Exploiting Catalytic Promiscuity for Biocatalysis

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Carbon-Carbon Bond Formation by a Proline-Based Tautomerase

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"It is a slightly arresting notion that if you were to pick yourself apart with tweezers, one atom at a time, you would produce a mound of fine atomic dust, none of which had ever been alive but all of which had once been you."

Bill Bryson

A Short History of Nearly Everything

Paranimfen Harshwardhan Poddar
Jan-Ytzen van der Meer

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Aim and outline of this thesis

The history of enzymes as catalysts in organic synthesis dates back to 1858, when Louis Pasteur achieved the first kinetic resolution by treating a solution of racemic ammonium tartrate with a mold of *Penicillium glaucum* to enrich (-)-tartaric acid. However, the low accessibilities and relatively poor performances (i.e. narrow substrate scope, low activity and/or stereoselectivity) of enzymes under non-physiological conditions (when compared to other methodologies) limited their catalytic applications in the past. Due to the invention of recombinant DNA technology and the development of directed evolution techniques in the last few decades, nowadays enzymes can be produced in large quantities with tailor-made properties. In addition, the rapidly growing number of publicly available sequences in gene banks and protein databases has significantly expanded the enzyme toolbox for practical applications. These developments make enzymes increasingly appealing catalysts for use in organic synthesis.

In particular, enzymes that can efficiently catalyze important carbon-carbon (C-C) bond-forming reactions, like the Aldol, Michael, Henry, Mannich, Diels-Alder, and Knoevenagel reactions, are of great interest for synthetic applications. The development of enzymatic systems for these types of reactions is highly challenging but also very attractive, because enzymes can generally be used under mild reaction conditions, could provide high selectivity (i.e. chemo-, regio-, and stereoselectivity), and, in some cases, may even catalyze reactions that are otherwise not possible.

Although enzymes are known for their high specificities, many existing enzymes were found to catalyze reactions other than their biologically relevant ones. This remarkable property is defined as catalytic promiscuity. Enzyme promiscuity is thought to play a key role in natural enzyme evolution and has become an important source for new enzymatic activities that can serve as starting points for laboratory evolution of novel biocatalysts. A number of enzymatic systems have been established for synthetically useful C-C bond-forming reactions with satisfactory efficiency and stereoselectivity. Notably, several of these methodologies involve the use of catalytically promiscuous enzymes since enzymes that naturally catalyze these reactions are rare or have not been identified. In **Chapter 1**, we review recent advances in enzyme promiscuity for C-C bond-forming reactions, with a focus on enzymes that exhibit high enantioselectivities.

Recently, we have established a biocatalytic methodology for asymmetric Michael-type addition of acetaldehyde to *trans*-nitrostyrene using the proline-based enzyme 4-oxalocrotonate tautomerase (4-OT). This catalytic promiscuity of 4-OT was discovered via an envisioned catalytic mechanism: the N-terminal proline (Pro-1) of 4-OT forms an enamine intermediate with acetaldehyde, which acts as a nucleophile and adds to the double bond of *trans*-nitrostyrene yielding the corresponding Michael adduct. In **Chapter 2**, we describe the enamine donor scope of the 4-OT catalyzed Michael-type addition reaction. Surprisingly, linear aldehydes ranging from acetaldehyde to octanal are all accepted by 4-OT as enamine donors and added to the electrophile *trans*-nitrostyrene to form the corresponding Michael adducts.

Inspired by the success in extending the enamine donor scope of the 4-OT catalyzed Michael-type addition reaction, we further investigated the electrophile (i.e. Michael acceptor) scope of this synthetically useful reaction. In **Chapter 3**, we report the asymmetric Michael-type addition of acetaldehyde to a series of nitroolefins yielding enantioenriched γ-nitroaldehydes, which are valuable precursors of several marketed γ-aminobutyric acid (GABA)-based pharmaceuticals such as phenibut, baclofen and pregabalin. This novel enzymatic methodology is as efficient as the most potent organocatalytic procedure for this type of reaction and provides a greener alternative for the synthesis of GABA derivatives.

Although the 4-OT methodology for asymmetric Michael-type addition reactions is still far from industrial application, it is a so far unique approach for this type of reaction because the additions are enzyme-catalyzed, proceed in aqueous media, with high stereoselectivity, and include a broad range of substrates. In **Chapter 4**, we describe practical methods for the asymmetric synthesis of two important γ-nitroaldehydes using 4-OT. The protocol includes the expression and purification procedures for 4-OT, procedures for the preparative scale synthesis of the γ-nitroaldehydes using 4-OT, analytical methods for monitoring reaction progress and product characterization, as well as the materials and equipment that are required.

As the 4-OT catalyzed asymmetric Michael-type addition of acetaldehyde to nitroolefins gives convenient access to valuable chiral precursors for GABA derivatives, we are interested in further improving this biocatalytic approach by both protein and substrate engineering. In **Chapter 5**, we describe the 4-OT catalyzed Michael-type addition of acetaldehyde to a number of *ortho*-, *meta*-, and *para*-substituted β-nitrostyrene derivatives. We report that different electron donating (e.g. hydroxyl and methoxyl groups) and electron withdrawing (e.g. chloro and nitro groups) substitutions at the aromatic ring of the nitroolefin substrate can significantly influence the catalytic rates and enantioselectivity of the 4-OT catalyzed Michael-type addition reactions.

In addition to the Michael-type additions, we previously discovered that 4-OT promiscuously catalyzes the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde. Mechanism-inspired engineering provided an active site mutant (F50A) of 4-OT with strongly enhanced aldol condensation activity. In **Chapter 6**, we report that 4-OT and the 4-OT F50A mutant accept various carbonyl compounds as substrates for both inter- and intramolecular aldol reactions.

In **Chapter 7**, we summarize the work described in this thesis and present some future perspectives of this study.