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Bloomington, IN 47401 -3654

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Jared Lewis
UU	UU	UU	UU		19b. TELEPHONE NUMBER 812-855-6011

RPPR Final Report
as of 02-Mar-2022

Agency Code: 21XD

Proposal Number: 73767LS **Agreement Number: W911NF-19-1-0074**
INVESTIGATOR(S):

Name: Jared C. Lewis
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DUNS Number: 006046700 EIN: 356001673

Report Date: 14-Sep-2021 Date Received: 01-Mar-2022
Final Report for Period Beginning 15-Jan-2019 and Ending 14-Jun-2021
Title: Engineering Artificial Metalloenzymes for Selective Catalysis in Complex Media
Begin Performance Period: 15-Jan-2019 **End Performance Period:** 14-Jun-2021
Report Term: 0-Other

Submitted By: Jared Lewis Email: jcl3@iu.edu
Phone: (812) 855-6011

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STEM Degrees: 1 **STEM Participants:** 1

Major Goals: The originally stated goals of this proposal were as follows:

1. Develop immobilization-based evolution approaches to optimize dirhodium artificial metalloenzymes (ArMs) for selective C-H functionalization in complex aqueous solutions.
2. Evolve ArMs containing base metal cofactors that catalyze selective C-H functionalization of representative drug molecules and pesticides in aqueous solutions.
3. Develop cofactor activation approaches that allow the use of chemically inert cofactors to form reactive ArMs only upon scaffold activation.

Progress toward these goals was significantly impacted by my group's move to Indiana University during the first funding period and by COVID-19 related university shutdowns and lab occupancy reductions. This report covers research conducted under the original award made while I was at the University of Chicago (18-1-0034) and following transfer of this award to Indiana University (19-1-0074). Despite these challenges, we were able to make significant progress on all three of the originally proposed goals. We also expanded the scope of the first goal to include ruthenium-based ArMs and to pursue initial studies on ArMs containing genetically-encoded bipyridyl alanine residues.

Accomplishments: See uploaded document.

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Training Opportunities: Technical experience gained by students working on this project include advanced techniques in organic/organometallic synthesis, molecular biology, directed evolution, protein purification, bioconjugation methods, and high throughput experimentation.

All students involved in the project participate in formal bi-weekly research meetings. These involve presenting research from a formal written report and providing feedback on the progress of others. Students also present their research at one-two group meetings per year and receive a formal evaluation of their progress from me each year. We meet to discuss research progress, plans following graduation/completion of postdoc, and how research plans can be modified to better prepare individuals for next steps. These meetings were all conducted via Zoom for the last two years of this project.

In addition to the technical research talks noted above, students also give one-two group meeting presentations per year on topics outside of their research. They regularly present results from the literature prior to our group meeting presentations. Students and postdocs can speak at a student/postdoc-led seminar series affiliated with the IU NIH QCB training grant and a regular biophysics "supergroup" meeting sponsored by various faculty members (including the PI). Again, all of this was virtual for the last two years of this project. While students are normally encouraged to present their results at both ACS National Meetings and the Biocatalysis and Metals in Biology Gordon Research Conferences, these were virtual for most of this project, and no students were interested in participating in these events. All students are responsible for maintaining several instruments in the group and attending seminars (virtually) in the Department of Chemistry and the Department of Molecular and Cell Biology at IU. Both Atreyi Bhattacharya and David Upp were also involved the NSF-funded CCHF, which allowed them to present their research with a range of researchers in the CCHF. Students write manuscripts describing their results, and I edit these to assist in their scientific writing development.

During the 3-month research shutdown at IU due to COVID-19, students involved in this project wrote up experimental information for publication, worked on their thesis, and learned a number of computational modeling tools that have already proven useful for the proposed research now that research has started up again. It should be noted that several group members did not return to lab for 1-2 months after they were allowed to by the University due to concerns for their own health. Our lab occupancy was capped at 50% for nearly a year, which significantly reduced our research efforts.

Results Dissemination: results were discussed at the following virtual talks in addition to the publications listed in the products section:

2021
Novartis Chemistry Lectureship, Basel, Switzerland, 6/16
AbbVie Platform Chemistry Technology Seminar, 5/12
University of British Columbia, Okanagan, 3/16

2020
San Diego State University, 10/16 (virtual)
Innovative Screening Strategies for the Directed Evolution of Enzymes, Pittcon, Chicago, IL, 3/4
University of Toledo, Department of Chemistry 2/24
RSC Applied Late-Stage Functionalization Symposium, University of Manchester, UK, 2/18-19
Third International Symposium on Carbene and Nitrene Chemistry, San Antonio, TX, 2/5-7

2019
ArtZymes Conference, Basel, Switzerland, 8/9
Telluride Science Research Conference (The Future of C-H Functionalization), 7/29-8/2
Society for Industrial Microbiology meeting, Washington D.C, 7/24
Novartis Chemistry Lectureship, Cambridge, MA, 7/18
International Symposium for Applied Bioinorganic Chemistry, Nara, Japan, 6/4
Marquette University, Department of Chemistry, 4/26

Honors and Awards: 2019Novartis Chemistry Lectureship

Protocol Activity Status:

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Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: PD/PI
Participant: Jared Lewis
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
Participant: Rui Huang
Person Months Worked: 4.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
Participant: Xinhang Yang
Person Months Worked: 10.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Undergraduate Student
Participant: Maxwell Bultman
Person Months Worked: 3.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: Alan Swartz
Person Months Worked: 6.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: David Upp
Person Months Worked: 12.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: Atreyi Bhattacharya
Person Months Worked: 6.00 **Funding Support:**
Project Contribution:
National Academy Member: N

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Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
Participant: Dipankar Sahoo
Person Months Worked: 4.00 **Funding Support:**
Project Contribution:
National Academy Member: N

ARTICLES:

