# Proposal 1

Title -Computational and Experimental Investigation of a Novel Catalytic Residue and in LmrR.

In collaboration with Roelfes Lab (University of Groningen)

## State Of the Art

Artificial enzymes are engineered biocatalysts that enable new-to-nature transformations with the precision and selectivity of natural enzymes, but often for reactions that are not found in biology. In the context of LmrR-based artificial enzymes, a dimeric transcription factor from *Lactococcus lactis* with a large hydrophobic pocket, which can be engineered to host abiological catalytic functionalities, which use non-natural amino acids such as para-aminophenylalanine (pAF) to catalyze reactions. LmrR\_pAF efficiently catalyzes Friedel–Crafts alkylation and hydrazone formation, with pAF acting as the nucleophilic residue that forms iminium intermediates with carbonyl substrates. However, the molecular mechanisms by which these mutations exert their effects remain incompletely understood.

The primary purpose of developing this artificial enzyme was to create a biocatalyst that could efficiently catalyze hydrazone and oxime formation reactions, transformations important in bioconjugation and chemical biology, but not naturally catalyzed by enzymes. By embedding pAF into the hydrophobic pocket of LmrR, the researchers aimed to. These choices represent typical aromatic aldehydes and nucleophiles commonly used in bioconjugation chemistry. The enzyme catalyzes the reaction between these two classes of compounds, forming either a hydrazone or an oxime linkage depending on the nucleophile present

LmrR is not an enzyme in its natural form. It is a transcriptional regulator protein, not a natural catalyst. LmrR naturally binds ligands. Its biological function involves binding various hydrophobic molecules, including antibiotics and other drugs, at its large, hydrophobic dimer interface.

The artificial enzyme is partially artificial: The protein scaffold (LmrR) is natural. The catalytic activity is artificial, created by introducing a non-canonical amino acid (such as p-aminophenylalanine). The resulting construct is an artificial enzyme with catalytic properties not found in nature.

## How Was It Generated?

Researchers used protein engineering to introduce a non-canonical amino acid (e.g., p-aminophenylalanine) at a specific position (such as V15) in the LmrR protein.

This was achieved by expanding the genetic code of the host organism to allow site-specific incorporation of the unnatural amino acid during protein expression.

The modified LmrR protein then serves as a scaffold, with the new residue providing the desired catalytic functionality.

## Role of pAF in the Reaction

The aniline group on pAF acts as a nucleophilic catalyst by attacking the aldehyde carbonyl group. This forms an iminium ion intermediate. This iminium ion is more reactive toward nucleophiles (like hydrazines) than the original aldehyde. The hydrazine can then attack this activated intermediate more efficiently. After the hydrazine addition, the aniline catalyst is released, completing the catalytic cycle.

## Chemical Reaction

Complete Reaction Sequence:

1. Step 1: Hemiaminal Formation
   * Benzaldehyde + pAF-NH₂ → Hemiaminal intermediate
   * Barrier: 15.2 kcal/mol (rate-determining step)
2. Step 2: Dehydration
   * Hemiaminal → Iminium ion + H₂O
   * Barrier: 8.7 kcal/mol
3. Step 3: Transimination
   * Iminium ion + NBD-H → Hydrazone product + pAF-NH₂
   * (This step regenerates the catalytic pAF residue)

The QM calculations specifically focused on steps 1 and 2 (the iminium ion formation pathway), showing how the evolved H93 residue acts as a general acid catalyst and how R92 provides stabilizing hydrogen bonds.

Hydrazone formation is a nucleophilic addition–elimination reaction between an aldehyde (or ketone) and a hydrazine (or hydrazide):

* Reactants: Aldehyde (e.g., 4-hydroxybenzaldehyde) + Hydrazine derivative (e.g., NBD-H)
* Product: Hydrazone + Water

## Question

Can a non-natural amino acid other than pAF, when incorporated at the catalytic site of LmrR enhance iminium-based catalysis of hydrazone formation?

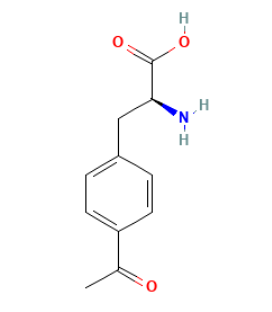
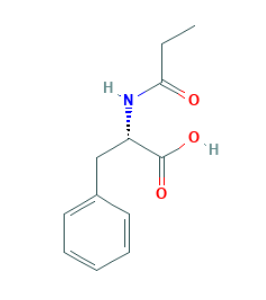
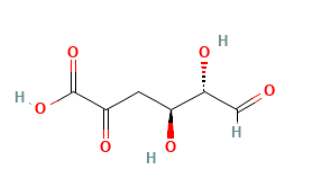
## Hypothesis

Substituting a computationally selected non-natural amino acid (nnAA) at the catalytic site of LmrR, might enhance the enzyme’s catalytic efficiency.

