CHEMICAL REVIEWS

REVIEW

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Enantioselective Oxidation of C−O and C−N Bonds Using Oxidases

Nicholas J. Turner*

School of Chemistry, University of Manchester, Manchester Interdisciplinary Biocentre, 131 Princess Street, Manchester M1 7DN, U.K.

CONTENTS

1. INTRODUCTION AND OVERVIEW OF ENZYMES THAT CATALYZE THE OXIDATION OF C−O AND C−N BONDS

Oxidation reactions represent a cornerstone of organic chemistry, providing access to a range of functional groups (e.g., aldehydes, ketones, carboxylic acids) that allow further functionalization of building blocks used in synthesis. Traditionally, many of these oxidation reactions have been carried out using stoichiometric amounts of transition metal based oxidants[1] (e.g., chromium, manganese, ruthenium) that offer high levels of selectivity in terms of product purity. Increasingly, however, attention and effort are being devoted to the development of mild, selective, and catalytic processes for effecting these transformations, including the use of organocatalytic methods.[2] In this respect, enzymes also offer a potential solution, not only because of the exquisite selectivity, mild reaction conditions, and high rates of turnover that can be obtained but also because there are a truly diverse range of different enzyme types that catalyze oxidation reactions (Figure 1).[3−7] Enzymes are able to catalyze oxidation processes encompassing hydroxylation and dihydroxylation of both aliphatic and aromatic C−H bonds, epoxidation, heteroatom oxidation including sulfoxidation and amine oxidation, Baeyer−Villiger oxidation of ketones to lactones, and halohydrin formation from alkenes. In many cases, enzymes are able to activate unactivated C−H and C═C bonds and hence often provide access to products that would be difficult, if not impossible, to obtain in a single step using alternative nonbiocatalytic processes. Another feature of enzyme-catalyzed oxidation processes is the plethora of different mechanisms that nature has evolved to be able to oxidize a range of different substrates. Thus, enzymes involved in oxidation processes variously employ different metals (e.g., Cu, Fe, Mn, V), cofactors (e.g., NADH/NADPH, FAD, FMN), and oxidants (oxygen, hydrogen peroxide). Chemists wishing to use enzymes as biocatalysts for oxidation reactions have been provided with a veritable arsenal of different options. However, significant challenges remain in terms of tailoring these different biocatalysts for the various applications needed in organic synthesis. Increasingly, directed evolution methods are proving to be powerful tools for developing enzymes with the appropriate properties (i.e., substrate specificity, enantioselectivity, catalytic acitivity, organic solvent tolerance, and thermostability) for applications as practical biocatalysts in organic synthesis.[8−10]

Two important types of oxidation reactions are the oxidation of alcohols to carbonyl compounds (C−O to C═O) and the oxidation of amines to imines (C−N to C═N). These two processes are typically catalyzed by the large family of NADH/NADPH-dependent alcohol dehydrogenases (ADH) and amino acid dehydrogenases (AADH), respectively. Such reactions are redox processes, being freely reversible, and indeed in many cases these enzymes are typically used in the reductive direction to generate enantiomerically pure alcohols and amino acids from the corresponding ketones and keto acids. However, for both ADH- and AADH-dependent reactions, it is necessary to recycle the expensive cofactor, which is usually achieved by introducing auxiliary enzymes together with stoichiometric quantities of inexpensive reductants such as formate or glucose.

An alternative to the use of dehydrogenases for C−O and C−N bond oxidation is to employ flavin- or metal-dependent alcohol or amine oxidases. Oxidases have the common feature that they use molecular oxygen as the oxidant, which is reduced to hydrogen peroxide in the process. In cases where hydrogen peroxide causes problems with side oxidation reactions, then the addition of catalase results in the conversion to water and dioxygen. Compared to dehydrogenases, oxidases are much less abundant in nature and are often of eukaryotic origin, which can present challenges in terms of the development of suitable efficient overexpression systems.[11,12] Oxidases employ a range of different metals and cofactors that mediate the transfer of electrons from the substrate to the electron acceptor molecular oxygen. In the case of flavin-dependent oxidases, the substrate

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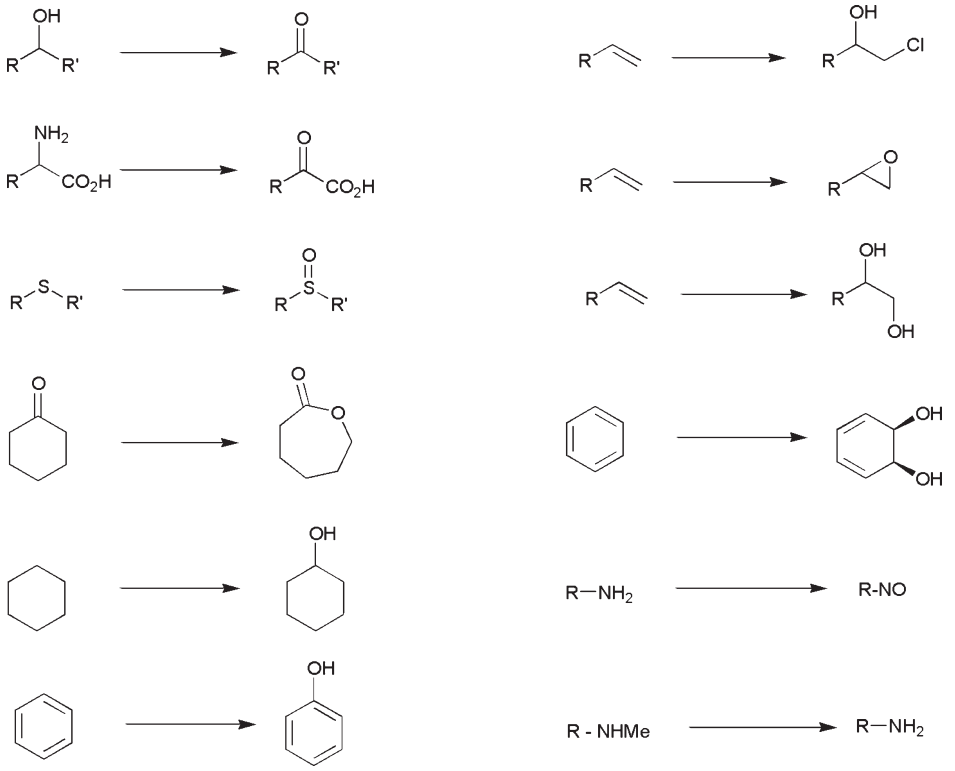

Figure 1. Range of different oxidation reactions catalyzed by enzymes.

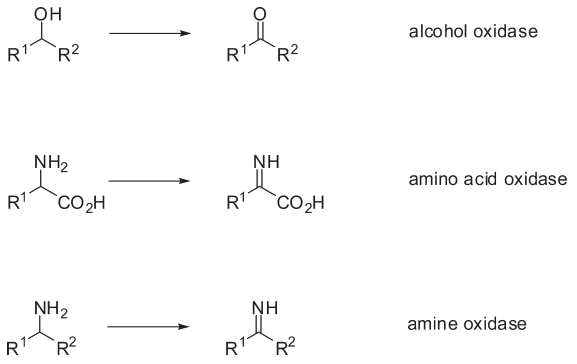

Figure 2. Oxidation of alcohols, amino acids, and amine catalyzed by oxidases.

interacts directly with the oxidized flavin (FAD or FMN) that is consequently reduced and then reoxidized with molecular oxygen, which itself is reduced to hydrogen peroxide. Metal-dependent oxidases (e.g., galactose oxidase) operate by a different process but also generate hydrogen peroxide from oxygen.

This review focuses on the application of alcohol, amino acid, and amine oxidases for the enantioselective oxidation of C—O and C—N bonds (Figure 2). Examples are presented that include both the kinetic resolution of racemic substrates and also the desymmetrization of symmetrical substrates. For alcohol, amino acid, and amine oxidases, deracemization processes have been developed in which the enantioselective oxidase is combined with an inexpensive nonselective chemical reducing agent or a second enzyme system. In many cases, these oxidase-catalyzed transformations are used to generate enantiomerically pure chiral building blocks for the synthesis of biologically active target molecules.

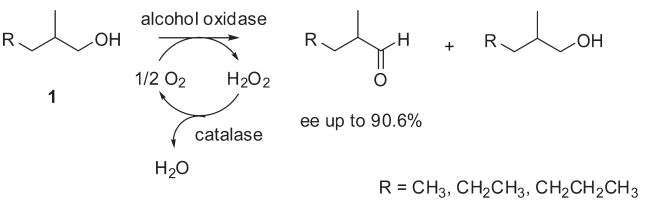

Figure 3. Enantioselective oxidation of racemic primary alcohols using aliphatic alcohol oxidases.

2. OXIDATION OF C—O BONDS

2.1. Aliphatic Alcohol Oxidase

The oxidation of several racemic 2-methyl-l-alkanols **1** by the alcohol oxidases from *Candida boidinii*, *Hansenula sp.*, *Pichia pastoris*, and *Torulopsis methanothermo* afforded reaction mixtures enriched in (*R*)-2-methyl-l-alkanol at incomplete conversions (Figure 3).[13] The alcohol oxidase from *C. boidinii* was found to have the highest enantioselectivity, leading to an enantiomeric excess for (*R*)-2-methyl-l-pentanol of 90.6% ee at 76% conversion (E = 4.8).