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Journal: Accounts of Chemical Research
Publication Identifier Type: DOI Publication Identifier: 10.1021/acs.accounts.8b00625
Volume: 52 Issue: 3 First Page #: 576
Date Submitted: 10/2/19 12:00AM Date Published: 3/1/19 5:00AM
Publication Location:
Article Title: Beyond the Second Coordination Sphere: Engineering Dirhodium Artificial Metalloenzymes To Enable Protein Control of Transition Metal Catalysis
Authors: Jared C. Lewis
Keywords: artificial metalloenzyme, prolyl oligopeptidase, dirhodium, directed evolution
Abstract: This Account outlines the development of a new class of ArMs based on a prolyl oligopeptidase (POP) scaffold. Studies conducted on POP ArMs containing a covalently linked dirhodium cofactor have shown that POP can impart enantioselectivity to a range of dirhodium-catalyzed reactions, increase reaction rates, and improve the specificity for reaction of dirhodium carbene intermediates with targeted organic substrates over components of cell lysate, including bulk water. Several design features of these ArMs enabled their evolution via random mutagenesis, which revealed that mutations throughout the POP scaffold, beyond the second sphere of the dirhodium cofactor, were important for ArM activity and selectivity. While it was anticipated that the POP scaffold would be capable of encapsulating and thus controlling the selectivity of bulky cofactors, molecular dynamics studies also suggest that POP conformational dynamics plays a role in its unique efficacy. These advances in scaffold select
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Volume: 10 Issue: 3 First Page #: 318
Date Submitted: 10/2/19 12:00AM Date Published: 1/1/18 5:00AM
Publication Location:
Article Title: Evolving artificial metalloenzymes via random mutagenesis
Authors: Hao Yang, Alan M. Swartz, Hyun June Park, Poonam Srivastava, Ken Ellis-Guardiola, David M. Upp, Gi
Keywords: dirhodium, artificial metalloenzyme, directed evolution, random mutagenesis
Abstract: Random mutagenesis has the potential to optimize the efficiency and selectivity of protein catalysts without requiring detailed knowledge of protein structure; however, introducing synthetic metal cofactors complicates the expression and screening of enzyme libraries, and activity arising from free cofactor must be eliminated. Here we report an efficient platform to create and screen libraries of artificial metalloenzymes (ArMs) via random mutagenesis, which we use to evolve highly selective dirhodium cyclopropanases. Error-prone PCR and combinatorial codon mutagenesis enabled multiplexed analysis of random mutations, including at sites distal to the putative ArM active site that are difficult to identify using targeted mutagenesis approaches. Variants that exhibited significantly improved selectivity for each of the cyclopropane product enantiomers were identified, and higher activity than previously reported ArM cyclopropanases obtained via targeted mutagenesis was also observed. Thi
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Volume: 58 Issue: 12 First Page #: 1616
Date Submitted: 10/2/19 12:00AM Date Published: 2/1/19 5:00AM
Publication Location:
Article Title: Crystal Structure and Conformational Dynamics of
Authors: Ken Ellis-Guardiola, Huan Rui, Ryan L. Beckner, Poonam Srivastava, Narayanasami Sukumar, Benoît F
Keywords: prolyl oligopeptidase, molecular dynamics, artificial metalloenzymes
Abstract: Enzymes in the prolyl oligopeptidase family possess unique structures and substrate specificities that are important for their biological activity and for potential biocatalytic applications. The crystal structures of Pyrococcus furiosus (Pfu) prolyl oligopeptidase (POP) and the corresponding S477C mutant were determined to 1.9 and 2.2 Å resolution, respectively. The wild type enzyme crystallized in an open conformation, indicating that this state is readily accessible, and it contained bound chloride ions and a prolylproline ligand. These structures were used as starting points for molecular dynamics simulations of Pfu POP conformational dynamics. The simulations showed that large-scale domain opening and closing occurred spontaneously, providing facile substrate access to the active site. Movement of the loop containing the catalytically essential histidine into a conformation similar to those found in structures with fully formed catalytic triads also occurred.
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Date Submitted: 9/4/21 12:00AM Date Published: 8/1/21 4:00AM
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Article Title: Engineering Dirhodium Artificial Metalloenzymes for Diazo Coupling Cascade Reactions**
Authors: David M. Upp, Rui Huang, Ying Li, Max J. Bultman, Benoit Roux, Jared C. Lewis
Keywords: artificial metalloenzyme, dirhodium, directed evolution, cascade catalysis
Abstract: Artificial metalloenzymes (ArMs) are now commonly used to control the stereoselectivity of catalytic reactions, but controlling ArM chemoselectivity remains challenging. In this study, we engineer a dirhodium ArM to catalyze diazo cross-coupling to form an alkene that, in a one-pot cascade reaction, is reduced to an alkane with high enantioselectivity (typically >99% e.e.) by an alkene reductase. The numerous protein and small molecule components required for the cascade reaction had minimal effect on ArM catalysis. Directed evolution of the ArM led to improved yields and E/Z selectivities for a variety of substrates, which translated well to cascade reaction yields. MD simulations of ArM variants were used to understand the structural role of the cofactor on large-scale scaffold structural dynamics. These results highlight the ability of ArMs to control both catalyst stereoselectivity and chemoselectivity to enable reactions in complex media that would otherwise lead to undesired side
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Publication Identifier Type: DOI Publication Identifier: 10.1039/D1SC05792H
Volume: Issue: First Page #:
Date Submitted: 1/25/22 12:00AM Date Published:
Publication Location:
Article Title: Controlling the optical and catalytic properties of artificial metalloenzyme photocatalysts using chemogenetic engineering
Authors: Yasmine S. Zubi, Bingqing Liu, Yifan Gu, Dipankar Sahoo, Jared C. Lewis
Keywords: photocatalysis, artificial metalloenzyme, ruthenium trisbipyridine, energy transfer
Abstract: Dynamic control over protein function is a central challenge in synthetic biology. To address this challenge, we developed an integrated computational and experimental workflow to incorporate a metal-responsive chemical switch into proteins. Pairs of bipyridinylalanine (BpyAla) residues were genetically encoded into two structurally distinct enzymes, a serine protease and firefly luciferase, so that metal coordination would bias the conformations of these enzymes, leading to reversible control of activity. Computational analysis and MD simulations were used to rationally guide BpyAla placement, significantly reducing experimental workload, and cell-free protein synthesis coupled with high-throughput experimentation enabled rapid prototyping of variants. Ultimately, this strategy yielded enzymes with a robust 20-fold dynamic range in response to divalent metal salts over 24 on/off switches, demonstrating the potential of this approach. We envision that this strategy of genetically encod
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Publication Type: Journal Article Peer Reviewed: Y **Publication Status:** 1-Published
Journal: ACS Catalysis
Publication Identifier Type: DOI Publication Identifier: 10.1021/acscatal.1c04748
Volume: 12 Issue: 2 First Page #: 935
Date Submitted: 1/25/22 12:00AM Date Published: 12/1/21 5:00AM
Publication Location:
Article Title: Controlling Non-Native Cobalamin Reactivity and Catalysis in the Transcription Factor CarH
Authors: Xinhang Yang, Benjamin H. R. Gerroll, Yuhua Jiang, Amardeep Kumar, Yasmine S. Zubi, Lane A. Bake
Keywords: B12, cobalamin, C?H functionalization, biocatalysis, non-native enzyme catalysis
Abstract: Dynamic control over protein function is a central challenge in synthetic biology. To address this challenge, we developed an integrated computational and experimental workflow to incorporate a metal-responsive chemical switch into proteins. Pairs of bipyridinylalanine (BpyAla) residues were genetically encoded into two structurally distinct enzymes, a serine protease and firefly luciferase, so that metal coordination would bias the conformations of these enzymes, leading to reversible control of activity. Computational analysis and MD simulations were used to rationally guide BpyAla placement, significantly reducing experimental workload, and cell-free protein synthesis coupled with high-throughput experimentation enabled rapid prototyping of variants. Ultimately, this strategy yielded enzymes with a robust 20-fold dynamic range in response to divalent metal salts over 24 on/off switches, demonstrating the potential of this approach. We envision that this strategy of genetically encod
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Publication Type: Journal Article Peer Reviewed: Y **Publication Status:** 4-Under Review
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Publication Identifier Type: DOI Publication Identifier: 10.33774/chemrxiv-2021-9ql74
Volume: Issue: First Page #:
Date Submitted: 1/25/22 12:00AM Date Published:
Publication Location:
Article Title: Metal-Responsive Regulation of Enzyme Catalysis Using Genetically Encoded Chemical Switches
Authors: Zubi, Y. S.; Seki, K.; Li, Y.; Hunt, A.; Liu, B.; Roux, B.*, Jewett, M. C.*, Lewis, J. C.*
Keywords: allosteric control, conformational dynamics, protein switch
Abstract: Dynamic control over protein function is a central challenge in synthetic biology. To address this challenge, we developed an integrated computational and experimental workflow to incorporate a metal-responsive chemical switch into proteins. Pairs of bipyridinylalanine (BpyAla) residues were genetically encoded into two structurally distinct enzymes, a serine protease and firefly luciferase, so that metal coordination would bias the conformations of these enzymes, leading to reversible control of activity. Computational analysis and MD simulations were used to rationally guide BpyAla placement, significantly reducing experimental workload, and cell-free protein synthesis coupled with high-throughput experimentation enabled rapid prototyping of variants. Ultimately, this strategy yielded enzymes with a robust 20-fold dynamic range in response to divalent metal salts over 24 on/off switches, demonstrating the potential of this approach. We envision that this strategy of genetically encod
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RPPR Final Report
as of 02-Mar-2022

