces griseus* aminopeptidase (***Sg*AP**) and Glycerophosphodiesterase (**GpdQ**)] metallohydrolases (Figure 1). Our integrated computational approaches either addressed or reconfirmed the multiple issues regarding their activities: (1) a low basicity of the ligand environment in enzymes such as **MMP** enhances the Lewis acidity of the metal ion and promotes hydrolysis; (2) a metal bound water is more suitable than a free water for the creation of hydroxyl nucleophile; (3) the water is more acidic in the bridging form than in the terminal form in binuclear enzymes; (4) the electronic nature of the metal and substrate predominantly controls the energetics of hydrolysis; (5) the different metal centers such as hetero- and homobinuclear in **GpdQ** and ***Sg*AP**, respectively, utilize distinct mechanisms for the hydrolysis of the same BNPP substrate; (6) heterobinuclear metal centers are generally more reactive than their homobinuclear counterparts; and (7) peptide hydrolysis is controlled by the nucleophilicity of the metal bound hydroxyl group, whereas phosphoester hydrolysis is controlled by double Lewis acid activation.

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67. Defining human cathepsin L: Mechanistic determination through steady state and pre-steady state kinetics

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Human cathepsin L (hCatL) is a member of the papain family of cysteine proteases. CatL is a lysosomal enzyme found ubiquitously in cells and tissues, and its dysregulation has been associated with metastatic cancers. In human cells permissive to infection by SARS-CoV-2, hCatL catalyzes cleavage of the coronaviral Spike protein which is an essential step to the establishment of infection. We have shown that several classes of existing and bespoke irreversible and reversible-covalent inhibitors of hCatL block infection by SARS-CoV-2 of human cells at sub-micromolar concentrations. To develop new classes of hCatL inhibitors, we seek to understand the catalytic mechanism of hCatL more fully in terms of the following questions: (1) what is the protonation state of the Cys-His catalytic dyad in the free enzyme form that binds both substrates and inhibitors? (2) For the double-displacement mechanism of cysteine proteases, is the acylation or de-acylation half-reaction of the mechanism rate-limiting? Previous studies have shown that cysteine proteases employ an Cys-His active-site dyad that is either neutral (Cys-SH:His) or a charged thiolate-imidazolium form (Cys-S^-:$HisH^+$), as this findings will influence inhibitor design. For hCatL, neither the protonation state of the Cys-His dyad in the free enzyme or the identity of the rate-limiting step in the reaction of hCatL have been defined. First, through inclusion body refolding, active protein was acquired for structural and kinetic studies. Effective inhibitor scaffold structure, and warhead placement, within the active-site pockets of hCatL is being determined through structural results from ongoing x-ray crystallography in tandem with kinetic inhibitor analysis. pH-Rate profiles are used to determine the protonation state of the active site Cys-His catalytic dyad, as well as the rate limiting step of the chemical mechanism. Fluorogenic substrates have been evaluated, showing that Cbz-Leu-Arg-AMC and Cbz-Phe-Arg-AMC exhibit optimal activity for kinetic evaluation. We have evaluated Z-LR-AMC in pH-Rate profiles to determine the protonation state of the catalytic dyad and the effect of pH on enzyme activity. This work has been used to determine the pK_as of the active site residues and define the protonation of active site residues through the mechanism. Further work through solvent kinetic isotope effects lends support to the protonation state of the active site residues and the rate limiting step of the chemical mechanism. Pre-steady state kinetics, performed through stopped-flow usage combined with solvent kinetic isotope effects, define the presence of a tautomerization step for the catalytic dyad and its interaction with substrate binding, while further solidifying the identity of the rate limiting step in the chemical mechanism of hCatL by the presence of a pre-steady-state burst of product formation. Results from these studies will assist in the design of new reversible-covalent inhibitors of hCatL.

68. Uncovering the biosynthetic pathway towards the production of Ammosamide C

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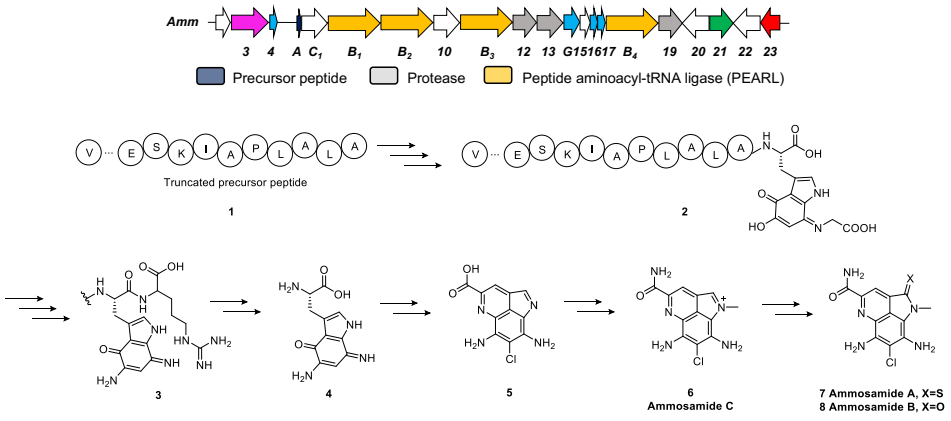
Ammosamide C (**6**) and its congeners A (**7**) and B (**8**) are marine natural products that contain a highly decorated pyrroloquinoline core. Ammosamides A and B were first isolated in 2003 from *Streptomyces sp*. CNR698 collected from sea bottom sediments in the Bahamas Islands.[1] These compounds were shown to be highly cytotoxic against diverse cancer cell lines and to target myosin.[2] In 2016, the ammosamide biosynthetic gene cluster (BGC) was identified by the Moore lab.[3] Through bioinformatic analysis, it was shown that this cluster did not contain the typical NRPS PKS architecture, instead elements from ribosomally synthesized and posttranslationally modified peptide (RiPP) gene clusters were present. Later work by the van der Donk lab[4-5] revealed that the starting point of the biosynthetic pathway was a truncated precursor peptide (**1**) as shown in the scheme below. Next, a peptide amino acyl-tRNA ligase (PEARL), $AmmB_2$, was shown to add a C-terminal tryptophan (W) that could be transformed into the pyrroloquinoline core. Then, a tailoring enzyme $AmmC_1$ trihydroxylated the indole of the C-terminal W and PEARL $AmmB_3$ gave an imine adduct with glycine appended to the 7th position of the W indole moiety (**2**). During this presentation, I will showcase the activity of the next acting enzymes from the ammosamide BGC elucidated through *in vivo* and *in vitro* studies. Briefly, three enzymes from the cluster including an oxidoreductase and two PEARL enzymes further modified the C-terminal W to produce **3** and then proteolytic cleavage and chlorination produced a chlorinated ammosamic acid intermediate (**5**) en route to ammosamide C (**6**.)



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69. Structural and Mechanistic Insights into the C–C Bond Forming Rearrangement Catalyzed by Heterodimeric Hinokiresinol Synthase

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Hinokiresinol synthase (HRS) from *Asparagus officinalis* consists of two subunits, α and β, and catalyzes an unusual decarboxylative rearrangement reaction of 4-coumaryl 4-coumarate to generate (*Z*)-hinokiresinol with complete stereoselectivity.[1] Herein, we describe the mechanism of the rearrangement catalysis and the role played by the heterodimeric HRS, through structural and computational analyses. Our results suggest that the HRS reaction is unlikely to proceed via the previously hypothesized Claisen rearrangement mechanism. Instead, we propose that the 4-coumaryl 4-coumarate substrate is first cleaved into coumarate and an extended p-quinone methide, which then recombine to generate a new C–C bond. These processes are facilitated by proton transfers mediated by the basic residues (α-Lys164, α-Arg169, β-Lys168, and β-Arg173) in the cavity at the heterodimer interface. The active site residues, α-Asp165, β-Asp169, β-Trp17A, β-Met136, and β-Ala171, play crucial roles in controlling the regioselectivity of the coupling between the fragmented intermediates, as well as the stereoselectivity of the decarboxylation step, leading to the formation of the (*Z*)-hinokiresinol product.

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70. Maintaining the Nucleotide Pool by Archaeal Homolog MutT

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Ribonucleotide triphosphates and deoxyribonucleotide triphosphates are the building blocks of DNA and RNA and constitute the cellular nucleotide pool. Importantly, the nucleotide pool is subject to damage by a variety of damage agents, including reactive oxygen species and methylating agents. Damaged nucleotides, if left unrepaired, can be misincorporated during replication. Of particular interest is the oxidation of 2'-deoxyguanosine-5'- triphosphate (dGTP) to 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP) by reactive oxygen species, because of its mutagenetic properties. In the *syn* glycosidic conformation, 8-oxo-dGTP can be misincorporated opposite adenine during DNA replication, resulting in A·T→C·G transversion mutations[1]. While several enzymes target the repair of 8-oxo-dG in the context of DNA, MutT is responsible for targeting oxidized guanines at the nucleotide pool source. MutT belongs to the nucleoside diphosphate-linked moiety X (NUDIX) superfamily and hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP preventing A to C transversions[2]. MutT has been well characterized in *E. coli*, and several other bacterial and eukaryotic species[3]. For the first time, we have identified a putative archaeal homolog of MutT, TK2284 in *Thermococcus kodakarensis* (T. kodakarensis), which contains the highly conserved NUDIX motif. Here we present the biochemical characterization of TK2284 and investigate the *in vivo* biological role of TK2284 towards maintaining the nucleotide pool of *T. kodakarensis*. Taken together, these results provide context to how *T. kodakarensis* maintain oxidative damage while also contributing to our broader understanding of nucleic acid maintenance in archaea.

