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Recent progress in directed evolution of stereoselective monoamine oxidases

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Abstract

Monoamine oxidases (MAOs) use molecular dioxygen as oxidant to catalyze the oxidation of amines to imines. This type of enzyme can be employed for the synthesis of primary, secondary, and tertiary amines by an appropriate deracemization protocol. Consequently, MAOs are an attractive class of enzymes in biocatalysis. However, they also have limitations in enzyme-catalyzed processes due to the often-observed narrow substrate scope, low activity, or poor/wrong stereoselectivity. Therefore, directed evolution was introduced to eliminate these obstacles, which is the subject of this review. The main focus is on recent efforts concerning the directed evolution of four MAOs: monoamine oxidase (MAO-N), cyclohexylamine oxidase (CHAO), *D*-amino acid oxidase (pkDAO), and 6-hydroxy-*D*-nicotine oxidase (6-HDNO).

Keywords: Monoamine oxidase, Directed evolution, Stereoselectivity, Chiral amine

Introduction

When performing catalytic stereoselective transformations, organic chemists can choose between chiral chemical catalysts (Noyori 2002; Sharpless 2002; Walsh and Kozlowski 2009; Zhou 2011; Blaser and Schmidt 2004; MacMillan 2008; List 2010; Allen and MacMillan 2012; Atodiresei et al. 2015; Wang and Tan 2018; Vetica et al. 2017) and enzymes (Drauz et al. 2012; Tao et al. 2009; Gotor et al. 2008; Li et al. 2018b; Reetz 2016a). The well-documented advantages of using enzymes are their exquisite regioselectivity and stereoselectivity at ambient conditions in many chemical reactions (Drauz et al. 2012; Tao et al. 2009; Gotor et al. 2008; Li et al. 2018b; Reetz 2016a). Thus, the use of enzymes as catalysts in synthetic organic chemistry has experienced rapid growth during the last 4 decades. However, the truly broad application of enzymes still suffered from several long-standing limitations for non-natural substrates, e.g., limited substrate scope, poor selectivity, insufficient stability, and sometimes substrate or product inhibition (Reetz 2016b; Ni et al. 2014; Sheldon and Pereira 2017). Nowadays, all of these long-standing limitations of enzymes can be generally addressed by directed evolution (Drauz et al. 2012; Tao et al. 2009; Gotor et al. 2008; Li et al. 2018b; Reetz 2016a).

Directed evolution involves repeated cycles of gene mutagenesis, expression, and screening of mutant enzyme libraries, simulating natural evolution (Reetz 2011; Quin and Schmidt-Dannert 2011; Brustad and Arnold 2011; Bommarius et al. 2011; Siloto and Weselake 2012; Bornscheuer et al. 2012; Porter et al. 2016; Zeymer and Hilvert 2018; Arnold 2018; Denard et al. 2015). When focusing on stereo- and/or regioselectivity, screening is the labor-intensive step (bottleneck of directed evolution) (Acevedo-Rocha et al. 2014; Reymond 2006). As the first step in each cycle, gene mutagenesis is crucial for the success of directed evolution, and therefore, considerable efforts have been invested in the exploration of gene mutagenesis techniques. The most commonly employed gene mutagenesis techniques are error-prone polymerase chain reaction (epPCR) (Leung 1989; Cadwell and Joyce 1994; Chen and Arnold 1993; Reetz et al. 1997), saturation mutagenesis (Estell et al. 1985; Kirsch and Joly 1998; Vandeyar et al. 1988; Zheng et al. 2004), and DNA shuffling (Stemmer 1994). The three mutagenesis techniques

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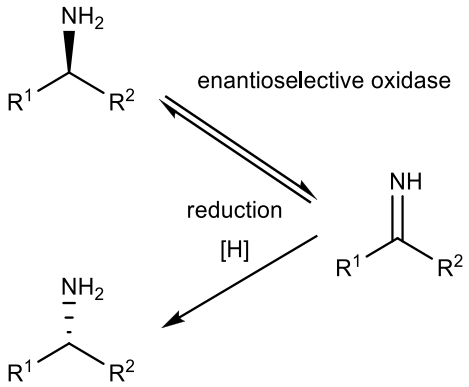
have been widely applied in directed evolution to address the limitations of enzymes. However, efficient approaches for delivering small and "smart" libraries needed in optimizing and inverting stereoselectivity of enzymes had to be explored. Along this line, Reetz et al. introduced Combinatorial Active Site Saturation Test (CAST) (Clouthier et al. 2006; Reetz et al. 2005, 2006a), according to which sites around the binding pocket are chosen for randomization. When the initial CAST libraries do not harbor fully optimized mutants, then iterative saturation mutagenesis (ISM) can be applied (Reetz and Carballeira 2007; Reetz et al. 2006b). The combination CAST/ISM has proven to be an efficient strategy in directed evolution, routinely applied by numerous groups (Acevedo-Rocha et al. 2018; Li et al. 2018a; Zhang et al. 2019a, b; Chen et al. 2018a, b).