Partners

,

I certify that the information in the report is complete and accurate:
Signature: jared lewis
Signature Date: 3/1/22 1:43PM

The originally stated goals of this proposal were as follows:

1. Develop immobilization-based evolution approaches to optimize dirhodium artificial metalloenzymes (ArMs) for selective C-H functionalization in complex aqueous solutions.
2. Evolve ArMs containing base metal cofactors that catalyze selective C-H functionalization of representative drug molecules and pesticides in aqueous solutions.
3. Develop cofactor activation approaches that allow the use of chemically inert cofactors to form reactive ArMs only upon scaffold activation.

Progress toward these goals was significantly impacted by my group's move to Indiana University during the first funding period and by COVID-19 related university shutdowns and lab occupancy reductions. This report covers research conducted under the original award made while I was at the University of Chicago (18-1-0034) and following transfer of this award to Indiana University (19-1-0074). Despite these challenges, we were able to make significant progress on all three of the originally proposed goals. We also expanded the scope of the first goal to include ruthenium-based ArMs and to pursue initial studies on ArMs containing genetically-encoded bipyridyl alanine residues.

I. Objective 1: Develop immobilization-based evolution approaches to optimize dirhodium artificial metalloenzymes for selective C-H functionalization in complex aqueous solutions

I. A. Immobilization Methodology and Performance

The original proposal outlined a plate-based approach to identifying solid supports for scaffold immobilization and ArM optimization (Fig. 1). These efforts focused on ArMs generated from a prolyl oligopeptidase (POP) scaffold from *Pyrococcus furiosus*, the structure of which we published in *Biochemistry* in earlier ARO-funded research (14-1-0334). We developed a robust protocol for POP ArM immobilization using Ni-NTA resin in 96 well plates. Several additional resins were also evaluated, but none of these led to significantly improved results (Fig. 2), so Ni-NTA remains the resin of choice for ongoing studies due to cost considerations.

Figure 1. Overview of screening procedure for identifying solid supports and conditions for POP immobilization via colorimetric assay of filtrate and resin activity. Supports that lead to high activity of bound POP and low filtrate background will be selected for ArM immobilization.

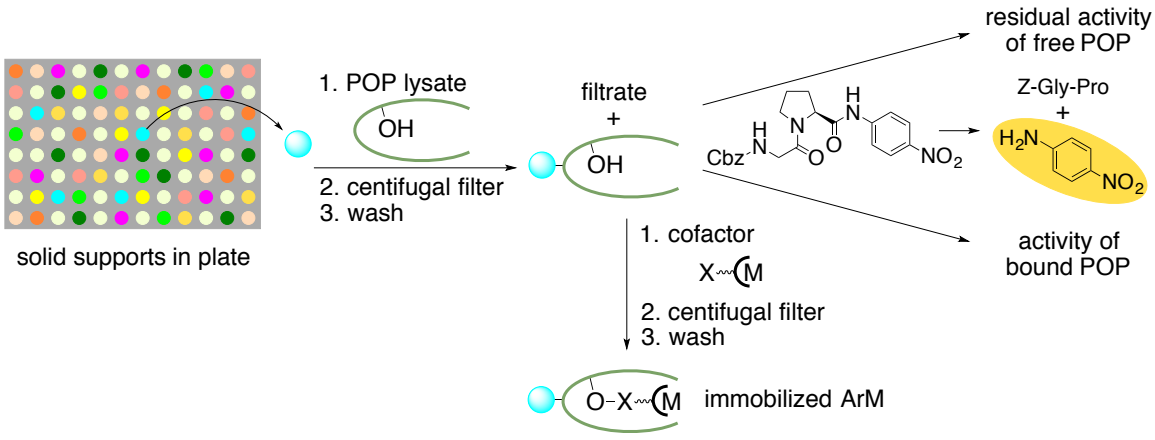


Figure 2. Yield and selectivity (e.e.) for styrene cyclopropanation (reaction 1) and THF C-H insertion (reaction 2) catalyzed by ArM 3-VRVH immobilized on different resins.

Entry	Resin	Reaction	Yield	e.e.
1	Fisher Ni-NTA	1	38%	48%
2	Enginzyme Opal	1	42%	31%
3	Enginzyme Ochre	1	12%	45%
4	Enginzyme Amber	1	35%	51%
5	ImmoBeads IB-COV-1	2	0%	-
6	ImmoBeads IB-ABS-1	2	0%	-

The original proposal also outlined the potential use of cell display to avoid the need for immobilization of ArM scaffolds entirely. While yeast display was originally proposed, careful consideration of different cell display platforms has led us to focus on *E. coli* display. Roughly 10^6 proteins can be displayed/yeast cell vs 10^4 for *E. coli*, but the 100x larger volume of yeast means that the volumetric display capacities of yeast and *E. coli* are similar. More importantly, far larger proteins (POP is a 70 kDa protein) and even some examples of ArMs have been displayed on *E. coli*. Several carrier proteins, including ompA, EstA, INPN, and AID-A, are currently being explored for *E. coli* display of POP-based ArMs.

I. B. Directed Evolution of Immobilized ArMs

Significant progress was made on the evolution of ArMs immobilized on Ni-NTA resin using the procedure shown in Fig. 1. Scaffold libraries of POP-ZA$_4$ (see original proposal for discussion of POP variants) were generated, picked, and cultured. Cell lysate from these libraries was added to filter plates containing Ni-NTA resin. Following incubation, the resin was filtered and washed via centrifugation. ArM formation was similarly accomplished via incubation with cofactor, filtration, and washing. ArM catalysis was readily evaluated via incubation with substrates, filtration, and analysis of the filtrate (containing reaction products) by LC/MS. This method (Figure 3), also supported by ARO award 14-1-0334, was published in *Nature Chemistry*.

Figure 3. Overview of ArM evolution via scaffold immobilization.

