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Highly engineered biocatalysts for efficient small molecule pharmaceutical synthesis

Jim Lalonde

Technologies for the engineering of biocatalysts for efficient synthesis of pharmaceutical targets have advanced dramatically over the last few years. Integration of computational methods for structural modeling, combined with high through put methods for expression and screening of biocatalysts and algorithms for mining experimental data, have allowed the creation of highly engineered biocatalysts for the efficient synthesis of pharmaceuticals. Methods for the synthesis of chiral alcohols and amines have been particularly successful, along with the creation of non-natural activities for such desirable reactions as cyclopropanation and esterification.

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In 2012, 'The Third Wave of Biocatalysis' [1] reviewed progress in engineering enzymes for the efficient synthesis of pharmaceuticals. Since that time, enzyme engineering has been accelerated by the integration of newly developed technologies for rational design and directed evolution. Creation of new enzymes with novel catalytic function, rare in the recent past, is now the norm. It is uncommon for wild-type enzymes to be used in the synthesis of a pharmaceutical given the dramatic enhancements in catalytic efficiency, stereoselectivity and stability that can be had through protein engineering.

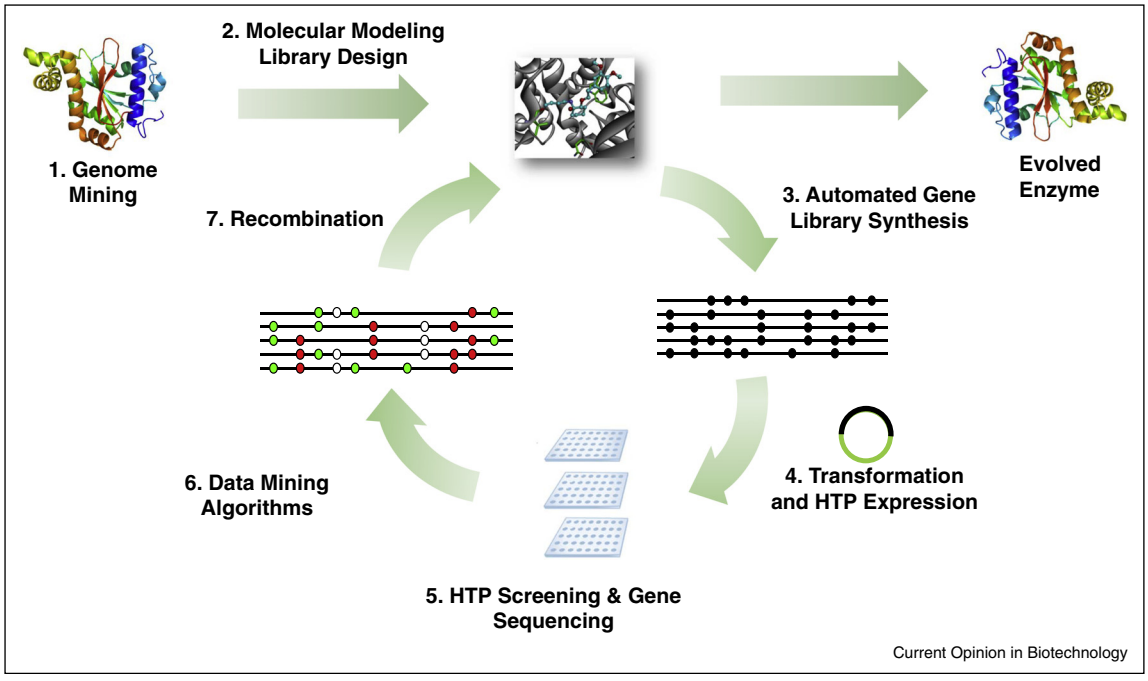
Advances in enzyme engineering

Advanced enzyme engineering technologies make possible the development of enzymes that are highly active on non-natural targets at high concentrations, in the presence of organic solvents, and even enzymes for chemical transformations not found in nature. These technologies help narrow the search of the immensity of protein sequence-activity space (estimated at $\sim 10^{11}$ for a 300 amino acid enzyme) [2] and focus on regions more likely to give improvements in enzyme activity and stability, to do so more rapidly and with much more information per experiment. The high levels of mutation needed to convert wildtype enzymes to suitable manufacturing catalysts means that simple random mutation cannot be used. Advanced engineering technologies, rather than being competitive or exclusive, can be powerfully combined (Scheme 1) to give enzymes with large changes in functional properties. Firstly, the exponential growth of genomic information provides starting points for enzyme engineering along with functional diversity from closely related homologs for recombination [3]. While we are far from the *de novo* design of enzymes [4,5], protein structural modeling, computational enzyme design [6,7] *in silico* substrate docking methods, and molecular dynamic simulation methods [8] serve to focus the design of libraries of variants. High through-put automation of enzyme test methods has been developed for activity, expression [9] protein analytics [10], and ultra high through-put selection methods [11•]. Advances in DNA technologies for low cost synthesis, PCR based library construction and microfluidics chip-based DNA sequencing methods [12], computational data analytics including machine learning algorithms [13•,14,15] substantially increase the information content/experiment. Application of these advanced engineering technologies to the creation of biocatalysts for pharmaceutical manufacturing typically starts with an integrated approach with establishment of initial catalytic activity for the reaction of interest through screening or design, followed by iterative cycles of mutation and selection to create highly stable and active enzymes with novel function.