2.2. Alditol Oxidase

van Hellemond and co-workers have explored the substrate specificity of the flavoprotein alditol oxidase (AldO) from *Streptomyces coelicolor*, which has been overexpressed in *E. coli*.[14,15] AldO was previously known to catalyze the oxidation of alditols to D-aldoses; however, the current study revealed that the enzyme is also active toward a wide range of aliphatic and aromatic alcohols. Alcohols containing hydroxy groups at the C-1 and C-2 positions such as 1,2,4-butanetriol, 1,2-pentanediol, and 1,2-hexanediol were accepted by AldO. The enzyme was

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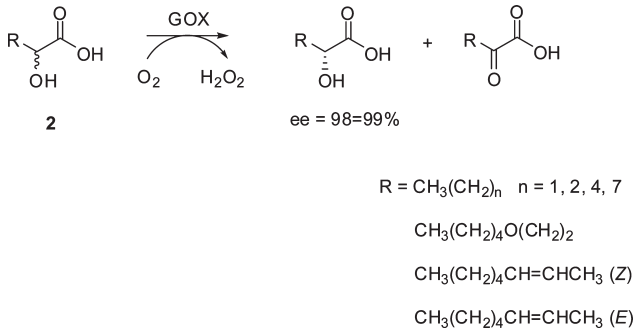

Figure 4. Enantioselective oxidation of α-hydroxyacids using glycolate oxidase.

shown to be highly enantioselective for the oxidation of 1,2-diols [e.g., for 1-phenyl-1,2-ethanediol, the (*R*)-enantiomer was preferred with an *E* value of 74]. Interestingly, for all 1,2-diols examined, the products were found to be the α-hydroxy acids instead of the expected α-hydroxy aldehydes. Labeling studies revealed that a second enzymatic oxidation step occurs via the aldehyde hydrate product intermediate.

Included here for the sake of convenience are cholesterol oxidase and vanillyl alcohol oxidase. Cholesterol oxidase isolated from *Rhodococcus erythropolis* has been employed for the selective oxidation of 2-cyclohexenyl-1-alcohols to the corresponding enones and was found to display (*S*)-selectivity at the secondary alcohol center.[16] Vanillyl alcohol oxidase displays broad substrate specificity and high stereoselectivity toward a wide range of phenolic compounds by catalyzing oxidation, deamination, demethylation, dehydrogenation, and hydroxylation reactions. For example, hydroxylation of 4-alkylphenols is highly stereospecific for the (*R*)-isomer, whereas dehydrogenation of these substrates is specific for the cis- or trans-isomer.[17]

2.3. Lactate Oxidase

Gao et al. have developed a process for the conversion of racemic lactic acid into a mixture of enantiomerically pure D-lactic acid and pyruvic acid by employing the lactate oxidase component of *Pseudomonas stutzeri* SDM. The biocatalyst was prepared from 9 (g of dry cell weight)/(L of cells), which were able to process 45 g/L of DL-lactic acid into 25.2 g/L of D-lactic acid. The D-lactic acid and coproduct pyruvic acid were separated by ion-exchange chromatography.[18] The same group has also reported the kinetic resolution of racemic 2-hydroxybutanoate using the NAD-independent L-lactate dehydrogenase, which is a flavin-dependent enzyme although not an oxidase.[19] Using this system, D-2-hydroxybutanoate was produced at a concentration of 0.197 M, with an enantiomeric excess (ee) of 99.1%, and could be recovered in an overall yield of 84.7%.[20]

2.4. Glycolate Oxidase

Glycolate oxidase (GOX; EC 1.1.3.15) is a flavin mononucleotide (FMN)-dependent enzyme that catalyzes the oxidation in vivo of glycolic acid to glyoxylic acid.[21−23] The enzyme is oxygen-dependent and is located in the peroxisome. Adam et al. have shown that the enzyme from spinach (*Spinacia oleracea*) is able to catalyze the enantioselective oxidation of a range of racemic 2-hydroxyacids **2** to yield the corresponding enantiomerically pure (*R*)-2-hydroxy acids (Figure 4).[24] The enzyme is able to accept both short- and medium-chain 2-hydroxy acids as well as side chains containing oxygen atoms and alkenes.

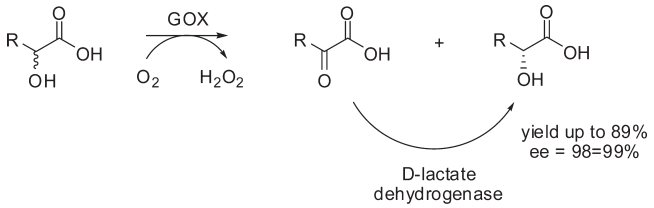

Figure 5. Deracemization of racemic 2-hydroxy acids by using a combination of glycolate oxidase and D-lactate dehydrogenase.

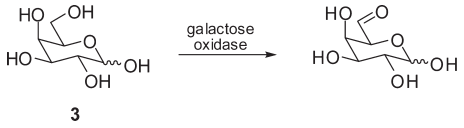

Figure 6. Oxidation of the C-6-OH of D-galactose **3** using galactose oxidase (GOase).

However, the more sterically demanding mandelic acid, 2-hydroxyisobutyric acid, and 2,3-dihydroxybutyric acid were not accepted as substrates. Das et al. have developed a system in which catalase is coexpressed with GOX in *Pichia* sp. to decompose the endogenous hydrogen peroxide produced in the reaction, thus attenuating any peroxide-based side reactions.[25] The oxidations of 3-phenyllactic acid, 3-indolelactic acid, 3-chlorolactic acid, 2-hydroxybutanoic acid, and 2-hydroxydecanoic acid were examined, resulting in a high degree of (*S*)-selectivity, leaving the (*R*)-isomers intact. The best substrates were 3-chlorolactic acid (110% relative to lactic acid) and 2-hydroxybutanoic acid (120%).

Adam et al. have combined GOX with D-lactate dehydrogenase to effect an overall deracemization of 2-hydroxy acids (Figure 5).[26] The enzymatic resolution of racemic chiral 2-hydroxy acids by enantioselective oxidation with molecular oxygen in the presence of glycolate oxidase from spinach (*Spinacia oleracea*) and subsequent asymmetric reduction of 2-oxo acids with D-lactate dehydrogenase from *Lactobacillus leichmannii* led to enantiomerically pure (*R*)-2-hydroxy acids in up to 89% yield based on the racemate.

2.5. Galactose Oxidase

Galactose oxidase (GOase; EC.1.1.3.9) is one of the most extensively studied alcohol oxidases with respect to both mechanistic investigations and also practical applications. Wild-type GOase is highly selective for D-galactose **3** and D-talose but will not oxidize other sugars (e.g., D-glucose, D-mannose) (Figure 6).[27]

GOase is a copper-dependent alcohol oxidase, isolated from *Fusarium* sp.,[28,29] and oxidizes galactose (Gal) residues either as monosaccharides or glycoconjugates that contain galactose at the nonreducing end.[30,31] GOase has been used in various applications including biosensors,[32] chemical synthesis,[33,34] and recently labeling of *N*-acetylglucosamine and mannose residues present in glycans and glycoconjugates.[35] Several variants of GOase with activity toward secondary alcohols, D-glucose (Glc), and D-fructose (Fru) have been developed by protein engineering.[36,37]

With respect to noncarbohydrate substrates, GOase possesses a fairly narrow substrate specificity.[38] A variant of GOase has been identified that has high enantioselectivity toward a broad range of racemic chiral secondary alcohols based upon the

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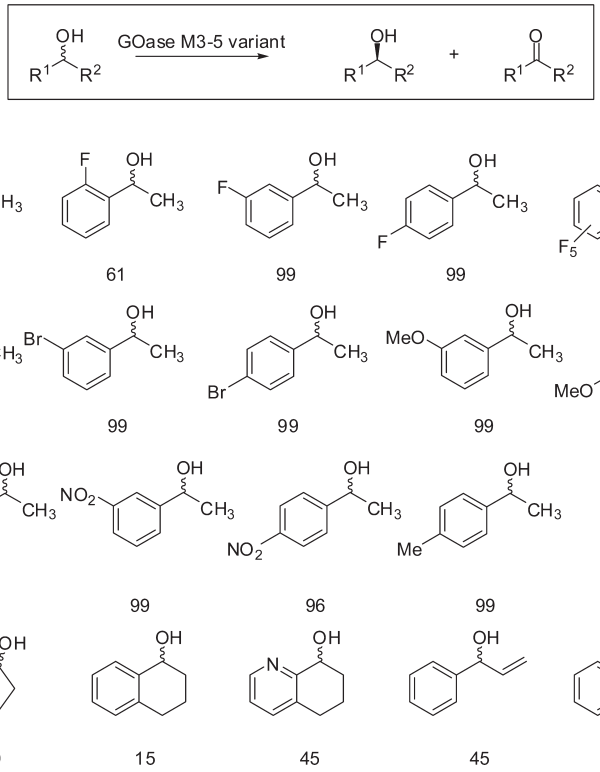
Table 1. Kinetic Resolution of a Range of Racemic Secondary Alcohols Using the M3-5 Variant of Galactose Oxidase; Values Shown below the Substrates Indicate the % ee of the Recovered Alcohol