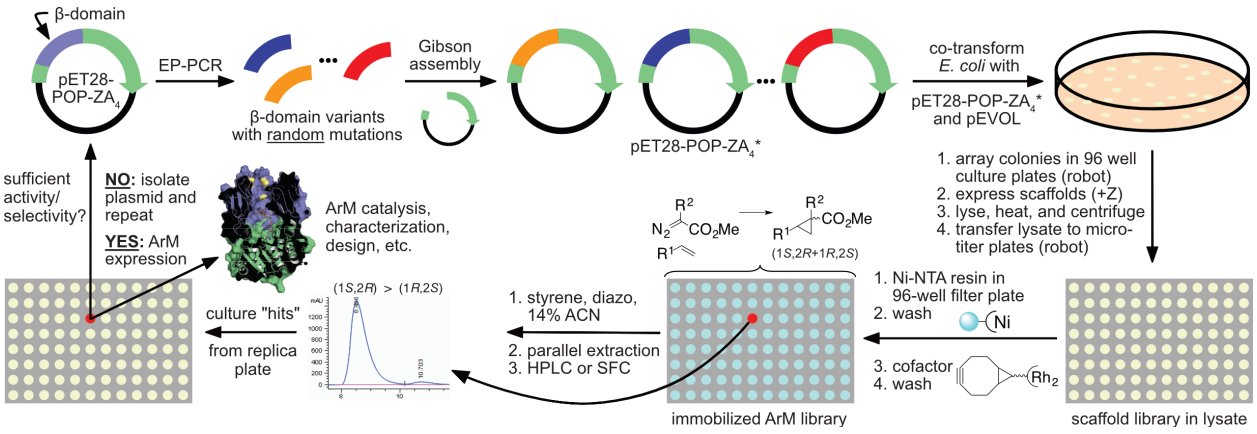


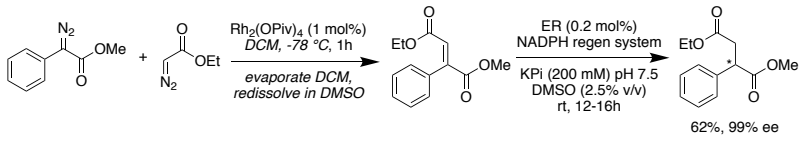
Table 1. Representative optimization steps conducted.

Change	Details	Results
Ni-NTA resin	Increase volume from 100 to 300 μL	Increase POP recovery efficiency by 2x
His tag	Increase the C-terminal His-tag number of fused POP from 6 to 12	Increase the wash tolerance of resin bound POP towards organic solvent wash
ArM preparation	Bioconjugation in cell lysate, two rounds of cofactor scavenging, then bind ArM to Ni-NTA resin	Eliminate the background reaction resulted from unbound cofactor
Product isolation	Remove the ethyl acetate evaporation step following product extraction	Avoid diazo side reactions following ArM catalysis
Temperature	Increase temperature from 4 to 23 ˚C	Increase C-H functionalization yield 2x
Co-solvent	Change from THF to ACN	Increase C-H functionalization yield 3.8x

Essentially every step of this protocol was further optimized as part of the current proposal effort to ultimately yield several-fold greater conversion for several reactions (Table 1). These modifications have greatly facilitated the evolution of ArMs for N-H insertion, Si-H insertion, and diazo cross-coupling (*vide infra*). While this procedure used the previously reported Ni-NTA resin, it demonstrated that ArM evolution on solid support provides a general means to improve ArM activity. Indeed, it has become the default procedure used for ArM evolution in my lab.

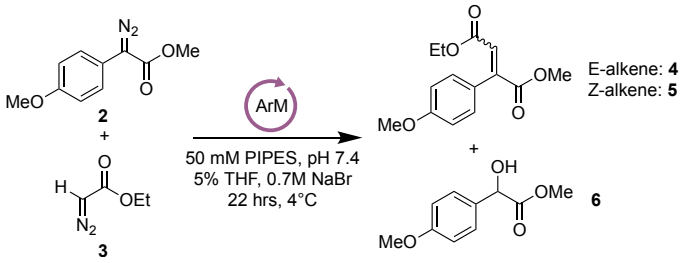
We first used this optimized evolution protocol to engineer ArMs for selective diazo cross-coupling. This reaction provided a particularly impactful example of the benefits of enabling transition metal catalyzed reactions in an aqueous environment. Dirhodium complexes react with donor-acceptor diazo compounds to generate highly reactive carbene complexes that react readily with nucleophiles, including water, thiols, amines, olefins, silanes, and even sp^3 C-H bonds. We envisioned that this promiscuous reactivity would allow studies on the extent to which a protein scaffold can be engineered to control the transition metal chemoselectivity in complex media. Indeed, previous studies in our laboratory have established that ArM chemoselectivity can be evolved to favor carbene addition to olefins (i.e. cyclopropanation) over undesired formal insertion into water O-H bonds. We also reasoned that this level of control over dirhodium reactivity could enable cascade reactions involving a variety of additional species in solution. For example, dirhodium-catalyzed diazo coupling has been used by Hartwig and Zhao (*ACS Cat.* **2017**, *7*, 2548) to generate maleic esters that are converted by alkene reductases to 2-substituted succinate derivatives (Figure 4). In this study, however, the dirhodium catalysis was conducted in organic solvent at cryogenic temperatures, and following this step, the solvent was evaporated and the residue dissolved in aqueous buffer to enable biocatalytic reduction. We evolved a dirhodium ArM with increased chemo- and regio-selectivity for diazo coupling and demonstrated that the resulting ArM can be interfaced with an alkene reductase in a one-post cascade reaction to produce substituted succinate derivatives.

Figure 4. Reported two-step procedure for sequential diazo coupling, ene reduction.



Five rounds of evolution led to a significant improvement in the production of E-alkene product **4** (Figure 5), which is required or the subsequent ene reductase step. This improvement (from 40% yield to 70% yield) resulted from both improvements in the stereo- and chemoselectivity of the reaction, clearly showing the impact that the POP scaffold has over dirhodium cofactor reactivity.

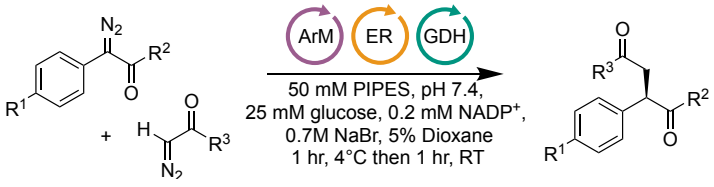
Figure 5. Directed evolution of dirhodium ArMs for improved diazo coupling to produce **4**.



Entry[a]	Variant	Mutagenesis method	Mutations from previous generation	% Yield[c]			E/Z	TTN of **4**
				4	**5**	**6**		
1	1-SGH	-	Parent	44	12	31	3.7	388
2	2-P	Q98NNK	Q98P	55	11	33	4.9	545
3	3-H	S99NNK	S99H	51	7	24	7.3	511
4	4-G	CCM	V71G	72	8	21	8.6	717
5	5-G	E283NNK	E283G	76	5	16	14.9	761
6[b]	5-G	E283NNK	E283G	45	16	11	2.8	44612

Importantly, we also established that the dirhodium ArM catalysis is unaffected by the ene reductase required for conversion of **4** to the corresponding chiral succinic acid esters. Good yields of the desired succinic acid derivatives resulting from cascade catalysis were obtained (Figure 6). Ensconcing the dirhodium cofactor within the POP scaffold thus eliminates the need for toxic organic solvents and cryogenic temperatures, improves reaction selectivity, and enables cascade reactions with other enzymes. MD studies conducted suggest that large-scale conformational dynamics regulate access to the active site, giving rise to the observed ArM chemoselectivity. A manuscript describing these findings was published in *Angew. Chem. Int. Ed.*

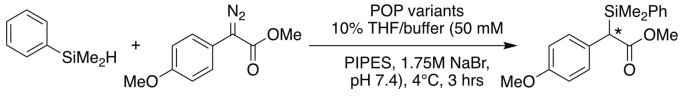
Figure 6. Cascade ArM-catalyzed diazo coupling/ene reductase catalyzed alkene reduction.