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71. The Formation of the ES Complex of 2,4'-Dihydroxyacetophenone Dioxygenase is Primarily Mediated by a Single Hydrogen Bond

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2,4'-dihydroxyacetophenone dioxygenase (DAD) catalyzes the oxidative cleavage of 2,4'-dihydroxyacetophenone (DHA) to form 4-hydroxybenzoic acid (4HB) and formic acid. Computational docking simulations suggest an important hydrogen bonding interaction between the 4'-OH group of DHA and the sidechains of tryptophan-62 and aspartate-64 of DAD. We have investigated the significance of this interaction in the formation of the DAD-DHA complex through the generation of active-site mutants and substrate analogs. Specifically, we have prepared DAD mutants W62F, D64N, D64L, and W62F/D64N and determined the values for k_{cat} and k_{cat}/K_M with DHA. While the W62F mutation had no appreciable effect relative to wild-type, the D64N and D64L mutations resulted in 2.5-fold and 70-fold decreases in k_{cat}, respectively, and 100-fold and 3700-fold lower k_{cat}/K_M values, respectively. These results demonstrate the relevance of aspartate-64 in the binding of DHA and, to a lesser extent, the catalytic reaction. We have also evaluated a variety of *para*-substituted analogs of DHA as substrates or inhibitors for the wild-type enzyme. In all cases, substitution of the 4'-OH moiety of DHA resulted in hundreds- to thousands-fold drops in k_{cat}/K_M with only modest effects on k_{cat}, supporting the requirement of the 4'-OH moiety in the formation of the productive complex. These results argue against the importance of tryptophan-62 in complex formation but support the direct interaction of aspartate-64 with the 4'-OH of the substrate as the primary driver for formation of the DAD-DHA complex.

72. Cryptic isomerization in diterpene biosynthesis and the restoration of an evolutionarily defunct P450

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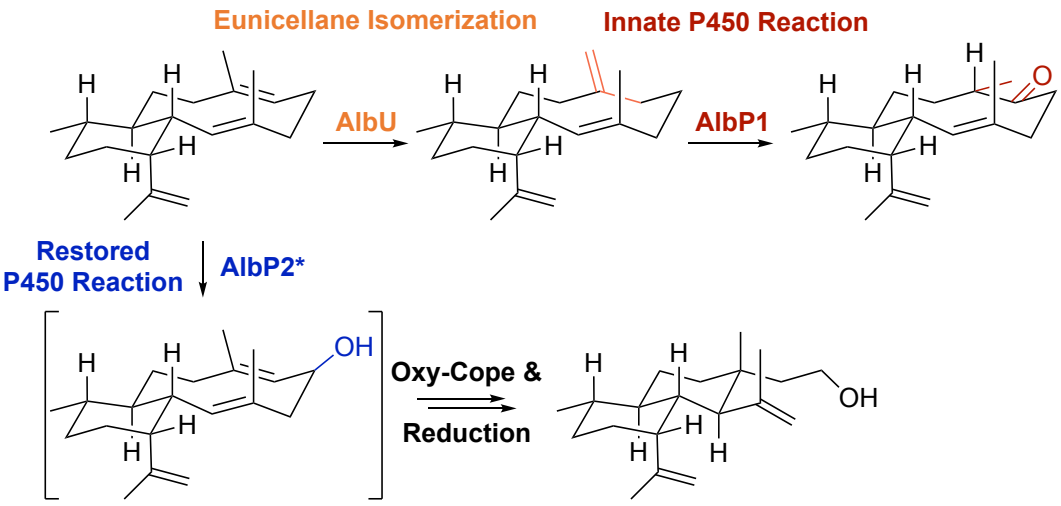
Terpenoids are the largest and most structurally diverse family of natural products. There are over 90,000 known terpenoids; however, less than 2% of these are of bacterial origin.[1] The eunicellane diterpenoids are a unique family of natural products seen in marine organisms, plants, and bacteria. Biosynthetic modifications of the 6/10-bicyclic hydrocarbon skeletons of the eunicellane family from any source are unknown. In the biosynthesis of a bacterial *trans*-eunicellane, albireticulone A, we here identified a novel isomerase that catalyzes a cryptic isomerization in the biosynthetic pathway and assigned functions of two cytochromes P450 that oxidize the eunicellane skeleton.[2] One of these P450s, AlbP2, was a naturally evolved non-functional P450 in the biosynthetic gene cluster, but after genetic repair catalyzed allylic oxidation of a competing pathway.



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73. Assembly and maintenance of the DOPA radical cofactor in metal-free class Ie ribonucleotide reductases from bacterial pathogens

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Ribonucleotide reductases (RNRs) are essential enzymes responsible for converting nucleotides into 2'-deoxyribonucleotides, the building blocks required for DNA synthesis and repair. All RNRs initiate nucleotide reduction with a thiyl radical located on a conserved cysteine residue (Cys_α) in the catalytic site of the enzyme.[1] In the aerobic class I RNRs, nucleotide reduction and radical generation occur in separate protein subunits, α and β, respectively.[1,2] Class I RNRs exhibit diversity within the β subunit, which typically houses a dinuclear metallocofactor responsible for generating the Cys_α radical.[3] Class Ie RNRs, found frequently in bacterial pathogens, differ from other class I RNRs in their use of a metal-free dihydroxyphenylalanine radical (DOPA•) for Cys_α oxidation. This novel radical cofactor likely employs unique assembly and maintenance pathways. Assembly of the Ie DOPA• cofactor is dependent on O_2 and the accessory flavodoxin, NrdI.[4,5] Unlike other class I RNRs, the active cofactor cannot be assembled with purified protein components and instead is obtained by heterologous co-expression of the Ie β subunit with NrdI inside *E. coli* cells.[4,5] The our inability to generate the Ie cofactor using purified protein components suggests that the assembly process requires an activation factor that is absent in our preparations. We assessed Ie β subunit interactions with first-row bioavailable transition metals. While none of the tested metals resulted in activity or Tyr modification after incubation with purified β subunits, Cu(I) was shown to bind the class Ie β subunit, coordinating near the site of DOPA• formation. Cu(I) exposure in activated Ie β results in a reductively inactivated cofactor that can be regenerated upon exposure to O_2 in the presence of reduced NrdI. Conversely, Cu(I) restriction during heterologous overexpression of Ie β appears to produce an overoxidized DOPA-quinone cofactor that is not readily rescued through redox cycling with NrdI and O_2. While Cu(I) does not play a role in cofactor maturation, our findings instead suggest that a Cu-mediated decay pathway may protect the class Ie cofactor from oxidative damage, a phenomenon not observed in other metal-dependent class I RNRs.

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74. Redesigning diterpene synthases with the TerDockin computational approach

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The complexity of the reactions catalyzed by terpene synthases has hindered enzymatic engineering. In most cases such efforts result in non-specific product outcome, with the targeted compound produced alongside others, hindering further use. Previous work with the structurally characterized ent-kaurene synthase from Bradyrhizobium japonicum (BjKS) identified a serine for alanine substitution (A167S) leading to premature deprotonation, yielding a pair of ent-pimaradiene double-bond isomers, with retrospective analysis by the TerDockin computational approach revealing the introduced hydroxyl acts as a catalytic base. Here this route to 'short-circuiting' the BjKS catalyzed reaction for ent-pimaradiene production was further explored, with two additional mutants (I166T and F72Y) found to also yield varying amounts of distinct ent-pimaradiene isomers in a non-specific manner. Solving the structure of BjKS:F72Y complexed with the pyrophosphate co-product and trio of divalent magnesium catalytic co-factors provided a high-resolution foundation for further engineering. Hence, TerDockin was then applied prospectively, via design-build-test cycles, enabling specific ent-pimaradiene production by addition of a single mutation. The resulting BjKS:F72Y/Y280S specifically yields the targeted ent-pimara-8(14),15-diene with reasonable catalytic efficiency, demonstrating the applicability of this approach to enzymatic engineering of terpene synthases. The application of this approach to reaction modifications beyond premature termination will further establish the effectiveness of this technique in redesigning terpene synthases. The redesign of BjKS to produce ent-rosadiene presents a good opportunity to explore such further modifications as it requires a hydride and methyl shift from the pimarane intermediate of BjKS.

75. Glutamate Decarboxylase of the human gut microbe can synthesize neuromodulatory molecules GABA, taurine, and β-alanine

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Alterations in the levels of neuromodulatory molecules and disturbances in the human gut microbiome are implicated as contributing factors in numerous neurodegenerative disorders. Despite this, the interconnection between these two crucial factors in the disease onset, progression, or exacerbation remains insufficiently understood. Conditions such as epilepsy, schizophrenia, ASD, ADHD, panic disorder, PTSD, major depressive disorder, progressive multiple sclerosis, and dementia have been linked to diminished levels of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). These observations demonstrate the need to understand the origins of fluctuations in GABA. Gut microbes of the genus *Bacteroides* are known producers of GABA. Here, we characterized the enzyme annotated as glutamate decarboxylase (GAD) from a prominent gut microbe - *Bacteroides fragilis,* and we show that *Bf*GAD is responsible for the formation of GABA from L-glutamate. Additionally, we explored the role of *Bf*GAD in the de novo formation of taurine and its derivatives. Lower taurine levels have been associated with cognitive deficits while taurine supplementation has demonstrated the ability to reverse cognitive impairment and enhance learning and memory in murine models. Taurine is a $GABA_A$ receptor agonist and exerts downstream effects similar to GABA and hence combined therapy with GABA and taurine together might have an additive effect in treating various neurological disorders. Our study demonstrates that a gut microbial glutamate decarboxylase (*Bf*GAD) can synthesize neuromodulatory molecules hypotaurine, taurine, homotaurine, and β-alanine. An engineered enzyme - *Bf*GAD_{D104N} with a single amino acid modification, produces two fold more taurine compared to the *Bf*GAD_{WT}. Additonally, directed evolution through the rational design of *Bf*GAD has yielded a range of engineered enzymes with specific characteristics, such as stringent substrate specificity towards L-glutamate (F81W), altered substrate prefernce (D104E), or increased catalytic tolerance at higher pH (T80S). Our findings suggest that fine-tuning of gut microbial glutamate decarboxylase activity could modulate levels of metabolites, offering a potential avenue for a novel therapy in various neurological disorders.

76. Phenelzine-based probes reveal Secernin-3 is involved in thermal nociception

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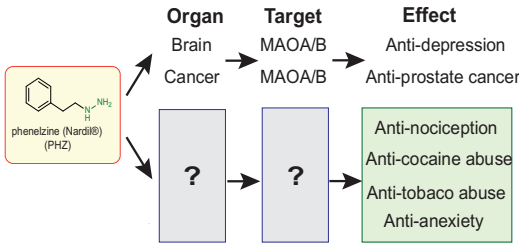
Phenelzine (PHZ, Nardil®) is an FDA-approved drug for treating depression. Since it was first introduced into psychiatry more than 70 years ago when tuberculosis patients showed 'side effects' of improved mood, its off-label use has had demonstrated efficacy in a broad range of diseases such as pain and drug abuse. PHZ's antidepressant activity has been attributed to its mechanism of action as irreversible monoamine oxidase A and B inhibitors (MAOIs), however, these versatile effects have not been explained by MAO inhibition (**Fig**).