Asymmetric ketone reduction (KREDs)

Syntheses of pharmaceutical intermediates and active pharmaceutical ingredients (APIs) have incorporated highly engineered enzymatic steps most commonly for the installation of chirality and the elaboration of scaffolds. The asymmetric reduction of ketones to chiral alcohols using engineered enzymes has been particularly successful. The efficiency and volumetric productivity of these biocatalytic routes to chiral alcohols is such that it appears that this technology has eclipsed all chemocatalytic methods for asymmetric reduction of ketones. The scope of the well-established enzyme platforms of ketone reductases has been expanded by numerous groups [16,17] and there has been broad adoption of these biocatalysts in synthetic routes to APIs. Development

Scheme 1



Integration of advanced technologies for enzyme engineering.

of a highly evolved KRED for the asymmetric reduction of a particularly challenging substrate; a near symmetrical ketothiolane [18•] for the antibiotic sulopenem is illustrative. The backbone of the KRED derived from a *Lactobacillus kefir* KRED and the closely related *L. brevis*, has since proven to be highly malleable with engineered variants providing efficient routes to several pharmaceutical targets such as S-licarbazepine [19], and montelukast [20]. Recent kinetic characterization and molecular dynamic studies on this family of enzymes provides some insight into the source of this malleability [21••]; a flexible loop encompasses one side of active site and its flexibility can be controlled through mutations in and near this loop. Similarly, Reetz has developed KREDs for the enantiospecific reduction of a near symmetrical ketooxolane by engineering the active site of the enzyme from *Thermoethanolicus brockii* [22].

Transaminases (TAs)