## What do we know about the other non-canonical amino acids

Goal - To Find Viable Alternatives to pAF for Iminium Catalysis

|  |  |  |  |
| --- | --- | --- | --- |
| Property | p-Acetylphenylalanine (pAF) | p-Propionylphenylalanine | Formylglycine (fGly) |
| Side chain functional group | para-acetyl (–COCH₃) | para-propionyl (–COCH₂CH₃) | aldehyde (–CHO) |
| Carbonyl group type | Ketone | Ketone | Aldehyde |
| Reactivity in hydrazone ligation | High (widely used) | High (slightly less than pAF) | Very high (aldehydes > ketones) |
| Steric bulk | Small | Larger (longer alkyl chain) | Minimal (smallest side chain) |

ncAAs that can participate in hydrazone formation are those that introduce a carbonyl group (aldehyde or ketone) into the protein, such as p-acetylphenylalanine (pAF), p-propionylphenylalanine, and formylglycine (fGly). These are the ncAAs described as suitable for hydrazone bioconjugation.

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## Objectives

1. Computationally select and model promising non-canonical amino acids (ncAAs) for iminium catalysis at the LmrR catalytic site.
2. Experimentally incorporate the selected ncAAs
3. Assess catalytic activity for hydrazone formation and compare to pAF-containing LmrR variants to wild type LmrR

## Workflow

## 1. Computational Selection

* Use LmrR structures (6I8N) to model incorporation of new (selected) ncAAs at V15.
* Parameterize EVB for hydrazone mechanism
* Perform Empirical Valence Bond (EVB) simulations on pAF\_LmrR and with the new ncAAs to estimate and compare activation energies with the wild type

2. Gene Design and Mutagenesis

* Use the LmrR structure (PDB: 6I8N) as a template, focusing on position V15, which is known to accommodate unnatural amino acids like pAF.
* Design gene constructs with an amber (TAG) codon at V15 for site-specific incorporation of the selected ncAAs.

3. Plasmid Construction and Preparation

* Clone the mutated LmrR gene into an appropriate expression vector.
* Prepare compatible plasmids encoding the orthogonal tRNA/synthetase pair for the selected ncAA.

4. Expression of LmrR Variants

* Transform E. coli with both the LmrR expression plasmid and the tRNA/synthetase plasmid.
* Grow cultures in media supplemented with the selected ncAA
* Induce protein expression under optimized conditions (e.g., IPTG induction, 18–25°C overnight).

5. Protein Purification

* Harvest cells and lyse using standard protocols (e.g., sonication or chemical lysis).
* Purify LmrR variants using affinity chromatography (e.g., His-tag purification).
* Analyze protein purity and confirm ncAA incorporation by SDS-PAGE and mass spectrometry.

6. Catalytic Activity Assays

* Set up hydrazone formation reactions using standard substrates (e.g., benzaldehyde and hydrazine derivatives).
* Monitor product formation by UV/Vis spectroscopy or HPLC
* Compare catalytic activity (turnover frequency, of new ncAA-LmrR variants to pAF-LmrR

6. Data Analysis and Iteration

* Analyze kinetic data to determine the effect of the new ncAA on catalytic efficiency.

## Timeline

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| --- | --- | --- |
| **Week(s)** | **Step** | **Tasks** |
| 6 | Computational Selection | - Model ncAA incorporation at V15 using 6I8N structure  - Parameterize EVB for hydrazone mechanism  - Run EVB simulations for pAF\_LmrR and new ncAAs  - Select top candidates based on activation energies |
| 3 | Gene Design and Mutagenesis | - Design gene constructs with amber (TAG) codon at V15  - Order primers and perform site-directed mutagenesis |
| 2 | Plasmid Construction and Preparation | - Clone mutated LmrR gene into expression vector  - Prepare/obtain orthogonal tRNA/synthetase plasmids |
| 3 | Expression of LmrR Variants | - Transform E. coli with both plasmids  - Grow cultures with ncAA supplementation  - Induce protein expression |
| 3 | Protein Purification | - Harvest and lyse cells  - Purify LmrR variants via affinity chromatography  - Confirm protein purity and ncAA incorporation (SDS-PAGE, MS) |
| 2 | Catalytic Activity Assays | - Set up hydrazone formation assays with standard substrates  - Monitor product formation (UV/Vis, HPLC)  - Compare activity to pAF\_LmrR |

Problems

* Specialized Resources: Incorporation of non-canonical amino acids (ncAAs) requires access to specific tRNA/synthetase systems, which may not be readily available or may have long lead times.
* The tight schedule leaves little room for troubleshooting, optimization, or repeating failed experiments.