Unknown pharmacological mechanisms of PHZ

Using our chemical proteomics discovery platform known as reverse-polarity activity-based protein profiling (RP-ABPP), a robust approach to discover enzymes, inhibitors and drugs, we demonstrated that the PHZ pharmacophore can functionally inhibit a wide range of non-MAO protein targets from diverse functional classes known to be important in neurological diseases, but were not previously known to have hydrazine-sensitive activities[1]. However, in order to evaluate the translational potential of these targets, we sought to expand our approach to be compatible in vivo.

In this work, we apply PHZ-based probes as in vivo pharmacological tools directly in animal models. We demonstrate that the probes are brain-penetrant following systemic delivery in mice and can be used to measure in vivo target engagement and map proteome- and organ-wide targets in the brain and other tissues. To illustrate how the approach can be implemented to deorphanize genes of unknown biological function, we further evaluated Secernin-3 (SCRN3), an uncharacterized metabolic enzyme and unexpected off-target of hydrazines in the brain. We show that SCRN3 expression is regulated by pro-inflammatory response pathways induced by lipopolysaccharide (LPS) in macrophages, and that SCRN3 is involved in thermal nociception in male mice[2]. The results illustrate the utility of these drug-inspired probes as in vivo pharmacological tools that direct the discovery of genetically-unpredicted protein function in neurological diseases.

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77. Experimental and Theoretical Investigation of Fungal Multi-functional Cytochrome P450 Monooxygenases

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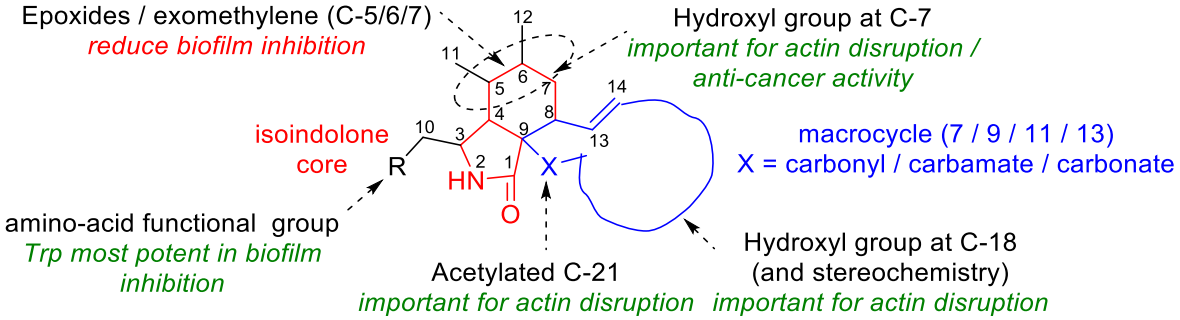
Cytochalasans are phytotoxic, cytotoxic and actin-binding natural products produced by fungi, with over 400 variants described. The reason for such structural diversity is partly explained by the flexible nature of the tailoring enzymes that introduce and modify functional groups in a site-selective manner, often on more than one substrate. The presence of certain functional groups at specific locations significantly impacts the biological activity of these molecules. Here, we have identified a diverse range of cytochalasan cytochrome P450 monooxygenase enzymes (P450s) using genome mining. We are confirming the function and scope of the P450s by heterologous expression in the fungus *Magnaporthe grisea*. In parallel we are using molecular dynamic simulations to understand the localization and conformational changes of these P450s. This project will generate fundamental knowledge on how to effectively engineer enzymes for efficient and rapid structural diversification of bioactive molecules with commercial potential.



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78. The First Transient State Analysis of Dihydroorotate Dehydrogenase Class 1B from *Lactococcus Lactis*

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Dihydroorotate dehydrogenase (DHOD) is a flavoenzyme that catalyzes the fourth step of the de novo pyrimidine base biosynthesis, the conversion of dihydroorotate to orotate. DHOD's are considered as targets for the treatment of specific diseases including rheumatoid arthritis, malaria, and cancer. There are two known families of DHODs. Members of family 1 are soluble cytosolic enzymes found in gram positive bacteria, archaea, and some lower eukaryotes while family 2 members are membrane bound from mitochondria of eukaryotes and in gram-negative bacteria. Family 1 DHODs, are divided into class 1A and 1B. X-ray crystal structures have shown that the Class 1A enzyme are homodimeric with one flavin mononucleotide (FMN) bound to each subunit (PyrDb). Class 1B is a heterotetramer consisting of two proteins (PyrDb:PyrK) with three cofactors spanning each dimer; an FMN and a flavin adenine dinucleotide (FAD) linked by one Fe_2S_2 cluster. We describe the first transient state analysis of the Class 1B DHODB from *Lactococcus lactis* (LlDHODB). During the purification of the enzyme two forms of the enzyme where resolved, the FMN dimer (PyrDb) and the native DHODB (PyrDb:PyrK). HPLC analysis confirmed the flavin cofactor occupancy. The fraction termed the FMN dimer had only a FMN cofactor while the native LlDHODB contained a 1:1 ratio of FMN and FAD. Using the native enzyme, transient state kinetics done anaerobically in the reductive half reaction with DHO shows that the forward reaction scarcely reduced the enzyme. Evaluation of the Native DHOD B reactions equilibrium position under anaerobic conditions shows that the reaction favors dihydroorotate and NAD^+ production by a factor of 10. Using NADH as the limiting reducing substrate, single turnover analysis shows four phases. In the first phase two electrons from NADH are taken up by the PyrK subunit (FAD site). In the second phase we see accumulation of a semiquinone species where one of the two electrons on the FAD populate the FMN site directly via the Fe_2S_2 cluster and the second follows resolving as one flavin reduced per PyrDb:PyrK dimer. The third and fourth phases show that that prior to reducing the pyrimidine the electrons separate to pass one at a time through the iron-sulfur center transiently forming semiquinones at either end of the protein. The rate of decay of the semiquinone species is more rapid in the presence of orotate.

79. Thioredoxin glutathione reductase (TGR): Transient State Characterization of a Complex Flavin Disulfide Reductase

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Parasitic flatworms of the genus *Schistosoma* contaminate water sources of subtropical and tropical regions and can infect mammals. Human schistosomiasis is a prevalent disease, afflicting nearly 200 million people yearly. Despite facing intense oxidative stress, schistosomes lack catalase and therefore are heavily reliant on reduced glutathione and thioredoxin to regenerate reducing forms of peroxiredoxins and ultimately ameliorate H_2O_2. While most organisms contain two separate enzymes (glutathione reductase and thioredoxin reductase) that are responsible for maintaining these reducing equivalents, schistosomes utilize a single enzyme, thioredoxin glutathione reductase (TGR). *Schistosoma mansoni* TGR (SmTGR) consists of one FAD and a chain of three cysteine-cysteine or cysteine-selenocysteine pairs per subunit. These disulfide (or selenide-sulfide) pairs act as an electron conduit, transferring electrons from NADPH through the enzyme, ultimately reducing the disulfide of one of the two substrates. For SmTGR, electrons will be passed from the flavin to the C154/C159 pair, then to the highly mobile C-terminus which contains the C596/U597 pair, and ultimately onto either the C28/C31 of the glutathione domain or thioredoxin. TGR's unique and extensive spectrophotometric reporter states are derived from the oxidation state of and proximity of reacting species with the flavin. These signals have allowed us to deduce the mechanistic details of its catalysis. These include the formation of several charge transfer absorption states and staged reoxidation of the flavin, resulting in a kinetic trace that includes five delineated events. We have utilized nontraditional transient state techniques along with 6 TGR variant enzymes to understand the catalytic and regulatory complexities of TGR. Specifically, we focused on the characterization of the U597C variant, which within our study, is representative of the wild type enzyme. We have also isolated several cysteine-to-serine variants, with the mutation being made to one of the cysteines in each of the proposed catalytic disulfide pairs, to halt the electron transfer beyond this point in the disulfide chain. Additionally, we have characterized Y296A and H571A which have been proposed to be respectively involved in the binding of NADPH and act as a base to promote electron transfer.

80. Enzymology of nicotinic acid degradation by soil *Bacillus niacini*

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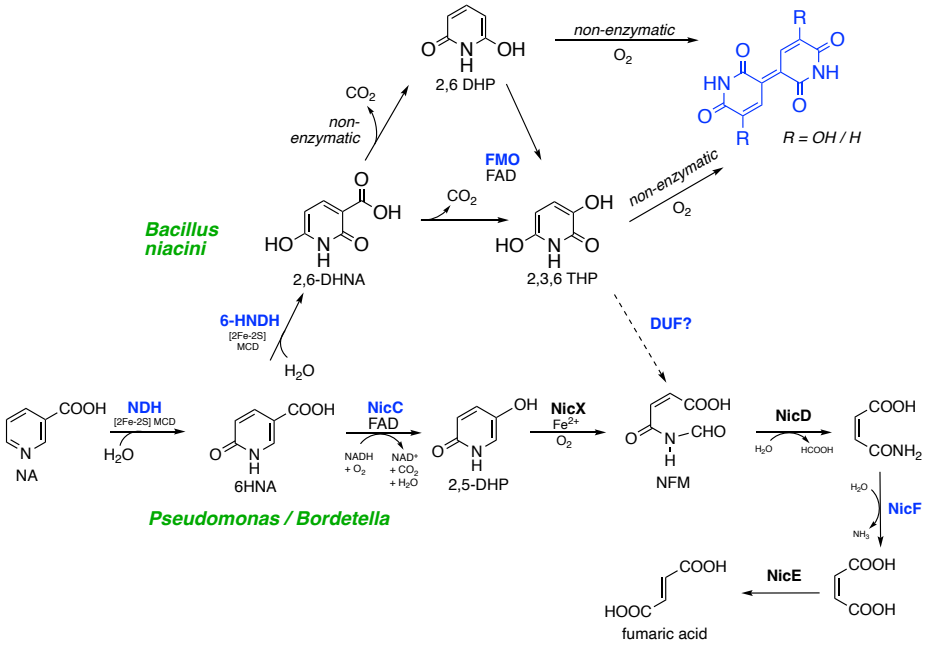
Nicotinic acid (NA) is a useful model for determining the enzymology of degrading *N*-heterocyclic aromatic compounds that have become pervasive contaminants in soil and ground water. Although the NA catabolic pathways in bacteria were determined decades ago, the recent discovery of the genes have enabled detailed structural and mechanistic studies of the recombinant enzymes, and have elucidated the pathway intermediates in *Bacillus* that remained elusive over the past 60 years. Our labs have determined the structure[1] of mechanism[2] of 6-hydroxynicotinate-3-monooxygenase (NicC), a class A flavoenzyme that catalyzes the hydroxylative-decarboxylation within the NA degradation pathway common to *Pseudomonas* and *Bordetella* species, and recently structurally and biochemically characterized a novel FMO[3] that appears to catalyze ring hydroxylation of either dihydroxypyridine (2,6-DHP) or dihydroxynicotinate (2,6-DHNA) within the NA degradation pathway in *Bacillus niacini*. In addition, we have determined the *Bacillus* genes required to reconstitute functional nicotinate dehydrogenase (NDH) and 6-hydroxynicotinate dehydrogenase (6-HNDH) complexes, homologues of xanthine dehydrogenase, that catalyze the pathway's initial ring hydroxylation steps. Current efforts are underway to elucidate the function of the final uncharaterized gene (encoding a domain of unknown function) that we hypothesize is necessary for ring-opening of 2,3,6-THP to *N*-formylmaleamate (NFM).