					% Yield[b] (e.e)[c]	
Entry[a]	ER	R^1	R^2	R^3	1-GSH	5-G
1	KYE1	OMe	OMe	OEt	25 (>99%)	61 (>99%)
2	YersER	H	OMe	OEt	35 (>99%)	56 (>99%)
3	YersER	Cl	OMe	OEt	18 (>99%)	47 (>99%)
4	YersER	Br	OMe	OEt	32 (>99%)	60 (>99%)
5	OPR1	OMe	OMe	NEt_2	22 (>99%)	40 (>99%)
6	OPR1	OMe	OMe	OBn	9 (>99%)	12 (>99%)
7	YersER	Cl	Me	OEt	34 (79%)	52 (78%)

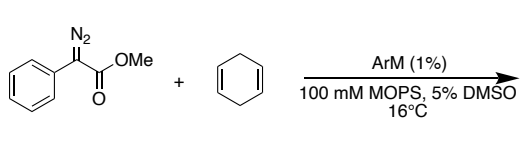
Notably, variants obtained from the diazo coupling evolution also led to improved selectivity for Si-H insertion (Figure 7). For example, enantiomeric excess of up to 89% was observed for the third-generation variant shown in Figure 5 (3-H), while the parent provided negligible selectivity. This bodes well for optimizing ArMs for stereoselective Si-H insertion, but it also provides a potential route to optimize C-H insertion. While ArM-catalyzed C-H insertion is possible (Figure 8), this reaction currently proceeds with insufficient conversion to enable detection under HT screening conditions. By improving ArM activity for Si-H insertion, we hypothesize that we will also improve activity for structurally and mechanistically related C-H insertion (Figure 9).

Figure 7. Improved Si-H insertion selectivity using ArMs evolved for diazo cross-coupling.



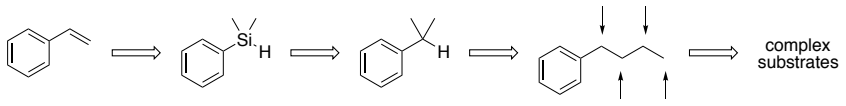
Entry	Variant	Yield	e.e. (%)
1	3'-VRVH	35	64
2	2-P	23	84
3	3-H	21	89
4	4-G	31	73

Figure 8. Representative ArM-catalyzed C-H insertion showing improved e.e. with evolved variant (3-VRVH) relative to parent (ZA4).



Variant	Yield (%)	TON	e.e.
ZA4	12	12	14
3-VRVH	15	15	57

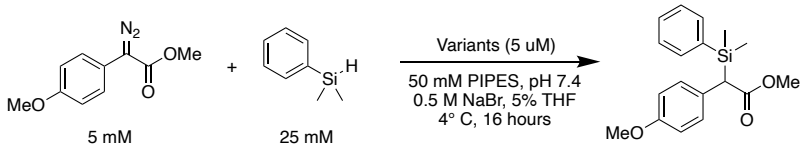
Figure 9. A substrate walking approach to evolve C-H insertion from Si-H insertion.



Toward this end, we have conducted four rounds of evolution to improve Si-H insertion efficiency starting from variant 3-H noted above in Figure 7. Because high enantioselectivity was already observed, the goal of this effort was to improve the ratio of Si-H insertion to undesired insertion into the O-H bond of water. Modest progress toward this goal has been made (Figure 10) using

mutagenesis targeted to the POP active site, and we are now moving to random mutagenesis of the entire POP beta propeller domain in analogy to our initial ArM cyclopropanation evolution to further improve Si-H and ultimately C-H insertion activity.

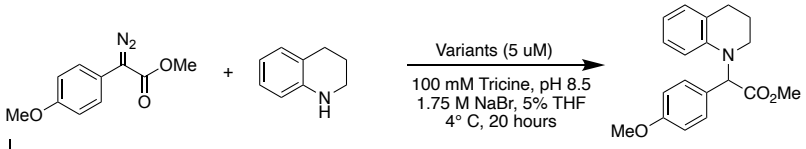
Figure 10. Progress toward directed evolution of an ArM for selective Si-H insertion.



			Si-H insertion		O-H Insertion		
Entry	Variant	Mutations	Yield (%)	e.e. (%)	Yield (%)	SiH/OH	TTN to SiH
1	3-H	Parent	24	93	71	0.34	241
2	2-SH	E241S/L328H	30	90	68	0.44	298
3	3-D	S241D	32	92	64	0.50	324
4	4-I	T211I	38	93	59	0.64	383

Finally, variant 1-SGH from our ArM cyclopropanation evolution effort has also been evolved to improve the selectivity of ArM-catalyzed N-H insertion (Figure 11). Ultimately, we envision that by evolving ArMs for these different reactions, we can study their unique structures to establish how different second sphere interactions within POP ArMs give rise to unique reactivity.

Figure 11. Progress toward directed evolution of an ArM for selective N-H insertion.



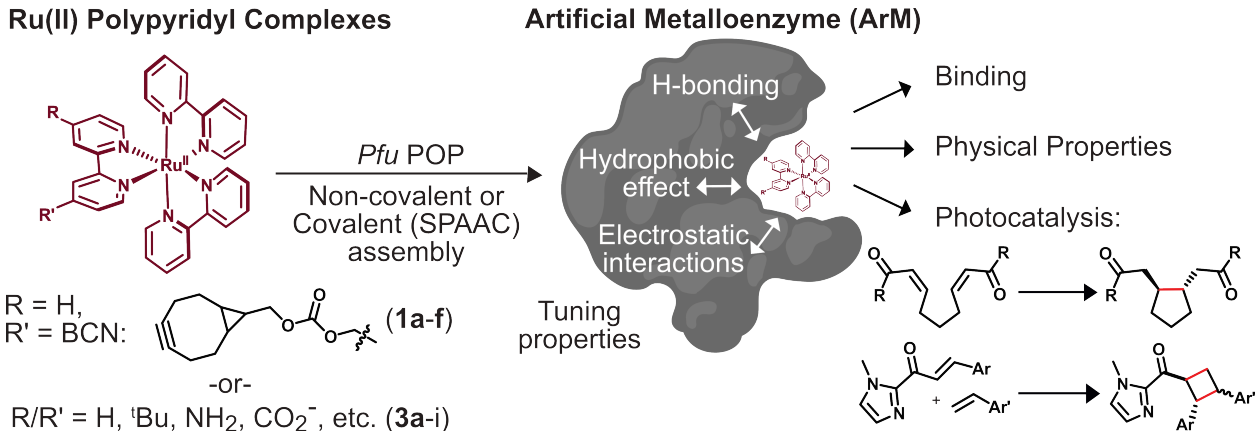
Entry	Variant	Mutations from Prevous Generation	Yield (%)	e.e. (%)
1	1-SGH	Parent	89	43
2	2-HH	Q98R/S99F/W142Y/T209V/W214Y/L328H	82	60
3	3-E	L194E	86	66
4	4-R	D119R	88	68

I. C. POP ArM Construction Using Metal-Polypyridine Cofactors

Building on the success of the dirhodium ArM efforts outlined above, we also explored analogous systems constructed from metal polypyridyl complexes. Such complexes are commonly used as visible light photocatalysts to enable a broad range of organic transformations that proceed via single electron or energy transfer. The photophysical properties of metal polypyridyl photocatalysts have been extensively studied and can be tuned by modifying the substituents on the pyridine ligands. On the other hand, ligand modifications that enable substrate binding to control reaction selectivity remain rare. Given the exquisite control that enzymes exert over electron and energy transfer processes in nature, we envisioned that ArMs created by incorporating

Ru(II) polypyridyl complexes into a suitable protein scaffold could provide a means to control photocatalyst properties (Figure 12).