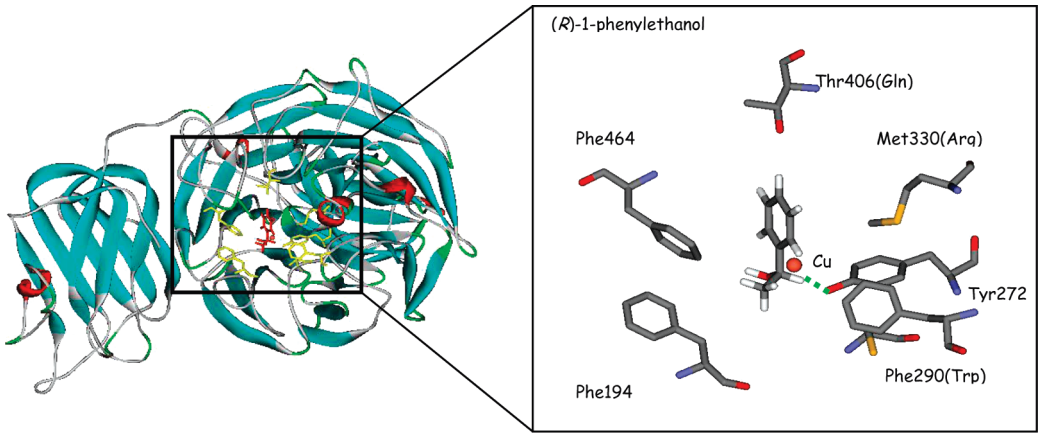


Figure 7. Model of (*R*)-1-phenylethanol at the active site of the M3-5 variant of GOase. The Met330 residue appears to interact with the hydrophobic aromatic ring of the substrate.

1-phenylethanol template (see Table 1).[39] Upon the basis of previous reports,[40] this group developed a high-throughput solid-phase colorimetric assay to identify a variant of the parent GOase with activity toward chiral secondary alcohols. The parent had previously been reported by the Arnold group[41,42] to have activity toward buten-2-ol, although no data on enantioselectivity was available. The M3-5 variant possesses a single mutation (Arg330Met) that is sufficient to confer broad substrate specificity on the variant toward a wide range of chiral secondary alcohols as shown in Table 1. In all cases, the enzyme displayed (*R*)-selectivity, leading to the production of enantiomerically enriched (*S*)-alcohols. A model of (*R*)-phenylethanol in the active site of the GOase M3-5 variant suggests that the Met330 residue is located close to the aromatic ring of the substrate. In the parent, this residue is Arg330 and is believed to make two important hydrogen bonds to the OH group of D-galactose (Figure 7).[43,44]

The same group has further exploited this novel (*R*)-selective alcohol oxidase in two ways. First, they have developed a rapid and sensitive screening method for determining the enantiomeric purity of chiral secondary alcohols (Figure 8).[45] Because the GOase variant is (*R*)-selective, the rate of oxidation of an

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(*R*/*S*)-mixture of unknown composition will depend upon the concentration of (*R*)-enantiomer. This rate can be easily measured by addition of horseradish peroxidase (HRP) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to the mixture to generate a colored product that can be detected spectrophotometrically.

Second, the GOase variant has been used to desymmetrize symmetrical atropoisomeric biarylethers **4** as shown in Figure 9.[46] Enantiomerically pure nonsymmetrical biaryl ethers are useful as chiral ligands for transition metal catalyzed reactions. The GOase variant catalyzed the enantioselective oxidation of the substrate to generate the (+)-*P*-enantiomer of the monoaldehyde product **5** in good yield and ee up to 95%. Interestingly, a secondary oxidation occurred to yield the dialdehyde **6**; this subsequent process is slower but selective for the (−)-(*M*)-enantiomer, leading to an improvement in the ee of (+)-(*P*)-**5** at the expense of yield (Figure 10).

Modeling the two enantiomers of **5** into the binding site of GOase reveals that the bulky *tert*-butyl group of the substrate is forced to occupy a position in which it is pointing away from the cleft and toward the surface of the protein. In such a binding mode, the hydroxymethyl group of *M*-**5** is placed close to the copper-containing reactive center poised for oxidation to the aldehyde. This model therefore predicts that *M*-**5** is the faster reacting enantiomer in the kinetic resolution process, an observation that is in agreement with the assignment of absolute configuration based on circular dichroism (Figure 11).

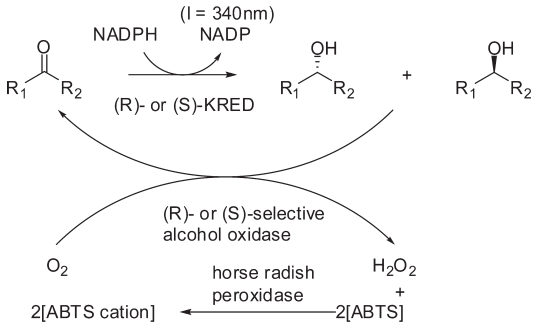

Figure 8. Determination of the enantiomeric purity of chiral alcohols derived from ketoreductase catalyzed reduction of ketones. The (*R*)-selective GOase M3-5 variant can be used as a reporter enzyme to determine the enantiomeric composition of the alcohols by use of a colorimetric read-out.

3. OXIDATION OF C−N BONDS

Oxidases are also well-documented as catalysts for the oxidation of C−N bonds, including examples in which this reaction occurs enantioselectively. The principal enzymes involved in this transformation belong to the flavin-dependent oxidases (1.4.3.*x*) including L-amino acid oxidase (1.4.3.2), D-amino acid oxidase (1.4.3.3), and the amine oxidases of which monoamine oxidase (1.4.3.4) has been most extensively studied. Flavin-dependent oxidases use molecular oxygen as oxidant, which is reduced to hydrogen peroxide at the expense of the 2-electron oxidation of a C−N bond to a C=N double bond. The oxidized form of the flavin (FAD) mediates the electron transfer process via its reduced form ($FADH_2$). The flavin can be either covalently or noncovalently

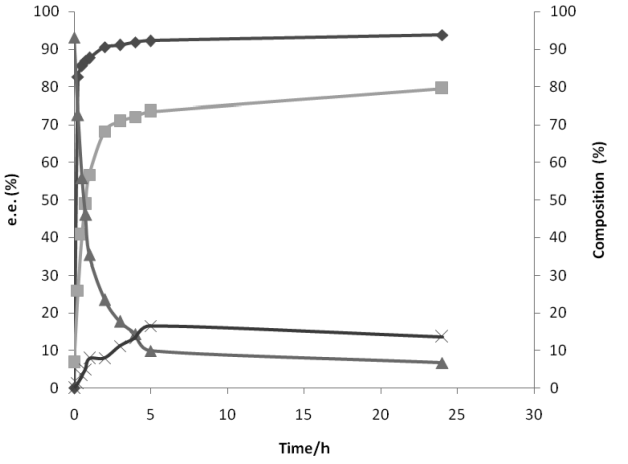

Figure 10. Enantioselective desymmetrization of diol **4** using GOase $M_{3\text{-}5}$ variant. % components: ▲= diol **4**; ■ = monoaldehyde **5**; X = dialdehyde **6**; ♦ = ee %.

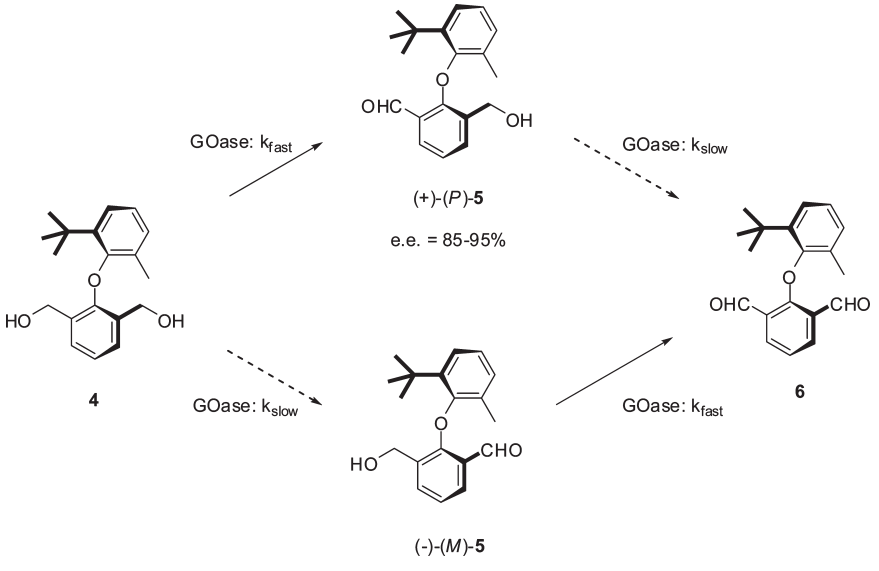

Figure 9. Desymmetrization of **4** using GOase M3-5 variant.