(Enzymes listed in blue type have been functionally characterized)

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81. Engineering hydroxylase activity, selectivity, and stability for a scalable concise synthesis of belzutifan

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Biocatalytic oxidations are an emerging technology for selective C-H bond activation. While promising for a range of selective oxidations, practical use of enzymes catalyzing aerobic hydroxylation is presently limited by their substrate scope and stability under industrially relevant conditions. Here, we report the engineering and practical application of an Fe/αKG-dependent dioxygenase for the direct stereo- and regio-selective hydroxylation of a non-native fluoroindanone *en route* to the oncology treatment belzutifan, replacing a five-step chemical synthesis with a direct enantioselective hydroxylation. Mechanistic studies indicated that formation of the desired product was limited by enzyme stability and product overoxidation, with these properties subsequently improved by directed evolution, yielding a biocatalyst capable of >15,000 total turnovers. Highlighting the industrial utility of this biocatalyst, the high-yielding, green, and efficient oxidation was demonstrated at kilogram scale for the synthesis of belzutifan.

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82. Divergent Regulatory Properties of Acetyl-CoA in Pyruvate Carboxylase

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Pyruvate carboxylase is subject to a wide range of allosteric regulation, with the degree and type of regulation differing between PC enzymes characterized from different sources. Nearly all kinetically characterized PC enzymes are allosterically activated by acetyl-CoA. However, PC from *Aspergillus nidulans* has been identified as an exception. Here, we present a detailed kinetic characterization of *A. nidulans* PC and compare it with an acetyl-CoA sensitive PC homolog from *Rhizobium etli* and *Staphylococcus aureus*. These homologous enzymes have very similar overall structures and maintain the same allosteric sites, but display remarkably different properties in response to allosteric activation by acetyl-CoA and allosteric inhibition by L-aspartate. In addition, studies on the allosteric inhibition of PC by L-aspartate revealed that the antagonistic effects of acetyl-CoA and L-aspartate on the regulation of PC activity results from their competitive binding at a partially overlapping binding site that is located at the biotin carboxylase (BC) domain dimer interface. The insensitivity to acetyl-CoA activation in *A. nidulans* PC is a function of intrinsic properties of the BC domain that is primed with high basal activity, rather than a consequence of different acetyl-CoA binding sites. We propose a new perspective of understanding the divergent regulatory properties inherited by homologous PC enzymes, which is a result of different "basal" activities that may be governed by the distribution of functional and nonfunctional conformational ensembles of the native enzyme.

83. Use of Mesophilic Prokaryotic Argonautes for Sequence Independent Scarless DNA Assembly

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Modern molecular and synthetic biology relies on the generation and manipulation of large DNA constructs ranging from kB sized genes to 100s of kB sized pathways, small genomes, and even artificial chromosomes. Current direct synthesis technologies do not allow for the direct writing of more than several hundred bases[1], many in vitro and in vivo methods have been devised to assemble shorter oligonucleotides into the final large targets. Homology directed methods (e.g., Gibson, NEBuilder) is largely sequence independent, but requires long overlaps, has issues with repetitive DNA and short fragments, can introduce mutations at fusion sites due to repeated exo/pol rewriting of the junctions, and is typically restricted to 4-6 fragments per assembly[1]. Ligation directed methods (e.g., Golden Gate assembly), have successfully been used to assembly 35 or more fragments in a single round and can assemble repetitive DNA, but typically requires that native Type IIS restriction sites be removed, requiring changes to natural DNA sequences to ensure method compatibility[2, 3].

Previous work has demonstrated the application of *Pyrococcus furiosus* argonaute (*Pf*Ago) as a programmable endonuclease and assembly tool[4, 5]. However, the enzyme is thermophilic (only functions at elevated temperatures above 85^{o}C) and not quite versatile in terms of reaction conditions. To address these limitations, we present progress towards the use of Clostridium butyricum argonaute (*Cb*Ago)[6, 7] as a mesophilic, versatile, programmable, DNA-guided assembly tool. We demonstrate that *Cb*Ago can be activated in vitro via the DNA strand unwinding activity of a nuclease-deficient mutant of RecBC DNA helicase from *Escherichia coli* (RecBexo^{-}C) and efficiently cleave linear double-stranded DNA[8]. We furthermore show that *Cb*Ago can generate arbitrary overhangs of virtually any length or sequence, which can be ligated with compatible overhangs to assemble up to five sequences efficiently and accurately. Our goal is to develop a platform based on *Cb*Ago-RecBexo^{-}C that enables rapid scarless construction of large DNA molecules from a diverse set of DNA components with varying lengths under mild temperature and buffer conditions without any need for Golden Gate-like sequence modification.

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84. Mutational and Conformational Analyses of Folate Binding and Catalysis in *E. coli* methylenetetrahydrofolate reductase (MTHFR)

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Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to 5-methyltetrahydrofolate (CH_3-H_4folate) utilizing NAD(P)H as a second substrate and enzyme-bound flavin adenine nucleotide (FAD) as redox coenzyme. The reaction is the sole source of CH_3-H_4folate, which is used in the synthesis of methionine. In humans, mutations in MTHFR have been correlated with elevated levels of homocysteine, a risk factor for cardiovascular disease, and with neural-tube defects in the fetus. We study MTHFR from *Escherichia coli* as a model for the catalytic domain of the human enzyme. Because the reaction has a ping pong kinetic mechanism, it can be divided into two half-reactions: a reductive half-reaction where NADH reduces the FAD and an oxidative half-reaction where CH_2-H_4folate returns the FAD to its oxidized form. In this talk, the focus will be on the oxidative half-reaction, where CH_2-H_4folate is proposed to accept a proton at the N10 position to open up the imidazolidine ring and form a 5-iminium cation intermediate, which then undergoes reduction by the reduced FAD. The X-ray crystal structure of the E_{ox}•CH_3-H_4folate complex[1] has revealed three active-site amino acid residues - Glu 28, Asp 120, and Phe 223 – with potential roles in folate substrate binding and catalysis in the oxidative half-reaction. To investigate these residues, we have prepared mutant enzymes containing amino acid changes at the site of interest and then measured the kinetic properties of the mutants compared to those of the wild-type enzyme. We have also performed hydrogen-deuterium exchange mass spectrometry (HDX-MS) to investigate the conformational changes upon CH_3-H_4folate product binding to the wild-type and select mutant enzymes. We hypothesized that Glu28, located next to a structural water molecule near N10 of the folate, could serve as the general acid catalyst to aid 5-iminium cation formation. Consistent with this role, mutant enzymes Glu28Gln and Glu28Asp are able to bind folate, but are completely inactive in catalysis. Differences in HDX between free and CH_3-H_4folate-bound Glu28Gln, Glu28Asp, and Glu28Gln/Phe223Leu enzymes allow us to determine how folate binding affects the local conformation. Among 13 reference peptides identified, residues near the folate hydrophobic binding pocket as well as near the protein-protein binding surface show the most changes in HDX, consistent with the proposed roles of Asp120 and Phe223 in facilitating folate substrate binding.

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85. Peering into the Donor Binding Site of Phosphoglycosyl Transferases Belonging to the "*Stealth Family*"

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The bacterial capsule protects bacteria from hosts immune system and is an important virulence factor. It is composed of a variety of monosaccharides joined through glycosidic or phosphodiester linkages. Synthesis of a capsule through formation of these chemical bonds is catalyzed by enzymes called glycosyl and phosphoglycosyl transferases, respectively. While glycosyl transferases (GTs) are relatively well researched, mechanistic, and structural information is lacking for phosphoglycosyl transferases (PGTs). Many of the bacterial PGTs render the organism invisible to host's immune system, they are therefore grouped into a family named "Stealth". Studying the mechanism and active site interactions of PGTs from stealth family can potentially translate into new vaccine production technologies and antibacterial treatments. CsxA from *Neisseria meningitidis* Serogroup X (NmX) is one such PGT, that catalyzes the synthesis of α(1→4)-linked N-acetylglucosamine (GlcNAc)-1-phosphate homopolymer using UDP GlcNAc as the donor substrate. Among the 6 disease-causing serogroups of *Neisseria meningitidis,* there is currently no vaccine against only NmX. This coupled with the recent outbreaks of meningococcal disease in Sub-Saharan Africa due to NmX, makes CsxA an important research target. We used equilibrium dissociation constants (Kd's) of sugar and base modified UDP GlcNAc analogs, measured using microscale thermophoresis (MST) to understand enzyme substrate interactions at the active site of CsxA. Using C-2 and C-4 modified UDP GlcNAc analogs, we were able to conclude that the presence of an acyl group at C-2 and a hydrogen bond donating hydroxyl group at C-4 are important for donor substrate binding. In addition to its role as an acceptor in capsular polysaccharide chain elongation, the C-4 hydroxyl group is important for donor substrate binding and recognition. By analyzing the Kd's from C-5' halogen substituted donor analogs, we hypothesize that electron density of uracil ring is important for donor substrate binding. We also identified a highly conserved aromatic side chain using mutational studies and docking experiments on Alpha-fold derived structure of CsxA. Although this aromatic side chain does not directly interact with the uracil group of the donor substrate, we noticed that it stabilizes a flexible region of the protein that interacts with the uracil group through hydrogen bond interactions. Additionally, substitution of a bulkier 5-formylthien-2-yl group at C-5' position resulted in an inhibitor that binds 3-fold tighter to CsxA than its natural substrate. Considering the lack of structural data for the stealth family of PGTs, this information adds to our understanding of how these enzymes catalyze the synthesis of capsules of virulent gram-negative bacteria.

