# Proposal 2

Title - Mapping Catalytic Hotspots in LmrR: A Synergistic Computational and Experimental Approach.

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. (evolutionary ref)

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

How do alanine substitutions around active site and distal residues, relative to the pAF (non-canonical amino acid) position in LmrR influences the enzyme’s catalytic activity.

## 

## Hypothesis

Carrying out free energy calculations by EVB with an alanine scanning approach will reveal which residues are essential for catalysis, as indicated by significant decreases in predicted activation energies.

## How This Proposal Advances Previous Enzyme Engineering Efforts

* Predictive Power for Unexplored Mutations  
  While earlier studies identified beneficial mutations through experimental screening, this proposal leverages EVB simulations to systematically predict the effects of alanine substitutions across both active site and distal residues, including those not yet explored in the lab.
* Mechanistic Insight Into Catalytic Contributions  
  By integrating EVB-based free energy calculations with alanine scanning. It quantifies how each residue, whether in the active site or at a distance, affects the enzyme’s activation barrier and overall catalytic mechanism. This mechanistic understanding explains why certain positions are important, not just which ones are beneficial.

## Objectives

1. Predict the impact of each alanine mutation on catalytic activity using EVB simulations to estimate changes in activation energy
2. Identify new key residues that are critical for LmrR catalysis using free energy barriers
3. Generate and express alanine mutants of LmrR experimentally and measure their catalytic activity using established assays

## Workflow

## Computational Alanine Scanning

* Identify target active site and distal residues for alanine scanning. (based on literature, and previous data).
* Use LmrR structures (6I8N) to model incorporation of new (selected) ncAAs at V15.
* Parameterize and run EVB for hydrazone mechanism
* Rank mutants by predicted catalytic impact and select top candidates for experimental testing.

## Mutagenesis & Cloning

* Design primers and perform site-directed mutagenesis for prioritized alanine mutants.
* Transform into E. coli and start cultures.
* Sequence-verify clones (use colony PCR and send for sequencing as soon as colonies are available).

## Expression & Small-Scale Purification

* Express LmrR alanine mutants in small-scale cultures

## Activity Assays

* Set up and run hydrazone formation assays for all purified mutants and controls.
* Analyze reaction rates and compare them to computational predictions.

## Data Integration & Reporting

* Correlate experimental activity data with EVB-predicted activation energies.
* Prepare summary

## Timeline

| **Week** | **Step** | **Key Actions** |
| --- | --- | --- |
| 6 | Computational Alanine Scanning | Model, simulate, and prioritize alanine mutants |
| 1 | Mutagenesis & Cloning | Generate and verify alanine mutants |
| 1 | Expression & Purification | Express and purify mutants (small-scale, rapid protocols) |
| 2 | Activity Assays | Run catalytic assays and collect data |

For Feasibility

* Focus: Limiting the number of mutants for experimental validation based on computational results.

## 