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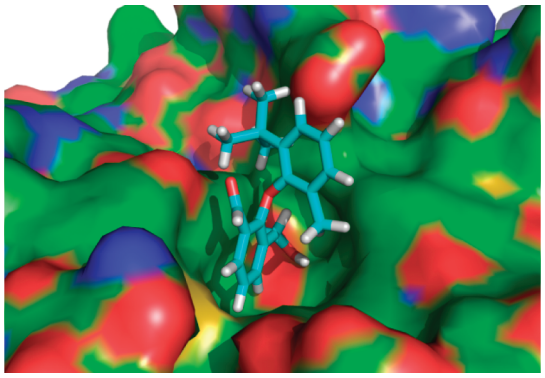

Figure 11. Modeling of the faster reacting enantiomer (*M*)-**5** into the active site of galactose oxidase.

bound to the oxidase enzyme. A separate class of copper-dependent amine oxidases operates via a different mechanism, involving the participation of the cofactor TPQ. In this case, the intermediate imine is enzyme-bound. The use of oxidases to catalyze the oxidation of C−N to C═N bonds is if anything more attractive than C−O bond oxidation given the paucity of alternative chemical catalysts, let alone stoichiometric reagents, for this conversion. Nonenzyme-catalyzed oxidation of C−N to C═N usually requires metal-based oxidants under fairly aggressive oxidative conditions. Such conditions often mitigate against selectivity, which is of particular interest in the oxidation of nitrogen-containing molecules because more than one oxidized product may be obtained (e.g., imine, *N*-oxide, nitroso, nitro products, etc.). Moreover, generation of imines via C−N oxidation provides a highly complementary synthetic strategy to classical methods based upon condensation of aldehydes with amines and thereby opens up the possibility of generating imines under aqueous conditions. Imines, although less reactive than aldehydes, are versatile synthetic intermediates, particularly for redox chemistry and multicomponent reactions as outlined below.

Other enzymes that variously catalyze the oxidation of C−N bonds include laccase, tyrosinase, and horseradish peroxidase. These enzymes are dealt with briefly toward the end of this review.

3.1. L-Amino Acid Oxidase

L-Lysine oxidase (EC 1.4.3.14) catalyzes the oxidation of the ω-amino group of L-lysine. This enzyme has been used in a one-pot, two-enzyme system for the synthesis of several unnatural cyclic amino acids from the corresponding linear precursors.[47] L-Lysine oxidase (alternatively L- or D-amino acid oxidase AAO could also be used in this step) was first used to convert the diamino acid **7** to the corresponding α-keto amino acid **8**, which underwent spontaneous cyclization in situ to the imino acid **9** (Figure 12). Addition of a second enzyme, namely, *N*-methyl-L-amino acid dehydrogenase (NMAADH), resulted in reduction of the imine to afford the cyclic amino acid **10** in good yield and excellent optical purity. A deracemization was also accomplished using racemic analogues of **7** in conjunction with D-amino acid oxidase (D-AAO) and NMAADH.

The enzyme lysine cyclodeaminase, which catalyzes the oxidative cyclization of L-lysine to L-pipecolic acid, has been identified in the rapamycin gene cluster. Cloning of the gene and subsequent overexpression and purification of the enzyme (Figure 13) yielded a biocatalyst that is able to convert L-lysine **11** to L-pipecolic acid **12**. However, the enzyme displayed much lower

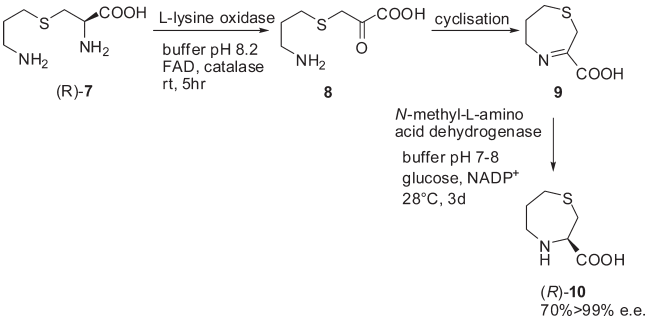

Figure 12. Use of L-lysine oxidase in combination with *N*-methylamino acid dehydrogenase for the conversion of (*R*)-**7** to cyclic amino acid (*R*)-**10**.

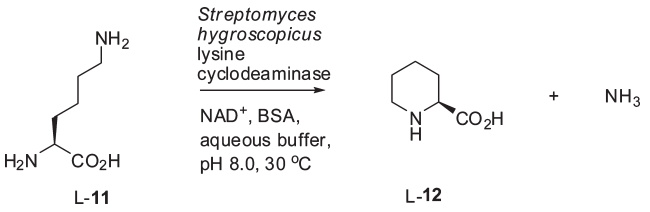

Figure 13. Conversion of L-lysine **11** to L-pipecolic acid **12** using a lysine cyclodeaminase obtained from *Streptomyces hygroscopicus*.

activity toward L-ornithine **5** than L-lysine (13 $mM^{-1}min^{-1}$), and D-lysine was not active. The enzyme mechanism was investigated via study of enzyme cofactor requirements and isotopic substrate labeling.[48]

3.2. D-Amino Acid Oxidase

D-Amino acid oxidase (EC 1.4.3.3) has been employed for many years as an industrial biocatalyst in the commercial production of 7-aminocephalosporanic acid **14** (7-ACA). The enzyme catalyzes the initial oxidative deamination of the D-amino acid side-chain in cephalosporin C **13**. Thereafter, glutarylacylase is used to cleave the modified side-chain to yield 7-ACA. Recently, a double mutant of glutaryl acylase from *Pseudomonas* sp. SY-77 was used in a triple-enzyme system to convert cephalosporin to 7-ACA (Figure 14).[49] The application of this three-enzyme, one-pot conversion gave the product 7-aminocephalosporanic acid **14** in high yield (80%).

Racemic 2,3-diaminopropionic acid **15** has been treated with a resin-bound D-amino acid oxidase from *Trigonopsis variabilis* to yield L-**15** with an enantiomeric excess of 98%. The reaction was carried out using 10% w/v resin-bound enzyme at 37 °C for a period of 24 h. The initial product of oxidation of the D-enantiomer, namely, 3-amino-2-ketopropionic acid, undergoes further transformation to yield glycine **16** as the byproduct (Figure 15).[50]

D-Amino acid oxidase from *R. gracillus* has been subjected to rational protein engineering to develop a variant enzyme that could be used to improve the bioconversion of D,L-naphthylalanine **17** and D,L-naphthylglycine (Figure 16). A single mutation, Met213Gly, led to a reduction in both reaction times and the quantity of catalyst needed. The V_{max} value was found to increase from 125 for the wild type to 870 min^{-1} for the Met213Gly variant. This improvement in turnover resulted in an improvement of conversion of substrate **17** to the α-keto acid product **18** from 96 to >99%. This rate enhancement led to a reduction in the reaction time from 400 to 100 min.[51]

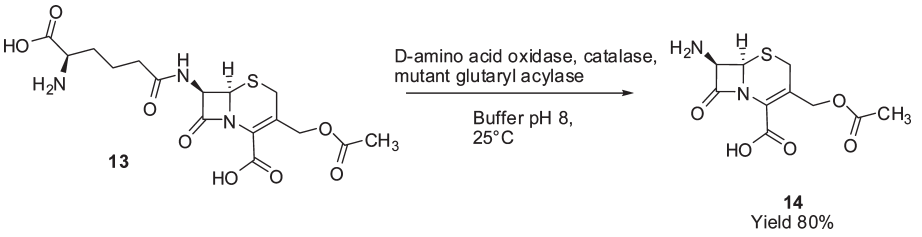

Figure 14. Conversion of cephalosporin C **13** to 7-aminocephalosporanic acid **14** using a three-enzyme system.

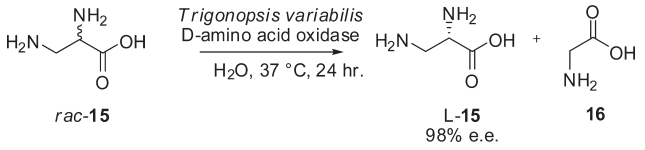

Figure 15. Enantioselective oxidation of racemic amino acid **15** using D-amino acid oxidase from *Trigonopsis variabilis*.

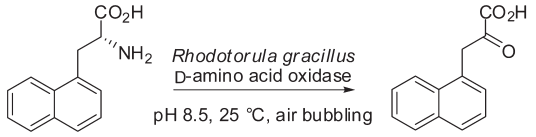

Figure 16. Oxidation of D-naphthylamine to the corresponding α-keto acid using D-AAO from *Rhodotorula gracillus*.

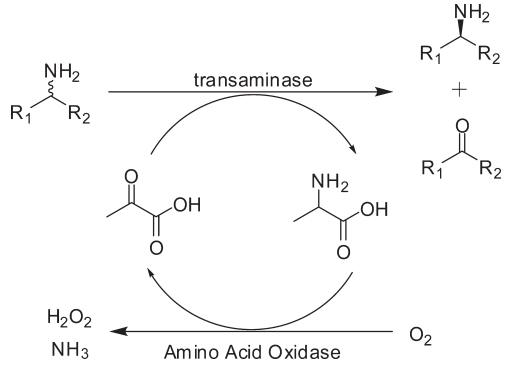

Figure 17. Dual transaminase/amino acid oxidase-mediated resolution of racemic primary amines.