86. Engineered Biocatalytic Synthesis of β-*N*-Substituted-α-Amino Acids

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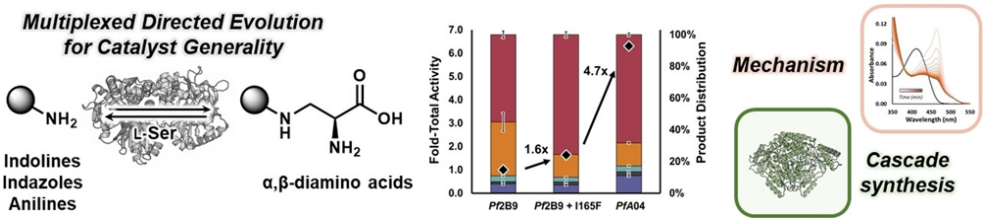
Non-canonical amino acids (ncAAs) are useful tool compounds for studying biological phenomena and are components in many pharmaceuticals. Altering the side chain structure of α-amino acids can impart new properties on compounds as diverse as small molecule catalysis and peptide therapeutics. In particular, bioactive molecules featuring β-*N*-substitution include intermediates in biosynthesis, antibiotic analogs of azafuranomycin and Aspergillomarasmine A, and protein kinase inhibitors. Traditional synthetic methods typically rely on protecting group manipulations to attenuate undesired reactions with the amine and carboxylic acid functionalities and must assiduously avoid epimerization of the chiral center. Recently[1-4], enzyme engineering has been leveraged to produce a suite of highly active enzymes for the synthesis of β-substituted amino acids. However, there are few examples of biocatalytic *N*-substitution reactions to make α,β-diamino acids. In this study[5], we used directed evolution to engineer the β-subunit of tryptophan synthase from *Pyrococcus furiosus*, *Pf*TrpB, for improved activity with diverse amine nucleophiles. These *N*-nucleophiles were previously used as inhibitors of TrpB enzymes from other organisms such as *Salmonella typhimurium*. Mechanistic analysis shows that high yields are hindered by product re-entry into the catalytic cycle and subsequent decomposition. Additional equivalents of L-serine can inhibit product reentry through kinetic competition, facilitating preparative scale synthesis. We show β-substitution with a dozen aryl amine nucleophiles, including a demonstration on a gram scale. Preparative scale reactions were often driven by the selective precipitation of products, which enabled the gram-scale synthesis of 2,3-dihydroiso-L-tryptophan. This reaction adds to a growing repertoire of *N*-alkylation reactions in biocatalysis, which operate independently to access desirable products and enable transformations for complex cascade catalysis.



The β-subunit of tryptophan synthase was engineered for efficient *N*-alkylation to access densely functionalized non-canonical amino acids. Mechanistic analysis guided preparative scale synthesis, adding a valuable new enzyme to the biocatalytic toolbox.

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87. Bioinformatics of Selenocysteine-Containing Ribosomally Synthesized and Post-Translationally Modified Peptide (RiPP) Natural Products

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Ribosomally synthesized and post-translationally modified peptide (RiPP) natural products (NPs) are a diverse class of molecules produced by microbes. The peptides are encoded by short open reading frames that colocalize with genes encoding modification enzymes (e.g., RiPP maturases) that post-translationally form the mature NPs. Previously, several bacterial RiPPs were proposed to contain selenocysteine (Sec) residues. Our laboratory has recently reported on a unique reaction catalyzed by a radical *S*-adenosyl-*L*-methionine (rSAM) RiPP maturase, SbtM, on the associated Sec-containing peptide, SbtMa. Here, we predict three more Sec residues in the biologically-relevant form of SbtMa, an additional paralog peptide, SbtMb, and provide biochemical evidence for the processing of SbtMb. Additionally, we investigate the diversity in the Sec-containing family of RiPPs using the associated homologs of SbtM to aid in peptide identification. The presence of Sec residues was predicted using IGV-Scanfold. Using our pipeline we identified over 70 related Sec-containing peptides and, when possible, updated the number of annotated Sec residues in the RiPPs found in the TIGR04081.4 protein family. We identified strongly conserved amino acid motifs flanking the site of Sec incorporation and have begun to establish a consensus model for the Sec-incorporating mRNA hairpins in these systems. Together, these findings will expedite the identification of additional Sec-containing RiPPs from this NP family and potentially provide a framework for *de* novo identification of other Sec-containing RiPPs.

88. An Adenylosuccinate Lyase in Antibiotic Biosynthesis and Biocatalysis

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The aspartase/fumarase superfamily of enzymes catalyze C-N or C-O bond cleavage from succinate containing compounds, releasing fumarate as the common product.[1] Adenylosuccinate lyases are members of this superfamily that typically catalyze C-N bond cleavage of adenylosuccinate leading to formation of adenosine monophosphate (AMP) in purine biosynthesis.[2] Our lab identified FlcB, an adenylosuccinate lyase in the biosynthesis of a copper-containing antibiotic, fluopsin C, from the bacterium *Pseudomonas aeruginosa*. In the first step of fluopsin C biosynthesis, FlcB catalyzes the formation of a C-S bond between cysteine and fumarate, generating an (*R*)-stereocenter.[3] The enantioselectivity of FlcB differs from other adenylosuccinate lyases and therefore makes it an intriguing subject for future study. We showed that FlcB is permissive toward various thiol- and amine-containing substrates. Kinetic parameters of FlcB were measured and essential catalytic residues were identified. Together, these findings shed light on the mechanism of FlcB. The enantioselective C-S bond formation catalyzed by FlcB has potential applications in biocatalysis.

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89. Diversifying Glycoconjugate Profiles: Substrate Specificity of PglJ Glycosyltransferase in *Campylobacter concisus*

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Members of the *Campylobacter* genus utilize the pgl biosynthetic pathway to produce N-linked glycoconjugates, essential for bacterial pathogenicity. The glycoconjugates produced by *Campylobacter* members vary significantly due to differing substrate specificities of glycosyltransferases within the pathway, namely PglA, PglJ, and PglH. In *C. concisus*, an emerging human pathogen, literature reports indicate intra-strain variability in PglJ substrate specificity between strains 13826 and 33237. Previous findings suggested that *C. concisus* 13826 PglJ reacted with UDP-GalNAc, while the 33237 PglJ reacted with a monosaccharide identified as a 217 m/z fragment in mass spectrometry glycan analyses, annotated as N-acetylhexuronic acid (HexNAcA).

Our study characterized the PglJ glycosyltransferases from both *C. concisus* strains. We established that both 13826 and ATCC 33237 strains utilize UDP-GalNAcA as their substrate. This determination was made via binding studies using nano differential scanning fluorimetry (nanoDSF) and enzyme activity assays, confirming the preference for UDP-GalNAcA over its epimer UDP-GlcNAcA. Sequence similarity network analysis of PglJ homologs across the *Campylobacter* genus enabled us to identify motifs associated with substrate preferences between HexNAcA and HexNAc. PglJs annotated to react with HexNAcA possessed the SER motif in their active site, while those reacting with HexNAc featured the NEC motif. This correlation was experimentally validated by engineering a variant of the PglJ from the 33237 strain where the SER motif was replaced with NEC. This variant demonstrated expanded substrate range, allowing reaction with HexNAc, thereby supporting our hypothesis regarding the role of these motifs in determining substrate specificity. Furthermore, we characterized a dehydrogenase within the *C. concisus* pgl operon, analogous to *P. aeruginosa* WbpO, responsible for the oxidation of UDP-GalNAc to UDP-GalNAcA. The co-occurrence of HexNAcA PglJs and dehydrogenases within the pgl operon of *Campylobacter* species was analyzed by genome neighborhood analyses. A consistent pattern of co-localization of dehydrogenases with *Campylobacter* PglJs preferring HexNAcA was found, while those predicted to utilize HexNAc substrates lacked a dehydrogenase within the operon.

Our study underscores the structural underpinnings allowing the diversity of glycoconjugates produced in these biosynthetic pathways. It also sheds light on the evolutionary trajectory of these enzymes, suggesting a shift from a more promiscuous to a more specific state.

90. Direct detection of the central α-carbon radical intermediate in OspD: Mechanistic insight into radical SAM peptide epimerization

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OspD is a radical *S*-adenosyl-L-methionine (SAM) peptide epimerase that converts isoleucine-4 (Ile4) and valine-13 (Val13) of the precursor protein OspA to D-amino acids during biosynthesis of the antiviral natural product landornamide A. OspD has been pro-posed to carry out this reaction via Cα H-atom abstraction to form a peptidyl Cα radical that is stereospecifically quenched by a conserved cysteine to provide inversion of configuration. Here we use site-directed mutagenesis together with freeze quench trapping, isotopic labeling, and electron paramagnetic resonance (EPR) spectroscopy to provide new insights into the mechanism of OspD catalysis, including the direct observation of the substrate peptide Cα radical. The putative quenching cysteine is changed to serine to generate the OspD C334S variant, which retains the radical SAM [4Fe-4S] cluster. OspD C334S is reacted with SAM and substrate OspA for 15 s prior to freeze-quenching, providing a doublet EPR signal with g-value and hyperfine coupling characteristic of a Cα radical coupled to a single β-H. Use of OspA in which both Ile4 and Val13 are deuterated provides a singlet EPR signal, identifying the radical as residing on one of these amino acid residues. When carried out with OspA deuterated at either Ile4 or Val13, only the deuterated Ile4 OspA gives rise to a singlet EPR signal, demonstrating that the initial radical intermediate forms exclusively in Ile4 of OspA. Longer quench times provide evidence for progression of the radical species from its initial site at Ile4 to the subsequent site at Val13, providing evidence for the N-to-C progression of epimerization by OspD.