Figure 12. Overview of initial ArM photocatalyst studies.

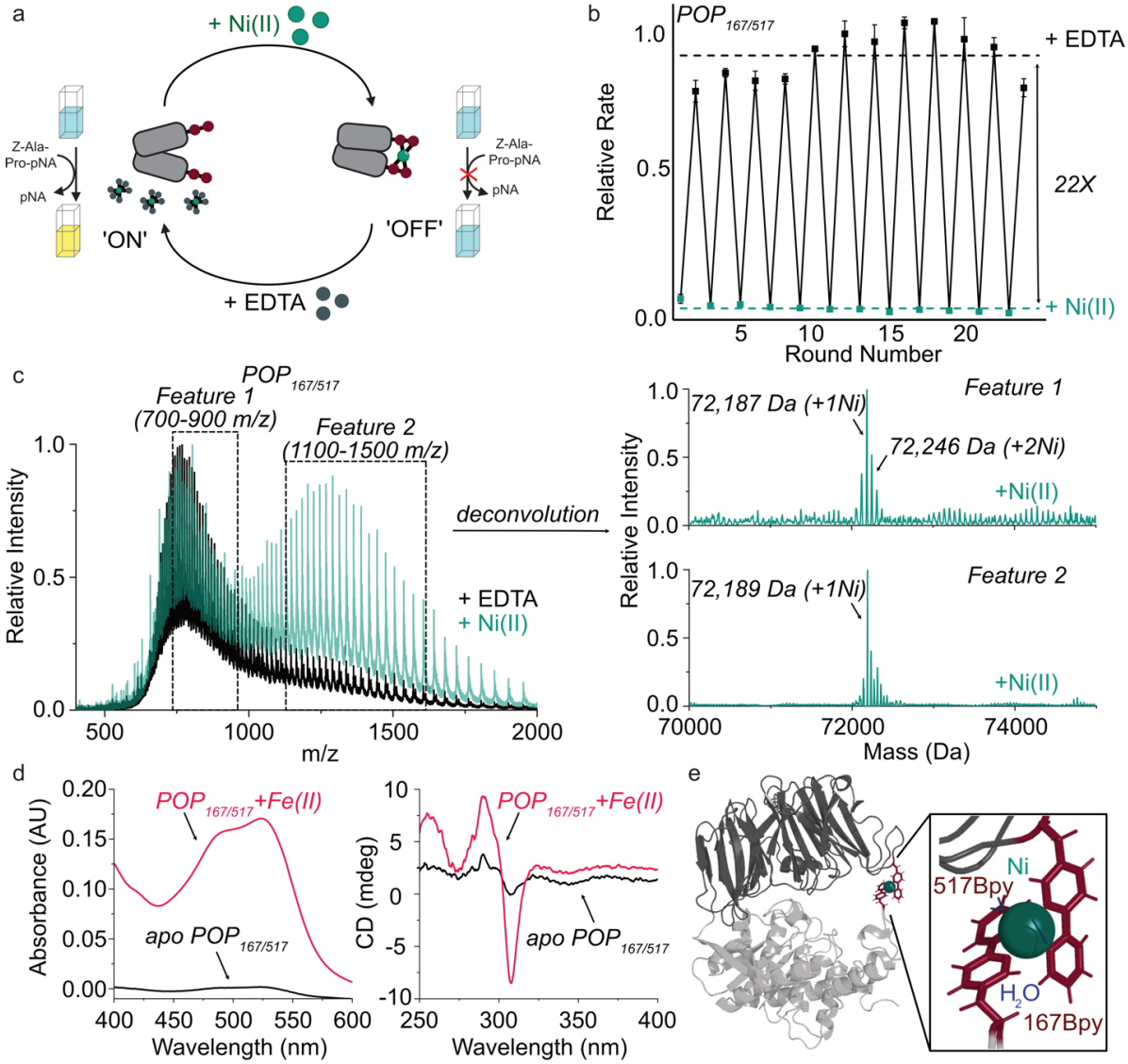


Our preliminary studies toward this end established approaches to create covalent and non-covalent ArMs from a variety of Ru(II) polypyridyl cofactors and the POP scaffold. A panel of ArMs with enhanced photophysical properties were engineered, and the nature of the scaffold/cofactor interactions in these systems was investigated. These ArMs provided higher yields and rates than $Ru(Bpy)_3^{2+}$ for the reductive cyclization of dienones and the [2+2] photocycloaddition between *C*-cinnamoyl imidazole and 4-methoxystyrene (Figure 12), suggesting that protein scaffolds could provide a means to improve the efficiency of visible light photocatalysts. This study was recently published in *Chemical Science*. Ongoing studies have established that mutagenesis of our initial constructs can be used to impart enantioselectivity to multiple energy transfer reactions, providing an exciting starting point for evolution of highly selective catalysts for a variety of photocatalytic transformations.

Funds from this award were also used to support initial studies aimed at constructing artificial metalloenzymes not by clicking alkyne-substituted cofactors into a scaffold containing a genetically encoded azidophenylalanine residue, but via first row transition metal binding to genetically encoded bipyridylalanine (BpyAla) residues. This study was expanded into a larger effort to control protein conformation via metal binding to pairs of genetically encoded BpyAla residues, which became part of a funded MURI proposal. A manuscript describing studies primarily supported by MURI funds, but also the initial studies supported this award, is currently in revision at *Nature Communications*. For this effort, we developed an integrated computational and experimental workflow to incorporate a metal-responsive chemical switch into proteins. Pairs of bipyridinylalanine (BpyAla) residues were genetically encoded into two structurally distinct enzymes, a serine protease and firefly luciferase, so that metal coordination would bias the conformations of these enzymes, leading to reversible control of activity. Computational analysis and MD simulations were used to rationally guide BpyAla placement, significantly reducing experimental workload, and cell-free protein synthesis coupled with high-throughput experimentation enabled rapid prototyping of variants. Ultimately, this strategy yielded enzymes with a robust 20-fold dynamic range in response to divalent metals over 24 on/off switches, demonstrating the potential of this approach (Figure 13). Extensive characterization was pursued

to confirm that the metal binding switch functioned as originally designed. This strategy of genetically encoding chemical switches into enzymes will enable applications where precise regulation of protein function is critical.