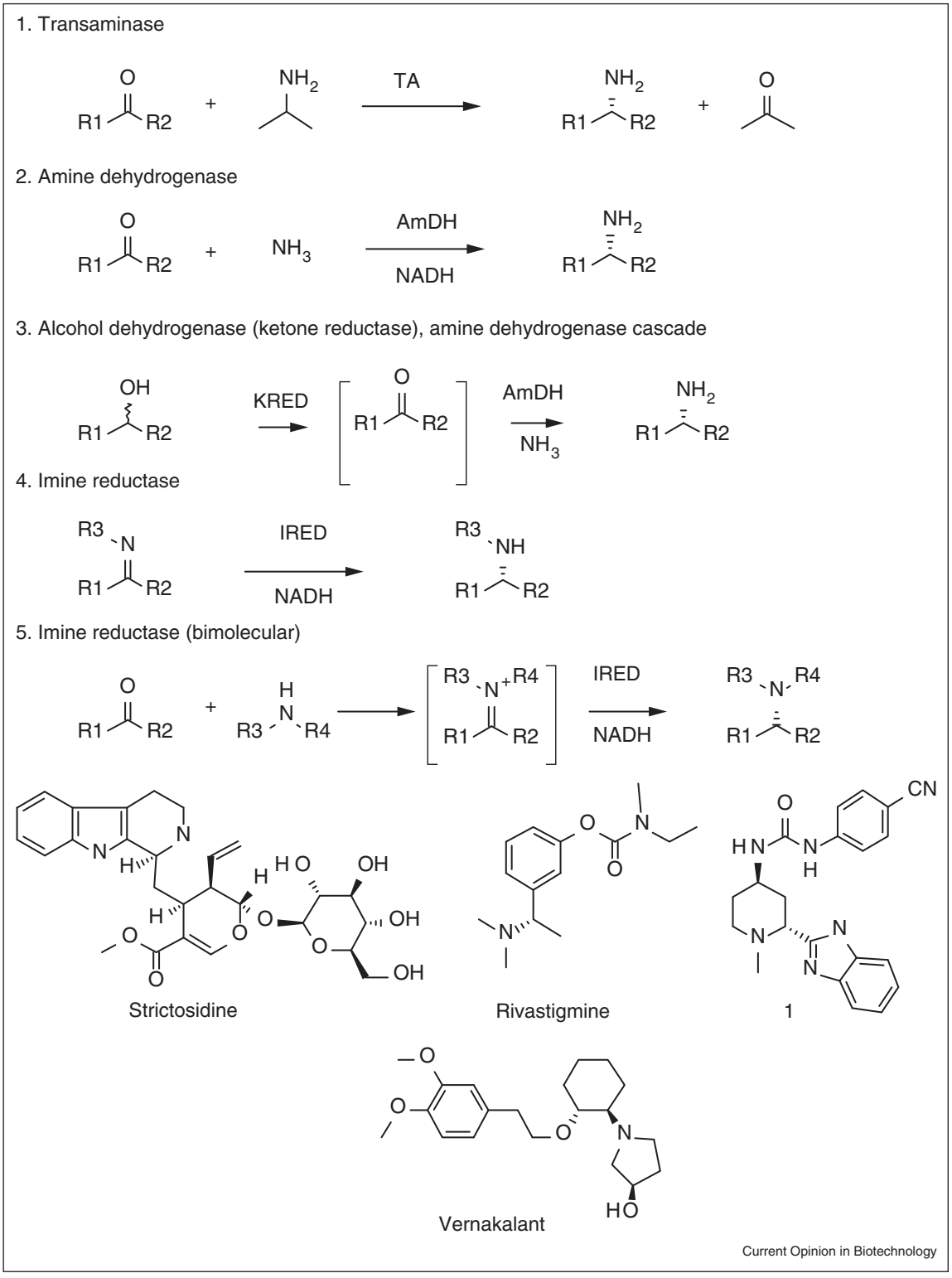
There has been a tremendous amount of activity in the development of enzymes for chiral amine synthesis in the last three years. While a large proportion of pharmaceutical actives contain chiral amines, most of these were synthesized using wasteful kinetic resolutions until very recently [23]. Novel biocatalytic approaches for the efficient asymmetric synthesis of chiral amines are outlined in Scheme 2. The most established of these is the use of engineered ω-transaminases [24], which catalyze the conversion of ketones to chiral amines using a donor such as isopropylamine and pyridoxidal phosphate as a cofactor as exemplified by the large scale manufacture of sitagliptin [25]. Such evolution programs can provide tens of thousands of variants as progeny for subsequent development. A highly selective transaminase for production of rivastigmine [26], for example, was derived by evolution of progeny from sitagliptin evolution.

Truppo *et al.* [27,28] have reported an improved asymmetric synthesis of vernakalant, an investigational antiarrhythmic agent, using an evolved transaminase dynamic kinetic resolution (DKR) process. Under alkaline conditions, the α-ether chiral center is rapidly equilibrating between the two enantiomers. The enzyme was evolved for selectivity for both the desired configuration at the α-ether center and the newly formed C–N bond to set both chiral centers in a single step. A DKR approach was also used by Wong *et al.* at Pfizer [29] using an evolved transaminase to enable a concise five step synthesis of an investigational leukemia treatment **1** in which the two chiral centers are set in the transamination step with >10/1 diastereoselectivity and >99% e.e.

Asymmetric reductive amination

Transaminases, while broadly used, suffer from the need to shift the relatively flat equilibrium. The position of equilibrium is such that a high degree of conversion is only possible if the equilibrium is shifted, for example, by removal of product. Reductive amination in contrast, in

Scheme 2



Biocatalytic routes to chiral amines.

which an intermediate imine is catalytically reduced, can be driven to high conversion by coupling regeneration of the nicotinamide hydride cofactor. Bommarius *et al.* [30] has developed amine dehydrogenases which catalyze the asymmetric reductive amination of ketones with ammonia. While this class of enzyme has long been used for the production of amino acids from alpha keto acids [31], these enzymes were inactive on simple ketones. Through domain shuffling of two amino acid dehydrogenases, Bommarius was able to develop engineered enzymes which can act on ketones having no α-carboxylate. Conversion is driven by coupling the reduction of the oxidized nicotinamide cofactor, for example, by the co-conversion of glucose to gluconate [32].