Amino acid oxidases have been combined with transaminases in a novel and interesting manner to develop a process for the kinetic resolution of racemic amines (Figure 17).[52] Initially a transaminase was used to enantioselectively convert one enantiomer of the racemic amine substrate to the ketone product. This resulted in the concurrent amination of the amine acceptor (pyruvate) to produce the coproduct alanine. Thereafter an amino acid oxidase (AAO) regenerates the amine acceptor in situ using molecular oxygen, thereby requiring only a catalytic amount of amine acceptor to be used (amine-to-amine acceptor molar ratio = 1000:1). The benefits of this system are (i) the oxidase can be used to overcome the unfavorable reaction equilibrium for the reaction by providing an irreversible step and (ii) inhibition of the transaminases, which is often a problem,

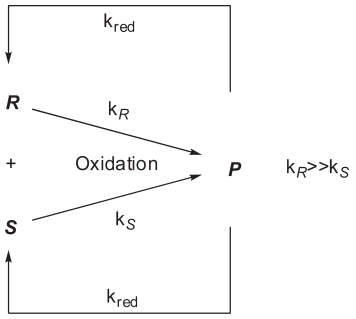

Figure 18. Deracemization of a racemic mixture of enantiomers by a cyclic oxidation and reduction sequence.

is significantly reduced by employing a very low concentration of amine acceptor to run the reaction.[53,54]

Recently a similar principle has been used to develop a sensitive and rapid method for screening transaminases for activity toward new substrates.[55] Activity of either (*R*)- or (*S*)-selective transaminases toward a panel of substrates was detected by addition of pyruvate as the amino group acceptor. Upon transamination, pyruvate is converted to either D- or L-alanine, which can be detected in a coupled assay system using either D- or L-amino acid oxidase, respectively. This method can be employed to measure both the activity and enantioselectivity of transaminases toward new substrates of interest, thereby complementing existing methods.[56]

3.3. Deracemization of Racemic Amino Acids Using Amino Acid Oxidases

Deracemization refers collectively to a set of processes in which a racemate is converted into a nonracemic product in 100% theoretical yield without intermediate separation of materials. Accordingly, dynamic kinetic resolution (DKR), dynamic thermodynamic resolution, stereoinversion, and enantioconvergent transformations of a racemate are all classified as deracemization processes.[57−66] Within this context, oxidases have been used for the deracemization of amino acids and amines by employing a cyclic oxidation and reduction sequence as shown in Figure 18.

In the first step of the catalytic cycle, enantioselective oxidation of the substrate ($k_R \gg k_S$) takes place such that one enantiomer of the starting racemate is oxidized to the achiral imine or imino acid intermediate. In the second step, the C=N of the imine/imino acid is reduced in a nonstereoselective manner to regenerate the amine of the substrate. Concerted oxidation and reduction cycles according to this principle lead to eventual deracemization of a racemic substrate.

It is interesting to note that, even after just four cycles of oxidation and reduction, the enantiomeric excess of the L-amino acid will be >93% from a starting racemate assuming a completely

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enantioselective oxidation and nonselective chemical reduction. After seven cycles, the ee of the amino acid has risen to >99%, at which point the reaction will stop because the enzyme has no remaining substrate to oxidize (Figure 19).

The potential for using this system to deracemize α-amino acids was first recognized by Hafner and Wellner, who reported the generation of L-alanine and L-leucine from the corresponding D-enantiomers by the combined use of porcine kidney D-amino acid oxidase and sodium borohydride.[67] Subsequently Soda and co-workers reported the application of this method to the deracemization of DL-proline and DL-pipecolic acid[68,69] also using D-amino acid oxidase and sodium borohydride (Figure 20).

A practical limitation of the Haffner and Soda system is the need to use sodium borohydride as the reducing agent. Aqueous solutions of sodium borohydride are unstable at pH 7.5, which is typically where the amino acid oxidases display maximum activity. Raising the pH to 10.0 stabilizes the solution, and although the amino acid oxidases are still active at this elevated pH, their stability is compromised. Subsequent studies led to the discovery of alternative reducing agents that could be used in place of sodium borohydride, such as sodium cyanoborohydride,[70] amine-borane complexes, and catalytic transfer hydrogenation[71] using formate as the hydrogen source with a metal catalyst. Catalytic transfer hydrogenation has been shown to be effective in the preparative deracemization of racemic amino acids with L-amino acid oxidase from *Proteus myxofaciens* to yield enantiomerically pure D-amino acids.[71]

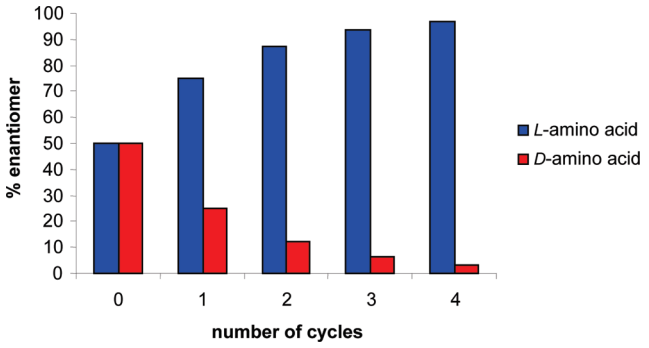

Figure 19. Enantiomeric excess as a function of the number of catalytic cycles during a deracemization process.

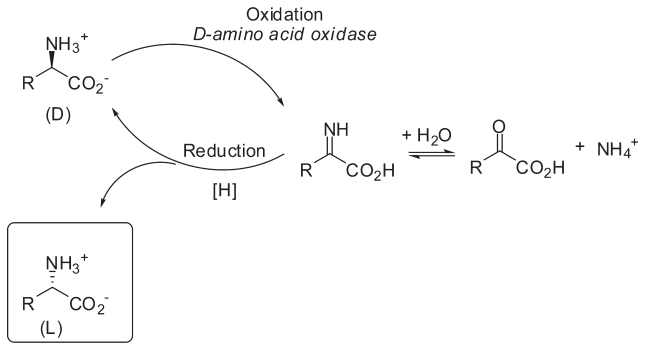

Figure 20. Deracemization of DL-amino acids to D-amino acids using D-amino acid oxidase in combination with a chemical reducing agent.

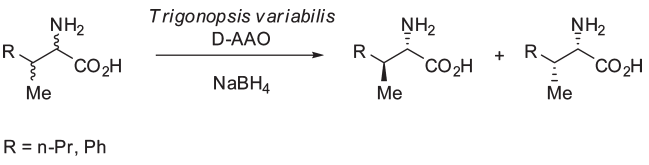

Figure 21. Stereoinversion of β-substituted α-amino acids using a combination of D-amino acid oxidase and sodium borohydride.

As outlined above, the oxidation−reduction deracemization process is in essence a stereoinversion process and hence could in principle be applied to substrates possessing more than one stereogenic center. Turner and co-workers demonstrated the interconversion of diastereoisomers of amino acids, e.g., the conversion of L-isoleucine to D-allo-isoleucine in high yield and ee/diastereomeric excess (de) (Figure 21).[72]

Furthermore, amino acid oxidase-based deracemization has been combined with the chemocatalytic asymmetric hydrogenation of dehydroamino acids to provide an efficient entry intro families of enantiomerically pure β-arylalanines **19** in high de and ee (Figure 22).[73] Specifically, a range of β-methyl-β-aryl alanine (2*R*,3*S*) and (2*S*,3*S*)-**19** diastereoisomers were synthesized chemoenzymatically using L-threonine methyl ester as the starting material. The (2*R*,3*S*) and (2*S*,3*R*) isomers were obtained via asymmetric hydrogenation of **20** chiral rhodium-DuPhos catalysts followed by hydrolysis of the amide and ester protecting groups. Thereafter, the enantioselective amino acid oxidases, in combination with ammonia borane, were used to stereoinvert one of the asymmetric centers to obtain the (2*R*,3*R*) and (2*S*,3*S*) β-substituted alanines with yields ranging from 68 −92% and ee's > 98%.

Interestingly the reduction step in these deracemization processes can also be performed electrochemically. Markle and Lutz have developed a method for deracemization of racemic leucine using D-amino acid oxidase in a batch reactor containing a graphite electrode at −1.5 V vs Ag/AgCl in pH 10 buffer. Reduction of the intermediate imine occurs at the cathode, yielding L-leucine in 91% ee with a space-time-yield of 3.5 mmol L^{-1} day^{-1} (Figure 23).[74]

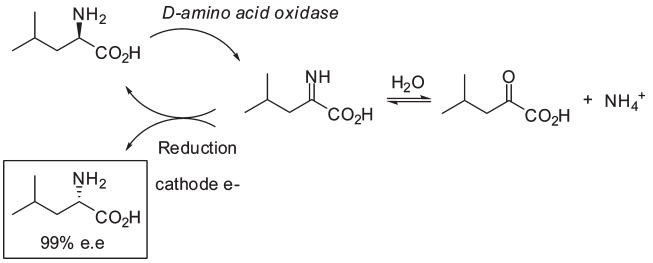

Figure 23. Electroenzymatic deracemization of D/L-leucine.

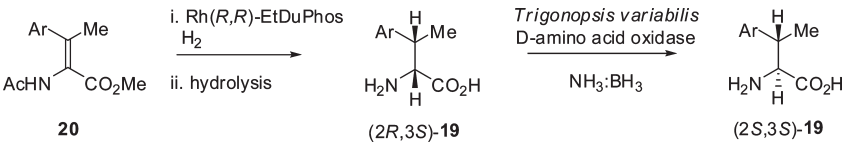

Figure 22. Chemo-enzymatic asymmetric hydrogenation and biocatalytic stereoinversion to generate β-substituted phenylalanines **19**.

