Enzymes

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Expanding the Enzyme Toolbox for Biocatalysis

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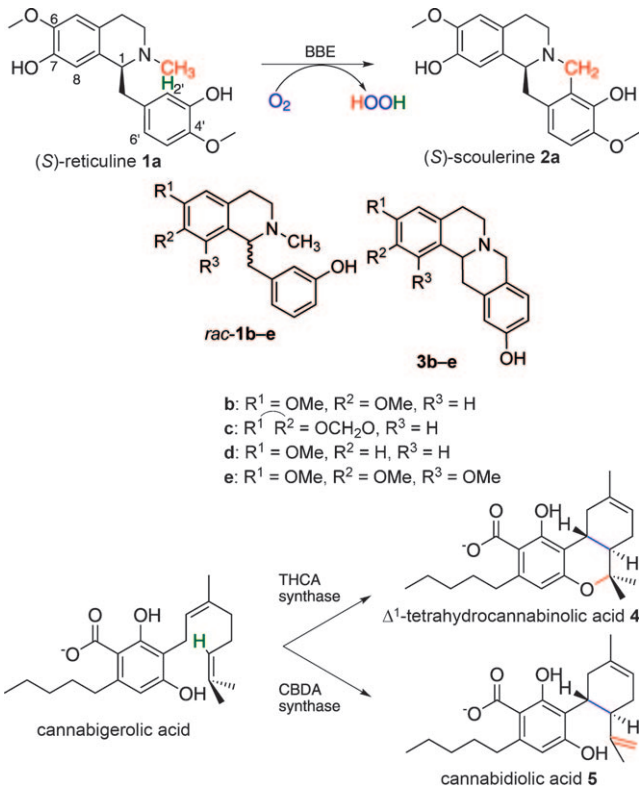
The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology maintains a database of enzymes (http://www.enzyme-database.org) for the enormous task of classifying and categorizing the 5174 currently identified distinct enzymatic activities by using guidelines established by the Enzyme Commission (EC).[1] This database is filled with enzymes that have not been explored for utilization of their unique catalytic activities.

An example of an enzyme that has been added to the enzyme database is the berberine bridge enzyme (BBE) [EC 1.21.3.3][2a,b] which catalyzes the conversion of the tetrahydroisoquinoline (*S*)-reticuline (**1a**) into (*S*)-scoulerine (**2a**; Scheme 1). BBE was first isolated from *Berberis beaniana* in 1985,[2c] but BBE activity was identified from cell extracts of *Macleaya microcarpa* in 1975.[2d] BBE is an FAD-dependent oxidase responsible for an intramolecular C–C bond coupling between the *N*-methyl group and the 2′-carbon atom of the benzyl moiety of **1a** (Scheme 1), thus forming the "berberine bridge" through a chemical reaction that is unique to enzymes. BBE does not utilize (*R*)-reticuline, the C1 enantiomer.

From a novel nonnatural products perspective, this is a very interesting enzyme. (*S*)-Scoulerine (**2a**) is a precursor to a variety of useful benzophenantridine alkaloid derivatives that posses antibacterial, antimicrobial, antihypertensive, analgesic, and sedative activities, and have potential for the treatment of schizophrenia, thus making the berberine family of alkaloids ripe for developing a series of novel nonnatural products. Similar bioactivities (e.g., antispasmodic, hypotensive activities) have been identified in the structurally related benzylisoquinolines family of alkaloids (see Schrittwieser et al.[2e] and references therein).

Recognizing this potential, Schrittwieser et al.[2e] set about to find out how promiscuous BBE can be. Chemical syntheses of berberines and benzylisoquinolines have been shown to be time consuming, low yielding, and rarely lead to optically pure products. Schrittwieser et al. began by synthesizing potential racemic BBE substrates in five steps with 40% yields. Then, by using these different substrates and BBE in vitro, they



Scheme 1. The novel C–C bond-forming oxidizing reaction catalyzed by BBE and reactions catalyzed by the presumed BBE enzyme homologues THCA and CBDA synthases.

showed that they can synthesize natural and nonnatural BBE products with good yields and in multigram quantities.

The nonnatural substrates used for BBE were racemic mixtures (C1) of the four (*S*)- and (*R*)-tetrahydroisoquinolines **1b–e** which all lacked the 4′-methoxy group of reticuline, and were substituted at the C6, C7, and/or C8 carbon atoms (Scheme 1). The *S* conformers of these four nonnatural tetrahydroisoquinolines all proved to be substrates for BBE whereas the *R* conformers were never utilized by BBE as seen for the natural substrates. Given the variety of natural (*S*)-scoulerine derivatives and their known pharmacological benefits, these four newly synthesized nonnatural products are excellent starting points for new drug discovery endeavors.