91. Human serine protease FAM111A is inhibited by the viral serpin SPI-1

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Proteases are essential enzymes required in many fundamental cellular processes. The human serine protease FAM111A (FAM111 trypsin-like peptidase A) was recently found to be important in two different processes: DNA replication[1] and antiviral protection[2,3]. FAM111A localizes to the DNA replication fork and is important for promoting DNA replication at DNA-protein crosslinks (DPCs)[1]. On the contrary, FAM111A is also an antiviral factor in host range mutant viruses of simian virus 40 (SV40) and orthopoxviruses. Within orthopoxviruses, serine protease inhibitor 1 (SPI-1), a member of the serpin family of protease inhibitors, is important for determining the host range. However, the host cell protease targeted by SPI-1 remains unknown. A previous study reported that knock down of the host cell serine protease FAM111A allows replication of a host range mutant virus lacking the SPI-1 gene[3]. It was hypothesized that SPI-1 inhibits FAM111A's protease activity, however this is yet to be demonstrated. Here, we show that FAM111A is directly inhibited by SPI-1 *in vitro*. Our biochemical study suggests that SPI-1 forms a covalent complex with FAM111A in an active-site-dependent manner. This demonstrates that SPI-1 inhibits FAM111A through the irreversible covalent bond formation, which is characteristic to serpin inhibition. Altogether, our findings identify SPI-1 as the first protease inhibitor of FAM111A and suggest that SPI-1 blocks FAM111A's ability to function as an antiviral protein.

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92. A New Biosynthetic Paradigm for NRPS Peptide Branching Through Ester and Amide Linkages on Serine

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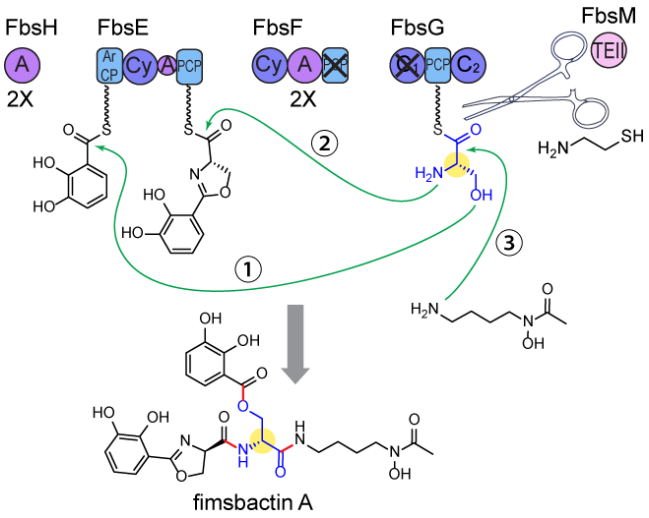

Branch-point syntheses in nonribosomal peptide assembly are rare but useful strategies to generate tripodal peptides with advantageous hexadentate iron-chelating capabilities, as observed in siderophore peptide scaffolds.[1] However, the chemical logic underlying the peptide branching by nonribosomal peptide synthetase (NRPS) often remain complex and elusive. Here, we report our biochemical investigation on the assembly mechanism of fimsbactin A, a branched mixed-liganded siderophore produced by human pathogenic strain *Acinetobacter baumannii*.[2] Our findings reveal the dynamic interplay between L-Serine and 2,3-dihydroxybenzoic acid-derived dipeptides, which can undergo partitioning between amide, oxazoline, and ester forms, ultimately leading to the formation of branched tetrapeptides through sequential condensation reactions between distinct NRPS modules.[3] Furthermore, we demonstrate the pivotal role played by a terminal condensation domain, FbsG, in mediating the release of the peptidyl intermediate by recruiting the soluble nucleophile *N*-acetyl-*N*-hydroxy-putrescine.[4] This study contributes to our understanding of the intricate biosynthetic pathways and chemical logic employed by NRPSs, shedding light on the mechanisms underlying the synthesis of complex branched peptides.

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93. Investigating Catalysis and Substrate Channeling in an Engineered Linkerless Construct of the Bifunctional Terpene Synthase PaFS

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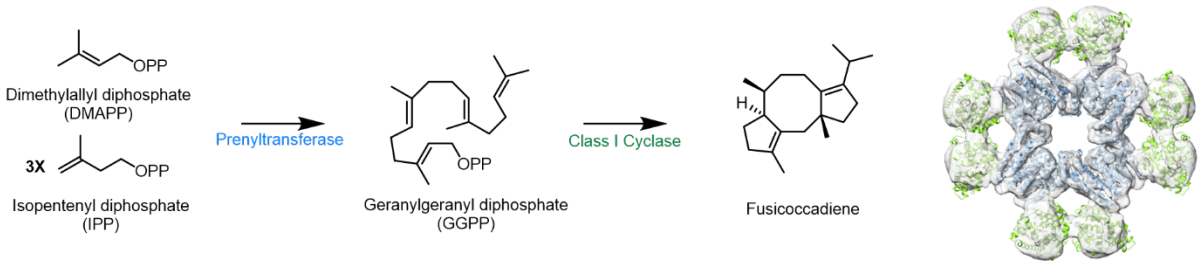
Fusicoccadiene synthase from *P. amygdala* (PaFS) is a bifunctional assembly-line terpene synthase containing a prenyltransferase domain that generates GGPP from DMAPP and three equivalents of IPP, and a cyclase domain that converts GGPP into fusicoccadiene, a precursor of the diterpene glycoside Fusicoccin A.[1] A disordered 69-residue polypeptide linker connects the two catalytic domains. The prenyltransferase domain mediates oligomerization to form hexamers or octamers, and cyclase domains are randomly splayed out around the prenyltransferase core.[2] Previous studies suggest that cluster channeling is operative in catalysis, since most of the GGPP formed by the prenyltransferase remains on the protein for the cyclization reaction.[3] Here, we characterize an engineered construct in which the 69-residue linker is spliced out and replaced with a short 3-residue linker. Structural characterization of this construct using cryo-electron microscopy reveals that cyclase dimers associate with all four sides of the prenyltransferase octamer. This construct cyclizes GGPP and exhibits substrate channeling with efficiency comparable to that observed for the wild-type enzyme. Taken together, these results suggest that optimal substrate channeling is achieved when the cyclase domain associates with the side of the octamer regardless of whether the two domains are covalently linked and regardless of whether this interaction is transient or locked in place.



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94. Characterization of the first Radical SAM Oxygenase for the Ether Crosslinking in Darobactin Biosynthesis

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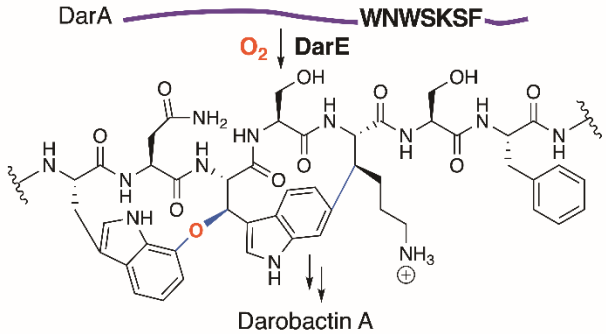
Darobactin A is a ribosomally synthesized, post-translationally modified peptide (RiPP) with potent and broad-spectrum anti-Gram-negative antibiotic activity[1]. The structure of darobactin A is characterized by an ether and C-C crosslinking. However, the specific mechanism of the crosslink formation, especially the ether crosslink, remains elusive. Here, using *in vitro* enzyme assays, we demonstrate that both crosslinks are formed by the DarE radical SAM enzyme in an O_2-dependent manner[2]. The relevance of the observed activity to darobactin A biosynthesis was demonstrated by the proteolytic transformation of the DarE product into darobactin A. Furthermore, DarE assays in the presence of $^{18}O_2$ or [^{18}O]water demonstrated that the oxygen of the ether crosslink originates from O_2 and not from water. These results demonstrate that DarE is a radical SAM enzyme that uses oxygen as a co-substrate in its physiologically relevant function. Since radical SAM enzymes are generally considered to function under anaerobic environments, the discovery of a radical SAM oxygenase represents a significant change in the paradigm and suggests that these radical SAM enzymes function in aerobic cells. Also, the study revealed that DarE catalyzes the formation of three distinct modifications on DarA; ether and C-C crosslinks and α,β-desaturation. Based on these observations, we will discuss the possible mechanisms of the DarE-catalyzed reactions.



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95. Insights into the Catalytic Mechanism and Substrate Specificity of Uracil DNA Glycosylase

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Uracil DNA glycosylase (UDG) is a pivotal enzyme in DNA repair pathways, responsible for excising uracil residues that arise from the deamination of cytosine or misincorporation of dUMP during DNA replication. UDG recognizes and excises uracil bases from DNA during base excision repair via "pinch-pull-push" mechanism[1]. Given that significant conformational changes occur in DNA during the UDG mechanism, DNA flexibility likely affects the efficiency of uracil excision by UDG. In support of this, Orndoff *et al.* showed that the efficiency of excision of uracil in steady-state reactions varied with DNA flexibility. The bases immediately adjacent to uracil are interconnected in an allosteric manner, exerting the most significant effect on DNA rigidity and UDG activity[2].

To determine why steady-state kinetics of uracil excision are influenced by neighboring bases, we explored pre-steady state kinetics of individual reaction steps. By employing a stopped-flow fluorometer, the adenine analog, 2-aminopurine base paired with uracil was used as a reporter to measure DNA binding and base excision by UDG. Rapid mixing-rapid quench experiments were used to measure pre-steady-state kinetics of uracil excision on the same substrates. We aim to capture transient intermediates during UDG catalysis, offering unprecedented insights into the early stages of substrate binding, conformational changes, and product release in different DNA sequence contexts to determine how DNA flexibility contributes to excision efficiency. Our proposed study aims to advance understanding of UDG dynamics, providing a comprehensive view of the rapid enzymatic events. The findings are anticipated to not only contribute to the field of UDG catalysis but also offer valuable insights with broad implications for the understanding of mutation hotspot genesis, molecular evolution, and the emerging field of base editing.

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96. Peptide extension after chain reversal during the biosynthesis of nonribosomal peptides

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Nonribosomal peptides are peptidic natural products with a broad range of biological activities, including antibiotic, anticancer, and immunosuppressant. Their peptide scaffold is biosynthesized by non-ribosomal peptide synthetases (NRPS), usually unidirectional, by loading amino acids onto the peptidyl carrier protein as activated thioester and subsequent peptide bond formation[1]. In some cases, the NRPS generates a ureido moiety at the chain initiation position, leaving an unactivated carboxylate group that cannot be further extended by the NRPS[2]. We discovered that an amino acid ligase extends the peptide chain at this inert carboxylate. The amino acid ligase is a homolog of peptide aminoacyl-tRNA ligases (PEARLs), enzymes that catalyze peptide bond formation using ATP and aminoacyl-tRNA[3-5]. The finding expands the chemical repertoire of peptide bonds formation during nonribosomal peptide biosynthesis.