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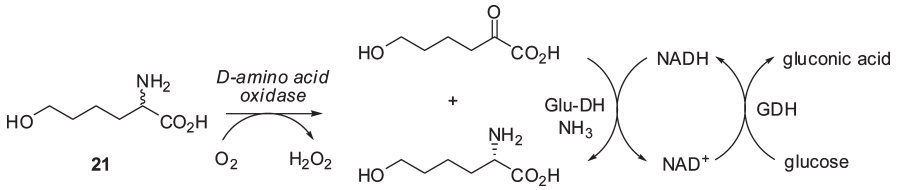

Figure 24. Combination of D-amino acid oxidase and L-glutamate dehydrogenase (Glu-DH) for the deracemization of racemic 6-hydroxynorleucine **21**.

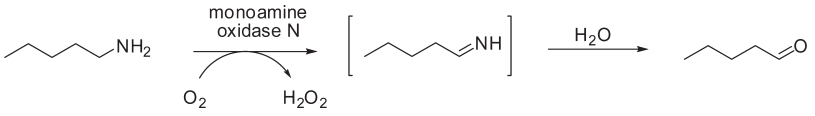

Figure 25. Oxidation of amines to aldehydes using monoamine oxidase N (MAO-N).

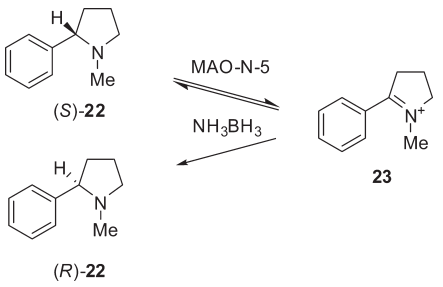

Figure 26. Stereoinversion of (*S*)- to (*R*)-nicotine **22**.

Hanson et al. have combined D-amino acid oxidase with L-glutamate dehydrogenase to generate a novel deracemization process.[75] Thus, racemic 6-hydroxynorleucine **21** was treated with D-amino acid oxidase and catalase (either porcine kidney D-amino acid oxidase and beef liver catalase or *Trigonopsis variabilis* whole cells) to yield L-6-hydroxynorleucine **21** and the corresponding α-keto acid (Figure 24). In the second step, L-glutamate dehydrogenase catalyzed the reductive amination step to convert the 2-keto-6-hydroxy hexanoic acid into L-6-hydroxynorleucine, yielding the product in >98% ee and a yield of 97% at 100 g/L. L-6-Hydroxynorleucine is a chiral building block required for the synthesis of Omapatrilat (Vanlev), an antihypertensive drug.

3.4. Monoamine Oxidase

Both D- and L-amino acid oxidases show broad substrate acceptability with respect to the amino acid side change but high specificity and enantioselectivity for either D- or L-α-amino acids depending upon the particular enzyme. For this reason, neither of these enzymes can be used in a more general manner for the enantioselective oxidation of C−N bonds present in a wider range of structurally different amines. Flavin-dependent amine oxidases, including monoamine oxidase, putrescine oxidase, diamine oxidase, etc., are known to catalyze the equivalent type of amine oxidation in substrates lacking the α-carboxyl group. However, although these enzymes have received considerable attention with respect to their mechanism and structure, and to some extend their substrate specificity, relatively little is known concerning their enantioselectivity toward racemic substrates.

The flavin-dependent monoamine oxidase N from *Aspergillus niger* (MAO-N; EC 1.4.3.4) was first identified in 1995 by Schilling and Lerch,[76] and subsequently the gene was cloned and overexpressed in *E. coli*.[77] The wild-type enzyme catalyzes the oxidation of simple amines such as butylamine, amylamine, and benzylamine to the corresponding imine using molecular oxygen as the oxidant (Figure 25). However, the native enzyme was found to possess very low activity when presented with chiral amines such as α-methylbenzylamine.

To broaden the substrate specificity of MAO-N, the enzyme has been subjected to several rounds of directed evolution using a combination of random mutagenesis and screening to identify variants possessing activity toward substrates of interest.[78] To facilitate the screening, a high-throughput screening method has been reported that can be used to assay *E. coli* colonies expressing variants of MAO-N. This colorimetric assay, which is based upon capturing the hydrogen peroxide produced as a biproduct, can be used to screen colonies directly on nitrocellulose membranes with a throughput of ca. 100 000 variants per round of evolution. Starting with the wild-type MAO-N, and using α-methylbenzylamine as the probe substrate, a number of variants were identified that possessed broader substrate specificity compared to the wild-type enzyme. These variants were also found to be highly (*S*)-selective and, hence, could be used for the deracemization and stereoinversion of various chiral primary amines.[79] For example, libraries of MAO-N were screened for variants that were able to deracemize chiral secondary amines resulting in the identification of a biocatalyst with improved catalytic properties toward cyclic secondary amines. This new variant had a single point mutation, Ile246Met, and was found to have improved catalytic properties toward a number of other cyclic secondary amines. The new variant was used in the deracemization of *rac*-1-methyl tetrahydroisoquinoline in high yield and ee.[80]

Another variant termed MAO-N D5 has been shown to display good activity toward a wide range of tertiary amines (Figure 26). For example, (*S*)-nicotine **22** was subjected to stereoinversion, via the intermediate iminium ion **23**, yielding (*R*)-**22** in 75% isolated yield and 99% ee within 24 h. The possibility of using a related approach for enantioselective intramolecular reductive amination reactions was also investigated.[81]

This D5 variant was also able to catalyze the enantioslective kinetic resolution of racemic *O*-methyl-*N*-hydroxylamines including substrate **24**, yielding the unreacted (*R*)-enantiomer with an ee = 99%. Interestingly the (*S*)-enantiomer of **24** was found to undergo oxidation only to the (*E*)-oxime product **25** (Figure 27).[82]

As an illustration of the potential for using MAO-N in the synthesis of building blocks for pharmaceuticals, the D5 variant has been applied to the deracemization of racemic crispine A **26**,

an alkaloid with potent biological activity.[83] To aid in the future predictability of suitable substrates for MAO-N variants, a desktop model **27** has been developed that not only allows prediction of the substrate specificity of the MAO-N variants but also rationalizes the observed (*R*)-configuration of the product (Figure 28).

A key aspect to broadening the substrate specificity of MAO-N has been the adoption of a substrate walking approach[84] in which the specific amine used in the screen is varied at each round of the evolutionary process (Figure 29).

Recently, the crystal structure of the MAO-N D5 variant has been solved to 1.8 Å resolution.[85,86] This variant contains 5 mutations compared to the wild-type enzyme, and examination of the structure has provided some insights into the nature of these mutations and how they may contribute to the broadened substrate specificity of the various MAO-N variants. For example, the enzyme, which exists as a dimer, appears to possess two substrate/product channels A and B that connect the surface of the protein to the active site where the flavin is located and where the oxidation reaction takes place. Interestingly, of the five mutations present in the D5 variant, four are located close to these channels suggesting that these mutations may in some way affect the volume or dynamics of the channels and hence exert their effect on substrate specificity by modulating either access of the substrate or perhaps release of the product (Figure 30).

In a recent extension of this work, the highly enantioselective MAO-N variants described above have been shown to be able to catalyze desymmetrization of a range of 3,4-substituted *meso*-pyrrolidines (Figure 31).[87] In most cases the ee's obtained were >98%, although the bicyclic pyrrolidine **28** gave only 94% ee. However, it was possible to improve the ee to >98% by recrystallization of the corresponding crystalline trimer, which exists in equilibrium with the monomeric imines. Interestingly, the rate of oxidation appeared to increase with increasing lipophilicity of the substrate.

The corresponding Δ^1-pyrrolines obtained from the MAO-N oxidation reaction were found to serve as useful building blocks for the synthesis of L-proline analogues and α-amino nitriles of high enantiomeric purity (Figure 32). The product imine **34** arising from the MAO-N catalyzed desymmetrization of amine **28** was isolated from the biotransformation and treated with trimethylsilyl cyanide (TMSCN), resulting in a highly diastereoselective trans-addition of cyanide to give α-amino nitrile **30**.

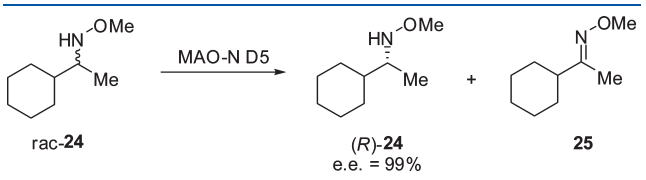

Figure 27. Enantioselective oxidation of racemic *O*-methyl-*N*-hydroxyamines **24**.

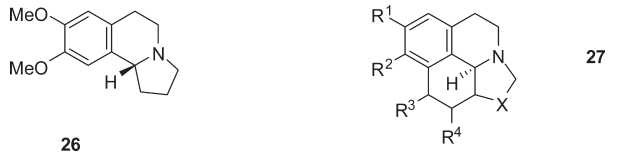

Figure 28. Deracemization of crispine A **26** uses a template-based model **27**.