Figure 13. (a) Assay for Ni(II)-dependent switching. **(b)** Ni(II)-dependent switching as measured by the relative rate of Z-Ala-Pro-*p*NA hydrolysis upon addition of Ni(II) (green points) or EDTA (black points); average rates are shown as green or black dashed lines, respectively. Data points are averages of triplicate reactions and error bars represent standard deviations. **(c)** Intact protein ESI-MS data for $POP_{167/517}$ in the presence of excess EDTA (black) or Ni(II) (green). Raw MS data (left) was deconvoluted (right) using m/z windows of either 700-900 or 1100-1500 for the Ni(II)-treated protein. **(d)** UV-Vis (left) and CD (right) spectra for apo (black) or Fe(II)-treated (pink) $POP_{167/517}$ showing expected MLCT transition and intraligand charge transfer, respectively, in the presence of Fe(II). **(e)** A representative structure of Ni(II)-bound $POP_{167/517}$ from MD simulation shows coordination of two BpyAla residues and two waters to the Ni(II) center.

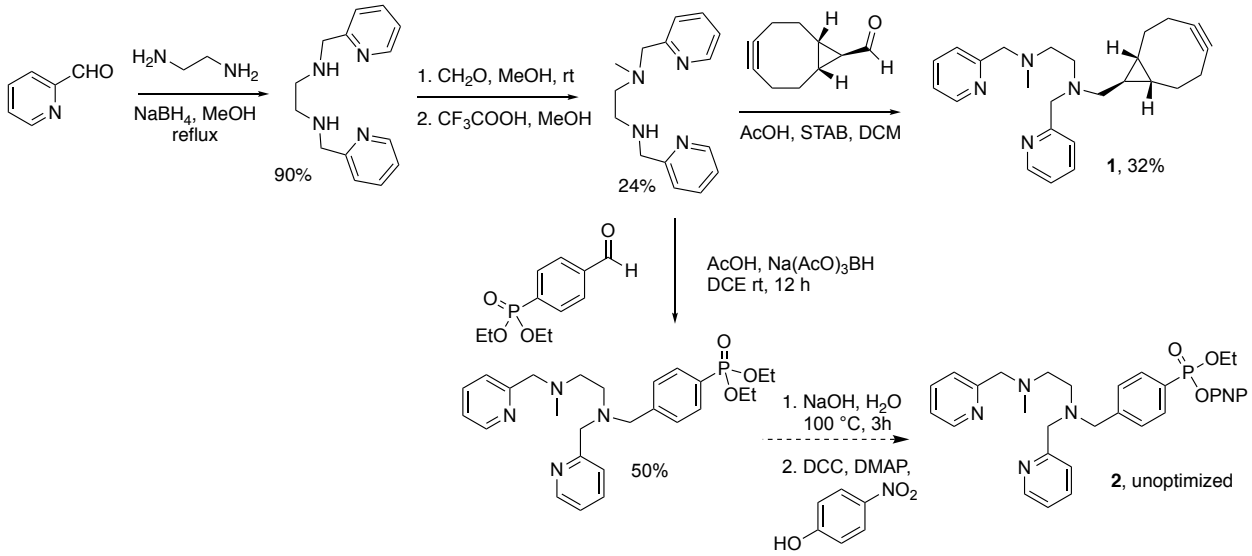


II. Objective 2: Evolve ArMs containing base metal cofactors that catalyze selective C-H functionalization of representative drug molecules and pesticides in aqueous solutions.

II. A. Cofactor Synthesis and Reactivity

Initial effort on objective 2 focused on the synthesis of base metal cofactors amenable to the ArM formation and evolution protocols outlined above. Toward this end, we completed the synthesis of BCN-substituted ligand **1** and phosphonate-substituted ligand **2** (Figure 14). Metallation of these ligands proved problematic due primarily to difficulties with characterization. Indeed, these difficulties led us to pursue the BpyAla approach to ArM formation outlined above.

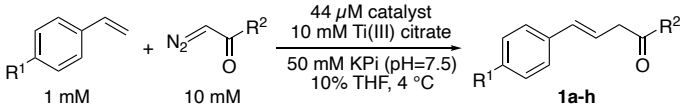
Figure 14. Synthesis of ligands **1** and **2**.



II. B. Non-native B12 Protein Catalysis

In considering different base metal cofactors that could be used for ArM formation, we were inspired to investigate the reactivity of naturally occurring B12-dependent proteins. B12, a Co-corrin complex, has been used to catalyze a number of synthetic transformations, including vinylic C-H functionalization, but none of this reactivity has been investigated using B12-dependent proteins. In early studies, we found that methionine synthase and methylmalonyl-CoA mutase could catalyze enantioselective olefin cyclopropanation. Notably, a different product that resulted from carbene insertion into a vinyl C-H bond of styrene was observed using the B12-dependent, light-activated transcription factor, CarH. Optimization of reaction conditions revealed that this reaction could be achieved in up to 60% yield with perfect selectivity for the E-product isomer (Figure 15). Several other electron rich styrenes were viable substrates for this reaction, and in all cases, CarH provided substantially higher conversion than B12 itself. A variety of kinetic isotope effect studies, solvent isotope effect studies, radical trapping experiments were then conducted to compare the reactivity of the B12 cofactor with CarH. Collectively, these studies suggest that the CarH scaffold can stabilize Co(III) alkyl intermediates along the catalytic cycle (e.g. Figure 16) to enable selective C-H functionalization, while B12 itself releases radical intermediates into solution where they promote non-selective styrene oligomerization (Figure 17). This study was recently published in *ACS Catalysis*.

Figure 15. Optimized CarH-catalyzed vinyl C-H insertion reaction.



Entry	**1**	R^1	R^2	Yield (%)[a]	
				B12	CarH*
1	**a**	OMe	OEt	22	57
2	**b**	OEt	OEt	11	22
3	**c**[b]	NMe_2	OEt	20	49
4	**d**	NHAc	OEt	15	97
5	**e**	Me	OEt	10	25
6	**f**[b]	H	OEt	8	29
7	**g**	OMe	OPh	13	32
8	**h**	OMe	NEt_2	14	68

Figure 16. A) Geometry-optimized structure of a ethyl carboxymethyl-B12 intermediate docked into a structure of CarH. B) Close-up of active site showing narrow, hydrophobic substrate binding channel between two helices of the 4-helix cap of CarH (van der Waals surface rendered).

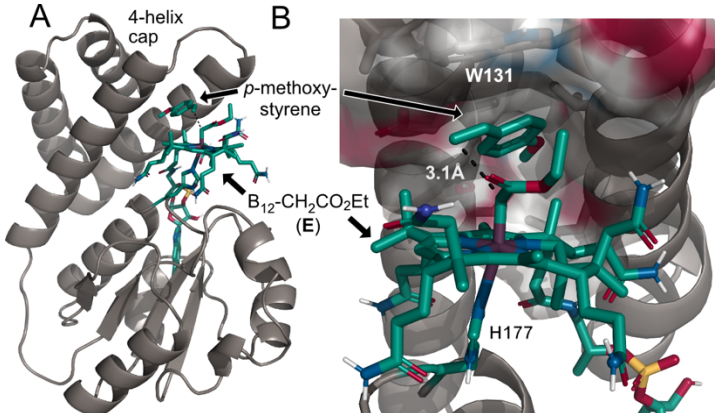
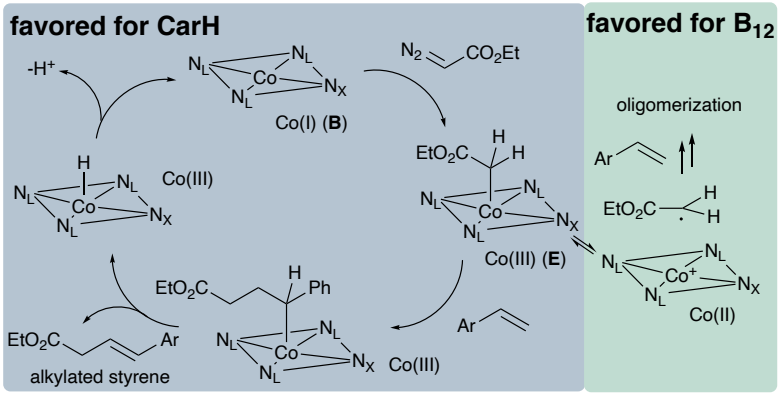


Figure 17. Mechanism of styrene alkylation accounting for differences in selectivity between CarH* and free hydroxycobalamin.