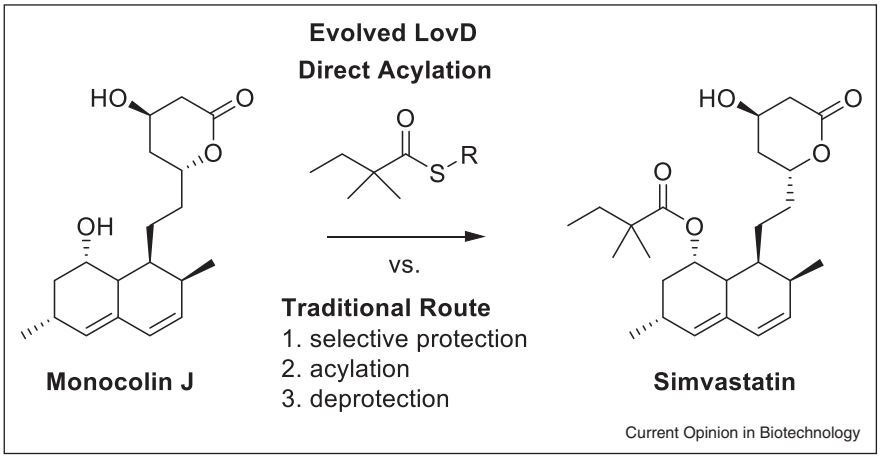
Enzyme cascades, or the use of two or more enzymes in a coupled process can extend the efficient synthesis of chiral amine drug targets to simple alcohols. Kroutil showed the use of a transaminase, alanine dehydrogenase and a KRED can afford the direct conversion of primary alcohols to primary amines [33]. Similarly, Faber *et al.* [34] have coupled amine oxidase for the oxidation, with amine dehydrogenase and cofactor recycling to convert alcohols to primary amines. Very recently, Turner *et al.* [35••] and Xu *et al.* [36] have extended the utility of these cascades to the direct conversion of secondary alcohols to chiral amines in high optical purity. In these inventive 'hydrogen borrowing processes', there is no need for an exogenous cofactor recycling system as the overall process is redox neutral. Turner *et al.* [37] also has developed a *chemoenzymatic* cascade to produce 2,5 disubstituted pyrrollidines in high stereoisomeric purity in one pot by coupling a transaminase catalyzed amination of a diketone with spontaneous cyclization to the imine, non-specific borane reduction in the presence of a selective amine oxidase to convert the undesired isomer back to the imine, thus enriching the optical purity for the targeted stereochemistry.

A newly developed class of biocatalyst, imine reductase, rather than simple conversion of ketones to primary amines, allows for the formation of chiral secondary and tertiary amines via asymmetric reduction of imines [38]. Imines can be preformed before the enzymatic step or formed concomitant with the enzymatic conversion in a one-pot intramolecular reductive coupling of an amine and ketone. Such reductive amination is rare in nature; however the opine dehydrogenase *Arthrobacter* sp. reductively couples amino acids with keto acids such as pyruvate in an intermolecular fashion [39]. In this regard, Mueller *et al.* [40] and Nestl *et al.* [41] have demonstrated activity for the intermolecular stereoselective reductive coupling of simple amines and ketones to give chiral secondary and tertiary amines with imine reductase genetic variants keto to give low yield. Using the *Arthrobacter* sp. opine reductase as a starting point, Merck and Codexis employed iterative rounds of mutation and recombination to create variants that catalyze reductive amination a range of amines and ketones [42,43••]. This ability to reductively couple amines and ketones, if proven a general approach, represents a biocatalytic convergent synthetic strategy with remarkable atom-economy.

Acylases

Chemospecific synthesis of highly hindered esters without the need for protecting groups is highly desirable. Tang *et al.* [44] demonstrated the adaptation of an acylase for the direct production of the blockbuster cholesterol treatment Simvastatin from Monocolin J (MJ) (Scheme 3). Traditional chemical routes to **5** require the use of protecting groups followed by either lithiation and methylation or acylation and deprotection. Using the acyl

Scheme 3



Direct acylation route to simvastatin.

donor activated as a thioester linked to a large protein (ACP), LovD from *Aspergillus terreus* catalyzes the alpha methyl butyrylation of MJ to give Lovastatin. Tang demonstrated initial activity replacing the acyl protein complex with a simple thiobutyryl ester and improved this initial activity fivefold using a directed evolution approach. Subsequently [45,46], the performance of the enzyme was improved a further three orders of magnitude in nine rounds of PROSAR guided evolution. Structural analysis of the evolution lineage gave no indication of the source of the dramatic increase in catalytic efficiency, however molecular dynamics studies [47••] implicated active site preorganization as a mechanism for catalysis. Interestingly, this active site preorganization is predicted in the formation of the acyl-ACP-LovD protein–protein complex in the native pathway, but was lacking in the low molecular weight thioester LovD interaction. Thus, blind evolution appears to have restored the active site preorganization catalytic mechanism to the evolved monomeric protein.