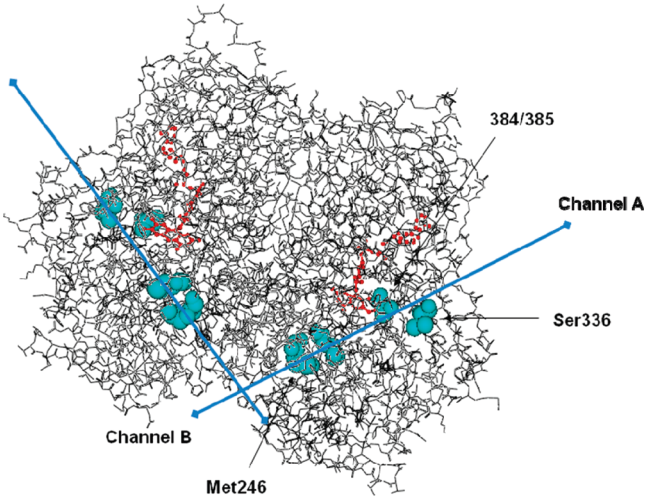

Figure 30. Structure (1.8 Å resolution) of MAO-N highlighting the presence of the channels A and B and also some of the residues that have been mutated to broaden the substrate specificity of the enzyme.

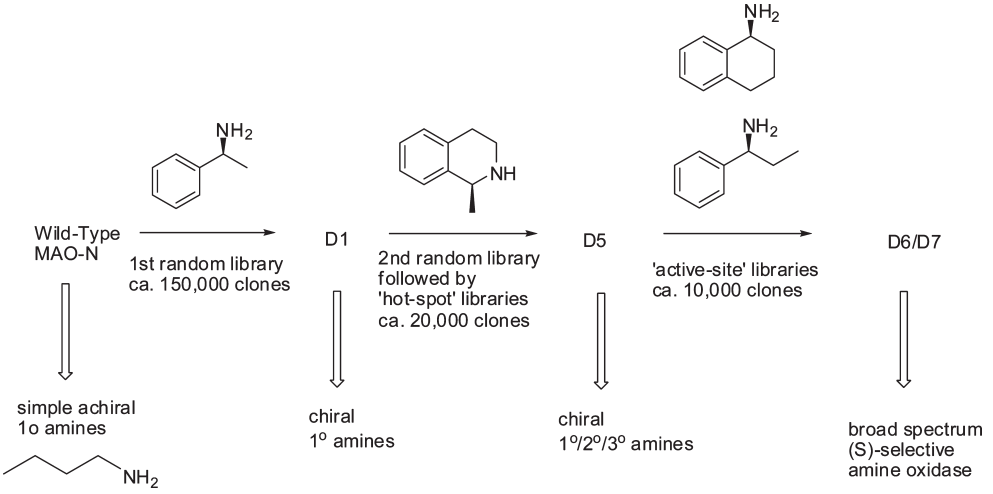

Figure 29. Summary of directed evolution of MAO-N to broaden its substrate range.

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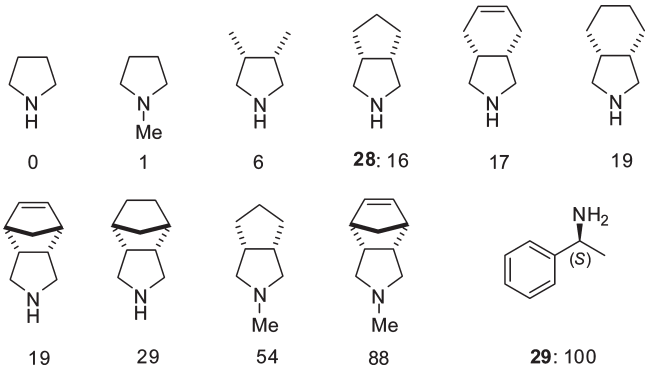

Figure 31. Desymmetrization of a range of symmetrical pyrrolidines using MAO-N. The numbers refer to the rates of oxidation relative to α-methylbenzyl amine **29**.

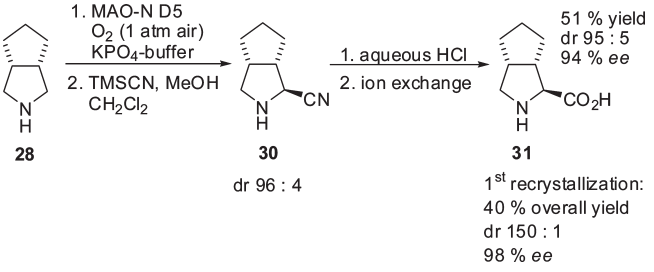

Figure 32. Conversion of the symmetrical bicyclic pyrrolidine **28** to an L-proline analogue **31** via initial MAO-N catalyzed desymmetrization followed by diastereoselective addition of cyanide and subsequent hydrolysis.

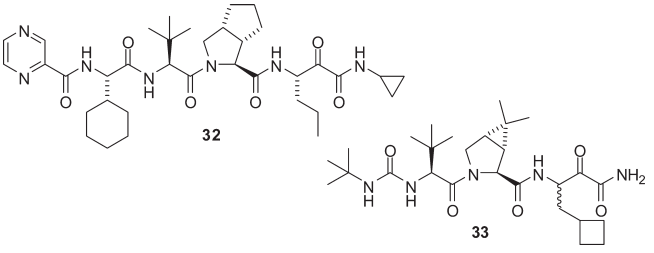

Figure 33. Structures of the hepatitis C viral protease inhibitors telaprevir **32** and boceprevir **33**, which are in phase III clinical trials.

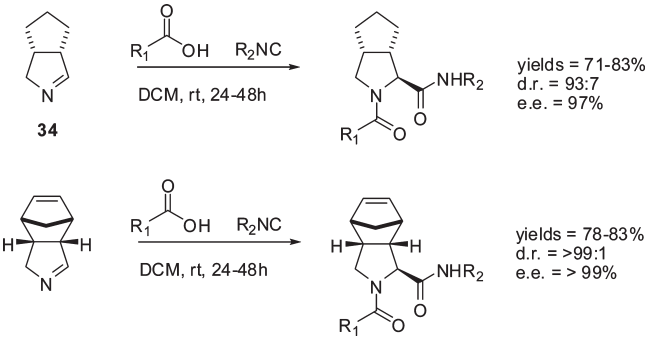

Figure 34. Generation of prolyl-containing peptides by employing imine **34** in a multicomponent Ugi reaction.

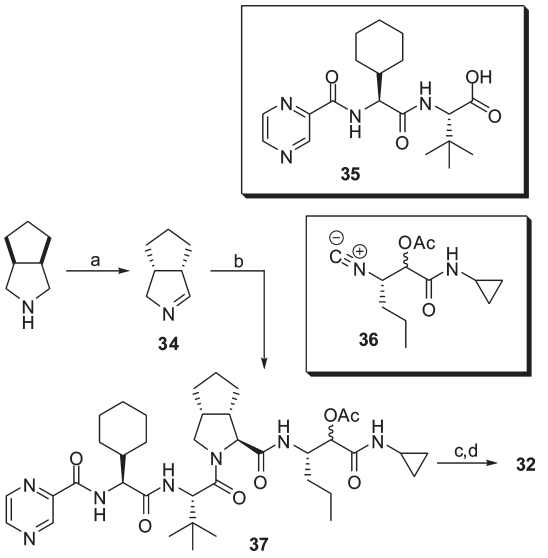

Figure 35. Synthesis of telaprevir **32** using the imine **34** in a multicomponent process. Reagents and conditions: (a) MAO-N, 100 mM KPO_4, pH = 8.0, 37 °C, then (b) **35**, **36**, CH_2Cl_2, 50%; (c) K_2CO_3, MeOH; (d) Dess-Martin, CH_2Cl_2, 50% over 2 steps.

Figure 36. Synthesis of (*R*)- and (*S*)-**38** using enantioselective copper-containing monoamine oxidases.

Subsequent hydrolysis and recrystallization yielded the L-proline analogue **31**. Such bicyclic proline analogues are components of the hepatitis C viral protease inhibitors such as telaprevir **32** and boceprevir **33**, which are currently in phase III clinical trials (Figure 33).[88,89]

The same bicyclic imine has also been used as a substrate in a 3-component Ugi reaction as shown in Figure 34.[90] Treatment of imine **34** with a carboxylic acid and isonitrile results in a highly diastereoselective reaction in which new C−N and C−C bonds are generated.

This strategy has been used to provide an alternative, and more efficient, synthesis of telaprevir **32** by direct coupling of imine **34** with the appropriate carboxylic acid and isonitrile as shown in Figure 35.[91] The imine **32** has also been used as a component of other multicomponent reactions to access a wider range of synthetic alkaloids of interest.[92]

3.5. Copper Amine Oxidase

The copper amine oxidases (CAOs) catalyze the oxidative deamination of primary amines to aldehydes at the expense of the reduction of O_2 to H_2O_2, through an active-site trihydroxyphenylalanine quinone (TPQ) cofactor-mediated pyridoxal-like transamination process.[93,94] Although less work has been reported on these nonflavin-dependent amine oxidases, the enantioselective oxidation of amphetamine by copper-containing amine oxidases from *Escherichia coli* and *Klebsiella oxytoca* was demonstrated in 2000 by Hacisalihoglu et al.[95] Moderate *E* values of ∼15 were obtained, opening up the possibility for future

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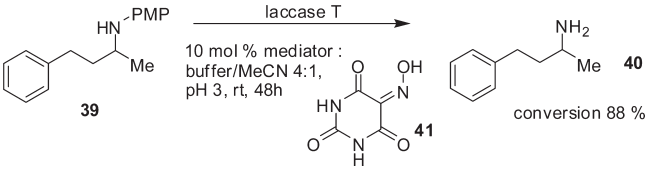

Figure 37. Removal of the *N-para*-methoxyphenyl (PMP) protecting group using laccase in the presence of a mediator.