III. Objective 3: Develop cofactor activation approaches that allow the use of chemically inert cofactors to form reactive ArMs only upon scaffold activation.

Fortuitously, our studies on non-native catalysis by CarH showed that this enzyme also catalyzes an N-H insertion reaction analogous to the ArM-catalyzed reaction shown in Figure 11. Unlike the dirhodium cofactor used in our ArM studies, however, B12 is *completely unreactive* toward this chemistry in the absence of its protein scaffold. We believe that this activation results from second-sphere H-bonding by an Asp/Thr chain to the His residue (H177, visible in Fig. 16) that binds to the B12 cofactor. We have found that mutating either the Asp or the Thr residues reduces CarH N-H insertion activity, and we are currently collaborating with a computational chemist to model these effects. Broad N-H insertion scope has been established, and we are also now isolating the resulting products to round out an initial publication on this system.

Importantly, B12 activation by CarH will greatly facilitate directed evolution of this enzyme. B12 must be added to the reaction medium to generate the holo enzyme, but because the cofactor is unreactive unless it binds to CarH, the scavenging strategy required for our dirhodium ArMs is not needed. While we proposed to achieve such activation for a truly artificial metalloenzyme, the current system, which involves a protein scaffold that natively acts as a transcription factor rather than an enzyme, and a cofactor that must be added to reconstitute the holo enzyme *in vitro*, is obviously very similar to our ArM designs. Initial evolution efforts will focus on enantioselective variants of the N-H insertion chemistry noted above.

Dissemination

The research outlined above was disseminated via publications and talks given by the PI and by graduate students. The work was also promoted on the PI's website and on Twitter.

Talks

2021
Novartis Chemistry Lectureship, Basel, Switzerland, 6/16
AbbVie Platform Chemistry Technology Seminar, 5/12
University of British Columbia, Okanagan, 3/16

2020
San Diego State University, 10/16 (virtual)
Innovative Screening Strategies for the Directed Evolution of Enzymes, Pittcon, Chicago, IL, 3/4
University of Toledo, Department of Chemistry 2/24
RSC Applied Late-Stage Functionalization Symposium, University of Manchester, UK, 2/18-19
Third International Symposium on Carbene and Nitrene Chemistry, San Antonio, TX, 2/5-7

2019
ArtZymes Conference, Basel, Switzerland, 8/9
Telluride Science Research Conference (The Future of C-H Functionalization), 7/29-8/2
Society for Industrial Microbiology meeting, Washington D.C, 7/24
Novartis Chemistry Lectureship, Cambridge, MA, 7/18
International Symposium for Applied Bioinorganic Chemistry, Nara, Japan, 6/4
Marquette University, Department of Chemistry, 4/26

Publications (citing either 19-1-0074 or 18-1-0034)

1) Zubi, Y. S.; Seki, K.; Li, Y.; Hunt, A.; Liu, B.; Roux, B.*, Jewett, M. C.*, Lewis, J. C.* Metal-Responsive Regulation of Enzyme Catalysis Using Genetically Encoded Chemical Switches. In revision at *Nature Communications*. Preprint available on ChemRxiv: 10.33774/chemrxiv-2021-9ql74.
2) Zubi, Y. S.; Liu, B.; Gu, Y.; Sahoo, D.; Lewis, J. C.* Controlling the Optical and Catalytic Properties of Artificial Metalloenzyme Photocatalysts Using Chemogenetic Engineering. *Chem. Sci.* **2022**, in press (DOI: 10.1039/d1sc05792h).
3) Yang, X.; Gerroll, B. H. R.; Jiang, Y.; Kumar, A.; Zubi, Y. S.; Baker, L. A.; Lewis, J. C.* Controlling Non-Native B12 Reactivity and Catalysis in the Transcription Factor CarH. *ACS Catal*. **2022**, *12*, 935-942.
4) Upp, D. M.; Huang, R; Li, Y.; Bultman, M. J.; Roux, B.*, Lewis, J. C.* Engineering Dirhodium Artificial Metalloenzymes for Diazo Coupling Cascade Reactions. *Angew. Chem. Int. Ed.* **2021**, 60, 2-7.

Honors

2019 Novartis Chemistry Lectureship

Training

Technical experience gained by students working on this project include advanced techniques in organic/organometallic synthesis, molecular biology, directed evolution, protein purification, bioconjugation methods, and high throughput experimentation.

All students involved in the project participate in formal bi-weekly research meetings. These involve presenting research from a formal written report and providing feedback on the progress of others. Students also present their research at one-two group meetings per year and receive a formal evaluation of their progress from me each year. We meet to discuss research progress, plans following graduation/completion of postdoc, and how research plans can be modified to better prepare individuals for next steps. These meetings were all conducted via Zoom for the last two years of this project.

In addition to the technical research talks noted above, students also give one-two group meeting presentations per year on topics outside of their research. They regularly present results from the literature prior to our group meeting presentations. Students and postdocs can speak at a student/postdoc-led seminar series affiliated with the IU NIH QCB training grant and a regular biophysics "supergroup" meeting sponsored by various faculty members (including the PI). Again, all of this was virtual for the last two years of this project. While students are normally encouraged to present their results at both ACS National Meetings and the Biocatalysis and Metals in Biology Gordon Research Conferences, these were virtual for most of this project, and no students were interested in participating in these events. All students are responsible for maintaining several instruments in the group and attending seminars (virtually) in the Department of Chemistry and the Department of Molecular and Cell Biology at IU. Both Atreyi Bhattacharya and David Upp were also involved the NSF-funded CCHF, which allowed them to present their research with a range of researchers in the CCHF. Students write manuscripts describing their results, and I edit these to assist in their scientific writing development.

During the 3-month research shutdown at IU due to COVID-19, students involved in this project wrote up experimental information for publication, worked on their thesis, and learned a number of computational modeling tools that have already proven useful for the proposed research now that research has started up again. It should be noted that several group members did not return to lab for 1-2 months after they were allowed to by the University due to concerns for their own health. Our lab occupancy was capped at 50% for nearly a year, which significantly reduced our research efforts.

Students
Alan Swartz
David Upp (Dissertation)
Atreyi Bhattacharya
Dr. Xinhang Yang
Dr. Dipankar Sahoo
Dr. Rui Huang