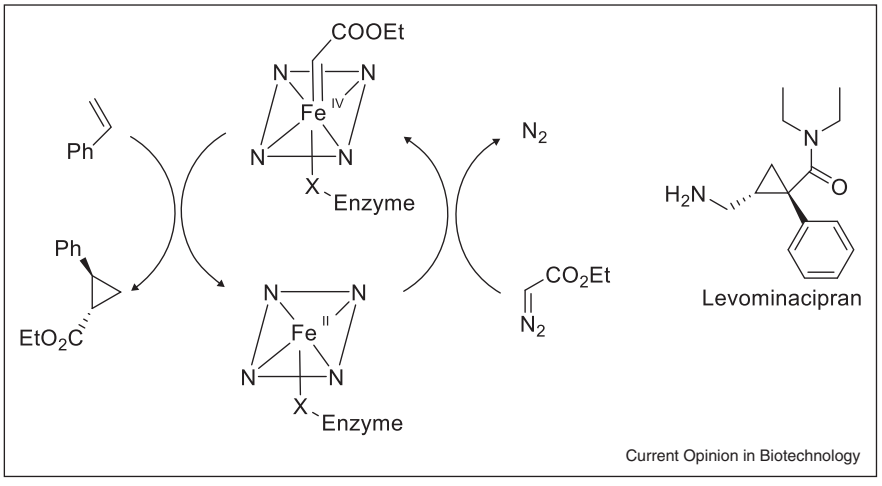
Creation of non-natural activity in enzymes

The engineering of enzymes with novel catalytic function often starts by taking advantage of an existing side activity of an enzyme from nature. Indeed, such catalytic promiscuity [48] is thought to be nature's method for creating new catalytic activity through the formation of intermediate enzymes with dual function; retaining the old function while developing a new activity giving an evolutionary edge [49].

Cofactor dependent enzymes, which employ low-molecular weight organic groups that resemble synthetic catalysts in function and structure, have been used to generate enzymes with non-natural activities that are desirable to synthetic organic chemists [50]. The Arnold lab [51,52] has used this chemomimetic approach to convert P450 monooxygenase enzymes to ones capable of carbene [53••] and nitrene [54,55] insertion reactions. Cyclopropanation, C–H amination, N–H insertion and aziridination, reactions that have no counter-part in nature, have been demonstrated with these P450 enzymes. The natural activity of P450 enzymes is formation of a reactive oxygen species capable of epoxidation of olefins and hydroxylation of unactivated C–H bonds. P450 monooxygenases were evolved to accept carbene and nitrene donors to form reactive carbene and nitrene Fe-porphyrin species (Scheme 4). An axial cysteine sulfur is ligated to the porphyrin bound Fe in all known P450 monooxygenases. By substitution of this cysteine with an axial serine (X=O) or histidine ligating residue variants capable of cyclopropanation can be generated. Mutation of an active site threonine, which is thought to assist in O–O bond cleavage in the oxidation cycle, also increased carbene and nitrene insertion activity. Subsequent improvement of catalyst turnover, substrate selectivity and stereoselectivity in variants were then optimized through extensive protein engineering. In one such example, an efficient synthesis of Levominacipran was developed [56••].

Lewis has developed a hybrid transition metal/enzymatic approach to construction of artificial metalloenzymes, using unnatural amino acids for site specific metal complex conjugation. A [57] cyclopropanation biocatalyst was developed utilizing an unnatural amino acid for linkage of the metal catalyst. An artificial dirhodium metalloenzyme was constructed by substitution of a 4-azido phenylalanine for an active site cysteine to provide a specific linkage site in an enlarged designed cavity of an oligopeptidase. Azide–alkyne coupling to a strained bicyclo alkyne provided an attachment site for a dirhodium carboxylate complex which resulted in an artificial metalloenzyme capable of catalyzing cyclopropanation of styrene using with diazocarboxylate donors in high e.e. [58].

Scheme 4



Enzymatic cyclopropanation and aziridination.

Future direction

Success in the development of biocatalysts for pharma such as we have seen in the last few years will likely continue unabated. Asymmetric reduction of ketones with KREDs and chiral amine synthesis with ATAs are well entrenched in API manufacturing, while platforms such as reductive amination will follow fast. The intersection of Chemical Development activities in pharma companies with advances in enzyme engineering will build upon promising initial efforts in development of desirable enzyme platforms such oxidation of unactivated C–H bonds and protecting group-free amide coupling [11•]. Synthetic biology allow creation of cell factories in which engineering pathways of enzymes, mostly limited to natural products (e.g. artemisin [59]) will be extended by the incorporation of non-natural transformations as seen in the production of semi-synthetic opioids in yeast [60••].

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have been highlighted as:

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- •• of outstanding interest

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