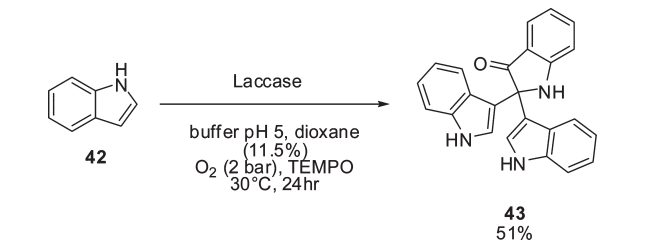

Figure 38. Oxidation of indole **42** to the trimer **43** using laccase in the presence of the mediator TEMPO.

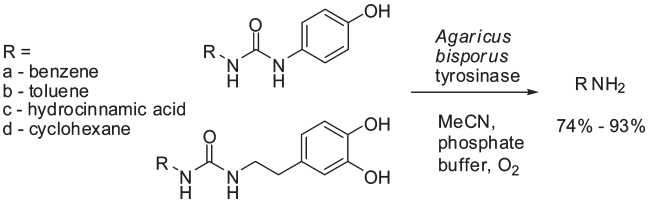

Figure 39. Removal of urea protecting groups using *Agaricus bisporus* tyrosinase in the presence of molecular oxygen.

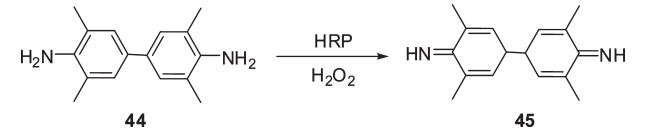

Figure 40. Oxidation of hydrogel precursor using horseradish peroxidase.

applications of these amine oxidase-catalyzed resolutions to generate both (*R*)- and (*S*)-amphetamine **38** (Figure 36).

3.6. Peroxidase, Laccase, and Tyrosinase

This final section deals with a group of metal-dependent enzymes that use either molecular oxygen (laccase, tyrosinase) or hydrogen peroxide (peroxidase) as co-oxidant for the oxidation of C−O and C−N bonds. Interestingly, these enzymes have found application in the removal of protecting groups used in organic synthesis. For example, a novel enzymatic procedure for the oxidative deprotection of *para*-methoxyphenyl (PMP) protected amines has been reported.[96] By using laccases under mildly acidic conditions, a range of PMP-protected amines, including N-protected 4-phenylbutan-2-amine **39**, were successfully deprotected to yield the corresponding free amine **40**. The scope of the reaction could be increased by using mediators such as **41** (Figure 37).

Laccase has also been found to catalyze the conversion of indole **42** into the trimer **43**.[97] Optimization of this reaction through the use of dioxygen overpressure and TEMPO as mediator delivered the trimer in moderate yield (51%) (Figure 38).

Tyrosinase from *Agaricus bisporus* has been used to catalyze the cleavage of the labile protecting groups 4-aminophenol and 3-hydroxytyramine, both of which are relatively stable to the more commonly used deprotection conditions.[98] A range of aliphatic and aromatic ureas were selected for protection yielding the corresponding amines, respectively (Figure 39).

Horseradish peroxidase (HRP) has been developed for a number of applications, including a simple and efficient method to prepare nanogels.[99] Compared with free HRP, the HRP nanogels exhibit similar biocatalytic behavior, as examined using 3,3′,5,5′-tetramethylbenzidine (TMB) **44** as substrate which undergoes oxidation to **45**. The nanogel enzyme demonstrated improved stability at high temperature and in the presence of polar organic solvent (Figure 40).

HRP has also been used for the oxidative coupling of hydrazones **46a** and **46b** with α-naphthol **47**.[100] The reproceeded under mild aqueous conditions, furnishing the corresponding adducts **48a** and **48b** in excellent yield (Figure 41).

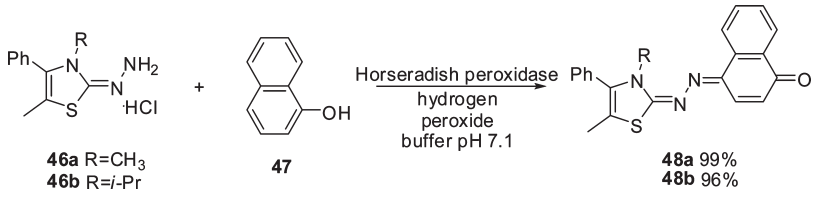

Figure 41. Oxidative coupling of hydrazones **46a** and **46b** with α-naphthol **47** using horseradish peroxidase.

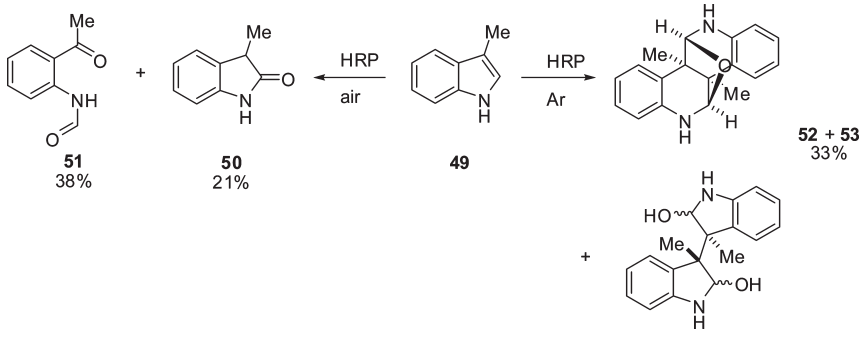

Figure 42. Treatment of 2-methylindole **49** with horseradish peroxidase (HRP) in air leads to a range of different oxidation products.

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The aerobic and anaerobic HRP-catalyzed oxidation of a number of substituted indoles has been investigated.[101] For example, using 2-methylindole **49** as a model substrate resulted in a range of different products being formed, all of which derived from the initial oxidation process. Both oxindole **50** and ring-cleavage products **51** were obtained in addition to the more structurally complex product **52** and **53** derived from dimerization of the substrate. The authors have also carried out additional experiments to gain insight into the mechanism of the reactions and have also examined the corresponding reactions and products using the ethyl substituted analogue (Figure 42).

4. SUMMARY AND OUTLOOK

The use of oxidases for the enantioselective oxidation of C−O and C−N bonds represents an attractive approach for the generation of aldehydes and imines under mild conditions. Dioxygen is a mild oxidant, and coupled with the fact that the reactions typically take place at room temperature and pH 7−8, these transformations are to be considered as green and environmentally friendly. Moreover, the nature of oxidation processes is such that often unwanted side-reactions can occur, particularly in substrates containing multiple functionality and one or more other potential sites of oxidation. Enzymes are inherently selective in the reactions that they catalyze and can often direct the oxidation to one particular part of the molecule. This point is exquisitely illustrated by a recent paper describing the enantioselective oxidation of a C−H bond by berberine bridge enzyme (BBE), resulting in oxidative formation of an intramolecular C−C bond.[102] Enzymes achieve this selectivity by specific alignment of the substrate within the active site such that one part of the molecule is exposed to the locus of oxidation within the enzyme.

As outlined above, the oxidation of C−N bonds leads to formation of imines and thus constitutes activation of C−H bonds α to an amine, a process that is both synthetically difficult to achieve and at the same time highly useful. Chiral imines are often somewhat unstable and moreover generally prepared in situ by reaction of a chiral amine with an aldehyde or alternatively a chiral aldehyde with an imine. The ability to generate imines via oxidation rather than condensation reactions opens up new opportunties for using oxidases and dehydrogenases in tandem with other bio- and chemo-catalysts. C−N bond oxidation using these enzymes can be carried out under considerably milder conditions than is typically required using transition metal based oxidants, suggesting that the former are likely to be more compatible with older reagents for further functionalization. Alcohol, amino acid, and amine oxidases have all been subjected to directed evolution to improve their substrate range and also selectivity. Suitable high-throughput screening methods have been developed that allow investigation of the relationship between sequence and function. In this respect it is likely that these enzymes will become more widely used in the future, particularly for the synthesis of chiral pharmaceutical intermediates and fine chemicals on a large scale.

AUTHOR INFORMATION

Corresponding Author

*E-mail: nicholas.turner@manchester.ac.uk. Telephone: +44 161 306 5173. Fax: +44 161 275 1311.

BIOGRAPHY

Nick Turner obtained his D.Phil. in 1985 with Professor Sir Jack Baldwin and from 1985−1987 was a Royal Society Junior Research Fellow, spending time at Harvard University with Professor George Whitesides. He was appointed lecturer in 1987 at Exeter University and moved to Edinburgh in 1995, initially as a Reader and subsequently Professor in 1998. In October 2004 he joined Manchester University, where his research group is located in the Manchester Interdisicplinary Biocentre (MIB, http://www.mib.ac.uk), as Professor of Chemical Biology. He is Director of the Centre of Excellence in Biocatalysis (CoEBio3, http://www.coebio3.org) and also a cofounder and Scientific Director of Ingenza (http://www.ingenza.com), a spin-out biocatalysis company based in Edinburgh. He is a member of the Editorial Board of Chemical Communications. His research interests are in the area of biocatalysis with particular emphasis on the discovery and development of novel enzyme-catalyzed reactions for applications in organic synthesis. His group is also interested in the application of directed evolution technologies for the development of biocatalysts with tailored functions.

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