Schrittwieser et al.[2e] also demonstrated that they could scale these reactions to conditions of commercial utility, that is, the substrates were turned over at concentrations of

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20 g L^{-1} using 1 g L^{-1} of BBE. To accommodate such high substrate concentrations, several obstacles had to be overcome. Firstly, to prevent the inhibitory effects of hydrogen peroxide building up during the reaction, catalase was included at 0.05 g L^{-1} to convert the hydrogen peroxide back into oxygen and water. Secondly, because at high concentrations the substrates were not very soluble in aqueous buffers, reactions with 20 g L^{-1} of substrates were performed in the presence of organic solvents. BBE was found to tolerate a variety of aqueous/organic solvent mixtures while still maintaining high catalytic activity, thus adding to BBE's acceptability for industrial applications.

As a result, under the reaction conditions developed by the authors BBE was selective for the *S* isomer of **1b**, thus resulting in 100% conversion of this stereoisomer after 24 hours. The obtained product also contained a minor side product (**3b**; Scheme 1), in which the C–C bond was formed using the 6′-carbon atom instead of the 2′-carbon atom—a not wholly unexpected result since BBE still has a pocket to accommodate the 4′-methoxy group of reticuline, which is missing from the nonnatural substrates, thus making rotation of the phenol group possible.

Additionally, the authors found that by varying organic solvents and ratios, they could alter the ratios of the desired **2b** product to the undesired **3b** side product. This **2b**:**3b** ratio ranged from 1:1 at its worst to 96:4 at its best. In comparison, a completely organic synthesis of **2b** results in a 2:3 ratio of **2b**:**3b** with only a 30% yield, unlike the 100% yield of (*S*)-**2b** from (*S*)-**1b** by BBE.

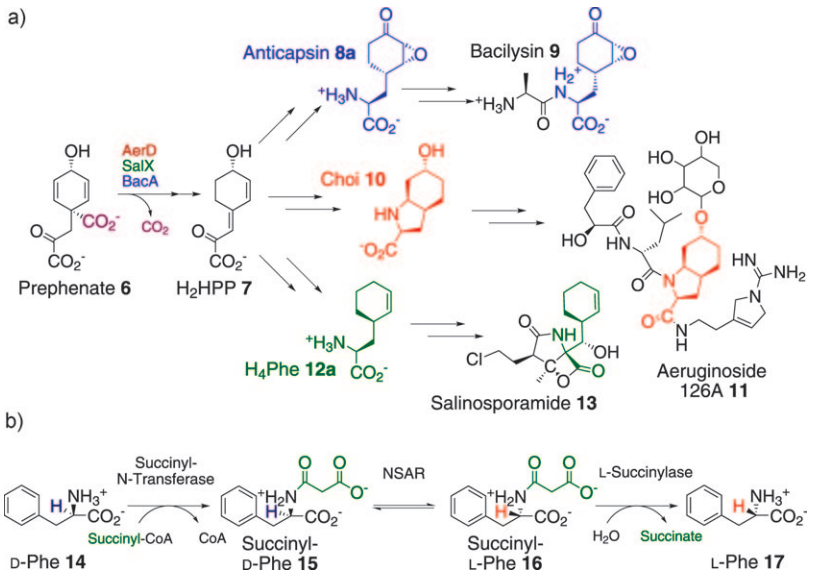
BBE appears to be an excellent enzyme for commercial exploitation. It is amenable to a variety of reaction conditions, it has a high turnover rate, gives excellent yields with high optical product purity, and can easily accommodate substrates.

What Schrittwieser et al.[2e] demonstrate with BBE is that there are many more enzymes out there beyond the relatively small number that are currently employed in biocatalysis and that these other enzymes have uniques activities and the potential to be utilized for synthetic applications once they have been characterized and their purifications have been established. For example, Δ^1-tetrahydrocannabinolic acid (THCA) synthase and cannabidiolic acid (CBDA) synthase are quite similar to BBE. Both enzymes use cannabigerolic acid to produce THCA[3a] (**4**) and CBDA[3b] (**5**; Scheme 1), respectively. Given the many bioactivities of cannabigerols,[3] these THCA and CBDA synthases appear to be excellent candidates for further exploration.

Another recently identified group of enzymes with potential for synthetic applications are the prephenate decarboxylases,[4a] the only known enzymes that decarboxylate prephenate (**6**) and transform the six-membered ring of prephenate into an aromatic ring. However, a newly discovered class of prephenate decarboxylases does not cause ring aromatization, but instead decarboxylates prephenate to form -4-hydroxydihydrophenylpyruvate, which then spontaneously isomerizes into the regioisomer H_2HPP (**7**; Scheme 2 a).