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97. Multi-step enzyme cascade to produce Vitamin B6 from renewable source

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Vitamin B6 is a fundamental nutrient involved in more bodily processes than any other vitamin. However, humans cannot synthesize it, so it must be obtained from foods or isolated supplements. Currently, vitamin B6 is produced through chemical or fermentative processes. Chemical synthesis employs expensive and hazardous resources. Biological routes that are environmentally correct still face producing low titers due to the toxic features of their intermediates. With this regard, the synthetic biochemistry approach is a potential way to provide biobased- vitamin B6. Therefore, this project proposes a cell-free process based on a 6-enzyme cascade to generate vitamin B6 using xylose as a substrate. The thermodynamic analysis demonstrated the cascade's feasibility, providing the initial conditions to test in vitro, and all the enzymes of the cascade were biochemically characterized. Limiting aspects were identified concerning the low thermal stability of the phosphoketolase alongside the low activity of the PLP synthase. The rational prospection of novel targets was performed using the sequence similarity network (SSN) tool and genome mining. This way, the final phosphoketolase and synthase presented melting temperatures 35 and 13 °C higher, and their activities improved by 21 and 2-fold, respectively. Throughout the development, the process elevated from 0.42 to 0.91 mM of vitamin B6 in a synthetic medium. Encouraged by these results, we tested and optimized the enzyme cascade in realistic substrates. As a result of the combined PB analysis, the process achieved similar titers of Vitamin B6 using sugarcane bagasse hydrolysate. Ultimately, the obtained productivity was higher than those reported by the state-of-the-art bio-based technology (0.073 *vs* 0.031 mmol L^{-1} h^{-1}).

98. Mechanism, Specificity and Biology of Archaeal Endonuclease V

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Hydrolytic deamination of the exocyclic amine on adenosine lead to the formation of hypoxanthine (i.e. inosine/deoxyinosine). Deamination can happen spontaneously, and results in altered base pairing preference[1]. For example, in the context of DNA, deamination of dA to dI leads to dA to dG point mutations[2]. Bacterial Endonuclease V is a well-characterized DNA repair protein which cleaves dI in duplex DNA and initiates the alternative excision repair pathway to remove and replace dI with canonical dA[3]. Endonuclease V contains a signature "D-E-D-D" RNaseH-like active site that is found in many DNA/RNA cleavage enzymes like RNases, Dnases, and argonauts[4,5]. In this work, we carry out *in vitro* biochemical and *in vivo* genetic experiments of archaeal Endonuclease V. Interestingly, biochemical kinetic cleavage assays reveal archaeal Endonuclease V has a strong preference for cleaving inosine in the context of RNA, even though the active site is identical to its bacterial Endonuclease V counterpart. Site-directed mutagenesis experiments are utilized to understand the residues responsible for RNA versus DNA substrate preference between archaeal and bacterial Endonuclease V, respectively. Finally, we genetically knocked out Endonuclease V from a hyperthermophilic archaeal species to understand the biological role of archaeal Endonuclease V in regulating cellular levels of rI in RNA. This work suggests the presence of RNA maintenance pathways present in archaeal organisms, and further broadens our understanding of divergent enzyme evolution that results in altered substrate preference of enzyme homologs.

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99. Elucidating how enoylreductase domains collaborate with downstream acyl carrier protein and ketosynthase domains within polyketide synthase modules

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Our lab is learning how to engineer modular polyketide synthases (PKSs) to produce stereochemically-dense polyketides. Compared to other domains that exert stereocontrol during polyketide biosynthesis, such as ketoreductases (KRs), enoylreductases (ER) are the least understood. ER domains are hypothesized to only share interfaces with two other domains in the module, through a small interface with the KR into which it is inserts and a transient interaction with the acyl carrier protein (ACP) domain that presents the polyketide intermediate to it for reduction. Despite recent advances in structural biology, the interface between ER and ACP remains unknown. Here, we present 50 ACP:ER docking AlphaFold2 solutions that indicate the high conservation of this interface and predict key residues in it. **P1-P5-P6-P7**, a model tetraketide lactone synthase comprised of the 1st, 5th, 6th, and 7th updated modules of the pikromycin synthase, was employed to test this interface in vivo. The decreases in activity observed for 20 **P1-P5-P6-P7** variants with mutations to PikER5 surface residues are consistent with the proposed interface. The conserved nature of the interface suggests ACPs can interface with ERs independent of the chemistry they perform and of the module or PKS to which they belong. Subsequent domain swapping studies using short, model PKSs constructed from the updated modules of the pikromycin synthase show the results of inserting ERs and cognate ketosynthases (KSs) proposed to gatekeep for the chemistries performed by the ERs into modules that only contain KR and DH. These results further our understanding of ER domains and how they can be employed to rationally engineer PKSs that produce stereochemically-dense, designer polyketides.

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100. Mechanistic manifold in a hemoprotein-catalyzed cyclopropanation reaction with diazoketone

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Engineered heme proteins have been successfully developed to effect various carbene transfer reactions. One recent experimental development is to use engineered myoglobin to catalyze cyclopropanation with diazoketone with excellent stereoselectivity (>99% de and ee).[1] Interestingly, our experimental collaborator's work revealed the existence of a complex mechanistic manifold, wherein the cyclopropanation pathway competes with alternative formation of an N-bound carbene adduct of the protein heme cofactor. This species is able to regenerate the active biocatalyst, thus constituting a non-productive, yet non-destructive detour from the main catalytic cycle.[2] However, mechanistic details of different alternative pathways have not been computationally studied before. Based on our recent computational work to reveal numerous reaction mechanisms of heme carbene cyclopropanations,[3-5] we investigated and compared the productive and non-productive heme carbene formation pathways via different kinds of coordination atoms (C,N,O) in the diazo precursors to determine the most favorable one. The C-coordinated productive pathway was found to have the lowest energy barriers. The study of both heme carbene formation step and cyclopropanation step shows a rate-determining step that is consistent with experiment. In addition, we identified an interesting novel mechanism for the facile formation of the N-alkylated species, which was found to be recycled back for catalytic cyclopropanations. The computed structural and Mössbauer parameters are in excellent agreement with experiments. These significant novel results shed light into the comprehensive mechanistic scenarios of heme carbene transfer reactions to facilitate future efforts toward sustainable carbene transfer catalysis using these systems.

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101. Ancestral Evolution of Oxidase Activity in a Class of Nicotine Degrading Flavoenzymes

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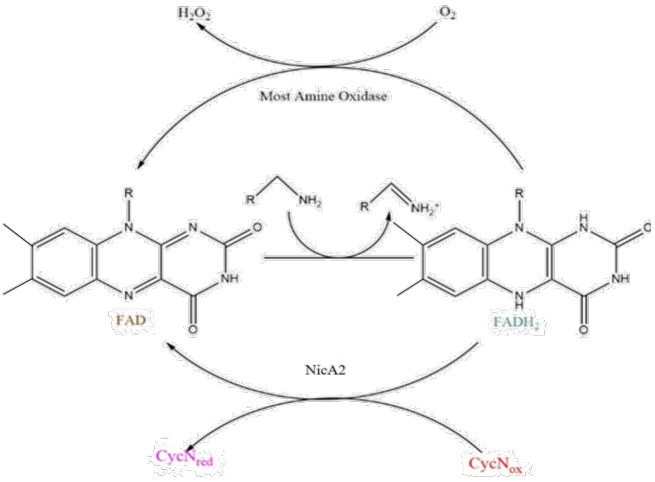

Flavoprotein amine oxidases (FAOs) are a group of enzymes that form a superfamily and catalyze a wide range of reactions that are important for living organisms. Although it's been accepted for years that molecular oxygen is the preferred electron acceptor for FAOs, our lab has discovered that nicotine oxidoreductase (NicA2), which phylogenetically belongs to the FAO superfamily, reacts poorly with oxygen, and prefers a kind of cytochrome C instead. Because of the odd oxidation reagent selection of NicA, we would like to investigate what is the molecular basis of this divergence. Through vertical comparison, obtained kinetic data suggested that in the NicA2-NctB-NdpX clade, oxidase reactivity was reemerged from a group of dehydrogenases. We successfully located the potential key residues by structurally and evolutionarily comparing the ancestral proteins resurrected. In the future, we are going to evolve one of our ancestral proteins for better oxidase activity.

102. Structural and mechanistic exploration of a thermostable Cystathionine γ-lyase reveals a new catalytic function

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We recently discovered a thermostable Cystathionine γ-lyase (CGL) from the archaeon, *Thermobifida fusca* (*Thfu*CGL) that operates on an unusually broad array of amino acid substrates. Here, we study the mechanism of this enzyme and the basis of its promiscuity. We show how the rate-limiting step of the γ-lyase mechanism depends on the pKa of the conjugate acid of the leaving group of a γ-substituted amino acid. We solved high-resolution X-ray crystal structures, including capture of a reactive intermediate. Lastly, we probed the role of the catalytic base in the reaction. To our surprise, mutation of Tyr to His had minimal impact and mutation to Phe yielded a slow enzyme that had new catalytic function. Together, these data answer unresolved questions about γ-lyase function and set the stage for future protein engineering campaigns to tune the catalytic specificity of these enzymes.

103. Mixed-linkage β-glucan utilization locus from herbivore gut microbiota has a gain of function towards substituted β-1,3-glucans

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Gut microbiota plays a pivotal role in the health and metabolism of mammals, having complex dietary glycans as major nutrients to thrive in this diverse ecosystem.[1] To cope with the sheer complexity of dietary glycans, the gut microbiota exploits elaborate multi-component systems comprising carbohydrate-active enzymes (CAZymes), recognition proteins and membrane transporters that are organized as polysaccharide utilization loci (PULs).[2] Most of these PULs have been elucidated from the human gut microbiota (HGM), remaining a knowledge gap regarding the functional conservation and/or adaptations of these systems from other ecological niches such as herbivores. Here, we have biochemically and structurally characterized all the encoded enzymes by a PUL identified in a metagenome-assembled genome from an herbivore, which resembles to that previously characterized from the human gut microbiota, revealing a gain of function towards substituted β(1,3)-glucans, a typical carbohydrate found in the diet of most herbivores. In the herbivore PUL, distinct types of β-glucans are first cleaved into oligosaccharides by an endo-glucanase with broad specificity and high catalytic rates. Then, the oligosaccharides produced are imported by TBDT SusC-like proteins into the periplasm, where two GH3 glucosidases with different selectivity act in concert to complete the hydrolysis. In contrast, the HGM PUL lacks a second functional glucosidase that enables the cleavage β(1,6)-glucosyl substitutions and the endo-glucanase is not active on β(1,3)-glucans, which restricts the function of this PUL from HGM over mixed-linked β-glucans, a remarkable functional differentiation between these two PULs from human and herbivores. These findings advance our knowledge regarding the current model for non-cellulosic β-glucans utilization by gut microbiota in mammals, highlighting the diverse molecular mechanisms exploited by commensal gut bacteria to cope with dietary glycans.