Through H_2HPP synthesis, these novel prephenate decarboxylases act as a gateway to a variety of known secondary metabolites, including the antibiotic bacilysin (**9**) as well as the protease inhibitors salinosporamide (**13**) and aeruginoside 126A (**11**).[4b] Given that these secondary metabolites are quite diverse, this new group of enzymes appears to be poised to help produce a wide variety of nonnatural products if they are amenable for nonnatural substrates, just as is the case with BBE. In conjunction, further elucidation of the secondary-metabolites pathways leading to salinosporamide and aeruginoside 126A must be continued, as is the case for bacilysin, to access an even larger variety of compounds.[4a]

Recently, Sakai et al. identified a three-member enzymatic pathway for the unidirectional racemization of D/L-amino acids[5] (Scheme 2 b). This path begins by adding a succinyl from succinyl-CoA to D-Phe (**14**) via a D-amino acid succinyl-N-transferase. Succinyl-D-Phe (**15**) is then racemized by *N*-succinylamino acid racemase (NSAR) into the corre-



Scheme 2. a) Three potential secondary metabolic fates for prephenate when initial catalysis is performed by AerD, SalX, or BacA. b) The irreversible racemization of D-Phe into L-Phe as catalyzed by three members of the diverse and promiscuous GNAT, enolase, and amidohydrolase enzyme superfamilies.

sponding succinyl-L-Phe (**16**). Lastly, the succinyl group is removed during hydrolysis by L-succinylase to leave the L-Phe product (**17**) in a net irreversible fashion, unlike the common TPP-dependent amino acid racemases. Using a single racemase to turn 50% useful substrate into 100% useful substrate is not a novel idea, but this work adds to the potential toolkit when an acceptable single racemase is not available by providing an alternative set of enzymes.

All three of these enzymes showed activity for a wide variety of substrates. The D-amino acid succinyl-N-transferase accepted 16 amino acids, the NSAR was able to racemize 17 succinylamino acids, and the L-succinylase was able to hydrolyse 19 *N*-succinyl-L-amino acids. Furthermore, succinyl-N-transferase, NSAR, and L-succinylase are members of the GNAT,[6] enolase,[7] and amidohydrolase superfamilies,[8] respectively. All three of these superfamilies are well characterized, catalyze a variety of reactions, and their enzymes are often quite promiscuous towards the number of substrates that are accepted.

Fortunately, many of the difficulties of finding the right enzyme for an application have been simplified by the PathPred program[9a] (http://www.genome.jp/tools/pathpred/) at the Kyoto Encyclopedia of Genes and Genomes (KEGG) database,[9b] which organizes its enzymes according to the EC standards. What PathPred allows one to do is input either a substrate for degradation or a desired natural product for production. PathPred will then produce a series of potential enzymatic paths to achieve the synthetic goal. For example, Figure 1 is a system map produced by PathPred for the synthesis of caffeic acid (C01197). In this example, Pathpred identified three different enzymatic pathways for the production of caffeic acid from an L-Tyr (C0079) starting point; as well as additional starting substrates and corresponding enzymatic paths to caffeic acid.

By considering that enzymes are often members of large enzyme superfamilies that often are well characterized, have a number of known functions, and whose members are frequently quite substrate promiscuous, one can conclude that there is a large untapped potential for the exploitation of more enzymes than those currently commercially available. Combine this with programs like PathPred that can help researchers, chemists, and engineers quickly identify useful enzymes and ever increasing database of the EC and one cannot help but to be optimistic about biocatalysis. We have the enzymatic tools, we have the talent, we just need to have the courage to use them.

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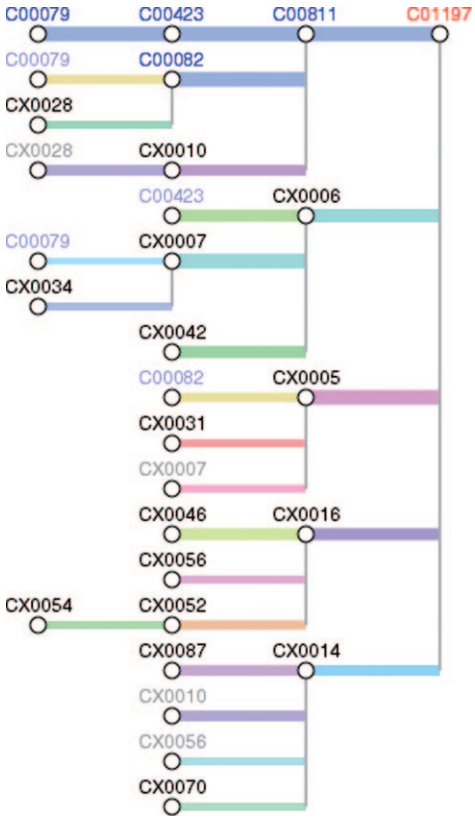

Figure 1. A system map for the production of caffeic acid obtained by the PathPred program at the KEGG database website. An open circle represents a chemical intermediate and colored horizontal bars represent enzymatic reactions. The grey lines are extensions of the open circles they hang from. For example, C00811 can be produced from three different substrates by two different enzymes (note the identical dark blue lines connecting C00811 to its neigbors C00423 and C00082, which indicates identical enzymes for these transformations).

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