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104. Development of a Series of Tetrazole Derivatives as Kynurenine 3monooxygenase (KMO) Inhibitors and Structure-activity Relationship (SAR)

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Kynurenine monooxygenase (KMO) is an essential enzyme for tryptophan degradation through the kynurenine pathway (KP). KMO catalyzes the conversion of L-kynurenine (L-kyn) to a neurotoxic metabolite, 3-hydroxykynurenine (3-HK) which is eventually converted to quinolinic acid (QUIN), a known N-Methyl-D-Aspartate (NMDA) receptor agonist. Both the above metabolites are associated with progression of several neurodegenerative diseases, such as Alzheimer's and Huntington's diseases. On the other hand, L-kyn is converted by kynurenine aminotransferase (KAT) to kynurenic acid (KYNA), a known NMDA receptor antagonist. Therefore, inhibiting KMO is thought to not only eradicate the above detrimental metabolites, but also to shift the pathway to a more neuroprotective metabolite KYNA. Here, we developed a series of tetrazole derivatives as potential KMO inhibitors and provide a new sight into the structure-activity relationship (SAR). Tetrazole is a well-known isostere of carboxylic acid. Our preliminary data shows that some tetrazole derivatives have remarkable Ki values for both Cytophaga hutchinsonii KMO (ChKMO) and P. fluorescens KMO (PfKMO). In these tetrazole derivatives, Structure-activity relationship (SAR) shows that 1-(3,4dichlorophenyl)-2-(1H-tetrazol-5-yl) ethan-1-one has Ki of 1.35 μM for chKMO and 3.96μM for pfKMO. Our crystallography data also suggests that the 1-(4-cyclohexylphenyl)-2-(1Htetrazol-5-yl)ethan-1-one has similar ligand protein interactions with the substrate. Developments of potent tetrazole derivatives and studies of SAR are our primary goal. The developments are based on three modifications: aromatics, ring and tetrazole modifications. The ongoing study will focus on looking at ligand protein interactions. Our future goal is to further narrow down from the tetrazole derivatives to lead compounds that will act as relevant inhibitors of KMO and provide promising therapeutic potential as drugs for treating neurodegenerative diseases.

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The EMC Catering Menu

Wednesday, January 3

6:00-8:00 pm

Opening Reception - Mangrove Pool

Pizza Napoli – Margherita, Pepperoni or Four Cheese Pizza, Seasonal Flatbread
Pasta – Pennetti with Vodka Sauce, Cheese Ravoli with Herbed Chicken and Butter Sauce, Gnocchi with Sun Dried Tomato, English Pea, Bacon, Parmesan, Spinach, EVOO
Taqueria – Beef Barbacoa, Mojo Pork, Mole Chicken
Cash Bar - Specialty beverage - Mojito - Mojito Cleland

Thursday, January 4

7:00-8:30 am

Breakfast – Royal Palm 6-8

GULF COAST
Seasonal Fruits & Berries
Individual Flavored Yogurts
Dry Cereals, Whole & Skim Milk
Oven-Fresh Bakery Specialties
Fruit Preserves, Butter
Oatmeal With Golden Raisins, Brown Sugar, Sliced Almonds
Scrambled Eggs
Egg white frittata with chive hollandaise buttermilk pancakes, warm maple syrup
Pork Link Sausage
Applewood Smoked Bacon
Herb Roasted Potatoes
Orange, Cranberry & Apple Juice
Coffee, Decaffeinated Coffee, Assorted Hot Tea

Poster Session 1

10:00 am-12:00 pm

Coffee Break/Poster Session - Royal Palm 1-3

Assorted Soft Drinks, Bottled Water, Fruit Infused Water, Coffee- Regular and Decaf, Hot tea, Iced Tea

12:00-1:00 pm

Lunch - Royal Palm 6-8

GULF COAST DELI
Baby kale & frisee salad - tomato, cucumber, carrots, red onion balsamic, ranch & citrus vinaigrette
Creamy coleslaw -traditional
German potato salad -red potato, whole grain mustard, bacon
Orzo pasta salad -spinach, pear tomato, preserved lemon
Cuban - roasted pork, ham, swiss, pickles roasted garlic aioli

Pastrami melt - swiss, caramelized onion, marble rye
Chicken parm sandwich - panko crusted chicken, pomodoro, provolone
Italian provision panini - chef's selection of meats & cheeses roasted peppers
Deli-style potato chips
Desserts - coconut macaroons pineapple upside down cake raspberry bars

3:00-7:00 pm
Free Time/Dinner (not provided)

Poster Session 2
8:50-10:00 pm
Drinks/Poster Session - Royal Palm 1-3, Royal Palm Foyer

SNACK LOVERS
Pretzels, Potato Chips, Terra Chips, Popcorn, Kit Kats, Reese's Peanut Butter Cups, Hershey Bars, M&M's
Specialty beverage - Strawberry Daquiri - The Michaelis Curve

Friday, January 5

7:00-8:30 am
Breakfast – Royal Palm 6-8

COUNTRY HARVEST
Seasonal Fruits & Berries Individual Flavored Yogurts Oven-Fresh Bakery Specialties Fruit Preserves, Butter
Dry Cereals, Whole & Skim Milk Scrambled Eggs
Pork Link Sausage
Applewood Smoked Bacon Potato Lyonnaise
Orange, Cranberry & Apple Juice
Coffee, Decaffeinated Coffee, Assorted Hot Tea

Poster Session 3
10:00 am-12:00 pm
Coffee Break/Poster Session - Royal Palm 1-3, Royal Palm Foyer
Assorted Soft Drinks, Bottled Water, Fruit Infused Water, Coffee- Regular and Decaf, Hot tea, Iced Tea

12:00-1:00pm
Lunch - Royal Palm 6-8

THE COOKOUT
Mixed green salad - buttermilk ranch & citrus vinaigrette
Roasted corn
& black bean salad cilantro, honey lime
From the grill - hamburgers, all beef hot dogs, hot links, grilled 8-cut bbq chicken
Traditional condiments & toppings - ketchup, mustard, mayonnaise, lettuce tomato, onion, pickle, sliced swiss, american & cheddar cheese

Sides - mac & cheese baked beans
Dessert - strawberry shortcake peach cobbler chocolate pecan tart

5:30-7:00 pm
Dinner – Royal Palm 6-8

CARIBBEAN VIBES
Bahamian seafood chowder
Chilled shrimp display - (3 pieces per person) rum cocktail sauce
Field greens calypso - key lime vinaigrette
Roasted corn & black bean salad - poblano, grape tomato, fresh lime
Grilled pineapple slaw - mango, bermuda onion, celery, cilantro citrus vinaigrette
Mojo roasted pork loin flank steak** - marinated in key lime, molasses & mustard
Jamaican jerk chicken - mango relish
Pepper seared mahi mahi - coconut rum sauce
Sides - assorted rolls, arroz con gandules, maduros seasonal vegetables
Dessert - banana cream tartlet, coconut chocolate cake pastelitos, caramel mango flan

Poster Session 4
8:50-10:00 pm
Drinks/Poster Session - Royal Palm 1-3, Royal Palm Foyer

BEACH SIDE TRAIL MIX
Granola, Cajun Snack Mix Yogurt Pretzels, Dried Cranberries Apricots, Banana Chips, Golden Raisins
Toasted Coconut, Chocolate M&M's Cashews, Almonds
Cash Bar - Specialty beverage - Lime Margarita - The Transition State

Saturday, January 6

7:00-8:30 am
Breakfast – Royal Palm 6-8

COUNTRY HARVEST
Seasonal Fruits & Berries Individual Flavored Yogurts Oven-Fresh Bakery Specialties Fruit Preserves, Butter
Dry Cereals, Whole & Skim Milk
Scrambled Eggs
Chicken Sausage
Turkey Bacon
Hash browns
Orange, Cranberry & Apple Juice
Coffee, Decaffeinated Coffee, Assorted Hot Tea

10:10-10:30 am
Coffee Break - Royal Palm Foyer

Assorted Soft Drinks, Bottled Water, Fruit Infused Water, Coffee- Regular and Decaf, Hot tea, Iced Tea

12:30-2:00 pm
Lunch - Royal Palm 6-8

Boxed lunches*
Veggie wrap - wheat wrap, grilled zucchini yellow squash, roasted peppers hummus, portobello mushroom
Italian sub - milano sub roll, ham, salami, pepperoni provolone, banana pepper, lettuce, tomato
Grilled chicken sandwich - brioche roll, dill havarti cheese, lettuce tomato, spicy pepper aioli
*Each includes: garden pasta salad, green apple, kettle chips chocolate chip cookie

Closing Banquet
6:00-7:00 pm
Reception with hors-d'oeuvres – Sunset Verandah

Bruscheta – Hummus, Baba Ganoush, Sun Dried Tomato & Olive Tapenade, Roasted Vegetable & Portobello, Grilled Pita, Naan & Focaccia Breads
Charcuterie – Proscuitto, Genoa Salami, oppa, Spanish Chorizo, Provolone, Fresh Mozzarella, Reggiano, Pepperoncini, Black & Green Olivess, Roasted Pepper, Marinated Mushroom, Crostinis
Crudite – Carrot, Celery, Caulifolower, Brocolli, Asparagus, Heirloom Tomato, Ranch & Garbanzo Hummus
Cash bar- Specialty Beverage - Cucumber Moscow Mule - The Induced Fit

7:00 pm
Banquet – Mangrove Pool

Crab boil – Crab Claws, Gulf Shrimp, Clams, Andouille Sausage, Sweet Corn, New Potato, Grilled Sourdough
Rissottos – Seasonal Roasted Vegetable & Roasted Chicken with tomato, leek and wild mushroom
Carved prime rib
Herbed fingerling potatoes, Cole slaw, Green beans
Viennese Dessert station – Mini chocolate eclairs, Napoleons, Seasonal fruit tarts, Petit gateau
Cash bar - Specialty Beverage - Cucumber Moscow Mule - The Induced Fit

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