Enantiomerically pure chiral amines are valuable synthetic intermediates for the preparation of pharmaceuticals; approximately one-third of the chiral pharmaceuticals on the market contain chiral amine functional groups (Blacker and Headley 2010; Harvey 2008). Traditionally, chiral amines have been obtained by resolution-based methods, for instance, crystallization of a diastereomer using a chiral acid to form a salt (Bálint et al. 2001) or kinetic resolution of a racemate with an enzyme (Guranda et al. 2001; Lee 1999; Van Langen et al. 2000; Skalden et al. 2016). Unfortunately, the maximal theoretical yield of a given enantiomer based on these methods is 50%, severely limiting the efficiency of such a kinetic resolution-mediated process. As a result, the development of efficient and widely applicable biocatalysts for the synthesis of chiral primary, secondary, and tertiary amines has received significant attention (Turner and Truppo 2010). For example, Bäckvall et al. developed a lipase-catalyzed process based on dynamic kinetic resolution (DKR) of primary amines, which requires a transition metal complex for the racemization step (Thalén et al. 2009). In 2002, Turner et al. reported a novel deracemization method for the preparation of optically active primary chiral amines, which involves the stereoinversion of one enantiomer to the other by repeated cycles of enantioselective enzymatic oxidation to the imine using an appropriate amine oxidase, followed by non-selective reduction to the racemic starting amines (Scheme 1) (Alexeeva et al. 2002). In the Turner's deracemization method, the key issue at the outset was to identify a highly enantioselective and active amine oxidase, which at that time was challenging. Turner et al. initiated directed evolution of MAO-N in conjunction with a colorimetric plate-based high-throughput screening assay (Alexeeva et al. 2002). After cycles of directed evolution based on random mutagenesis using a mutator strain, a "toolbox" of MAO-N variants was developed in the



Scheme 1 Process of the deracemization of chiral amines by employing recursive cycles of enantioselective oxidation using an amine oxidase coupled with a non-selective reducing agent

Turner group, which has been successfully applied to the deracemization of primary (Carr et al. 2003), secondary (Alexeeva et al. 2002), and tertiary amines (Dunsmore et al. 2006). The process was also applied to the synthesis of chiral heterocyclic amines such as substituted pyrrolidines (Köhler et al. 2010) and tetrahydro-isoquinolines (Ghislieri et al. 2013; Rowles et al. 2012). Prompted by these pioneering studies, more monoamine oxidases were identified and subjected to directed evolution for chiral amine synthesis; for instance, CHAO (Leisch et al. 2011; Li et al. 2014a), pkDAO (Yasukawa et al. 2014, 2018), and 6-HDNO (Heath et al. 2014).

The present review summarizes recent efforts regarding the directed evolution of monoamine oxidases for the synthesis of chiral amines. It is not meant to be comprehensive. Rather, a select number of representative studies are featured and analyzed, illustrating the viability of directed evolution for manipulating the catalytic properties of monoamine oxidases.

Directed evolution of monoamine oxidase from *Aspergillus niger* (MAO-N)

The most frequently reported monoamine oxidase is MAO-N, which displays high activity towards aliphatic amines, e.g., amylamine and butylamine, low but measurable activity to benzylamine and α-methylbenzylamine (Carr et al. 2003). Turner et al. chose this enzyme as a viable starting point for improving both the catalytic activity and stereoselectivity by directed evolution. At the time of the initial study, the X-ray data were not available; therefore, the method of random mutagenesis based on *E. coli* XL1-Red mutator strain was employed for generating a library of MAO-N variants. Facing the huge library size of variants created by this gene mutagenesis technique, the initial priority was the development of an

effective high-throughput screening assay based upon capture of the generated hydrogen peroxide by a peroxidase in the presence of 3,3′-diaminobenzidine, giving a dark pink, insoluble product (Fig. 1) (Carr et al. 2003). With this plate-based colorimetric assay in hand, a subset of the library (about 150,000 clones) was screened, finally identifying an improved mutant (Asn336Ser). Relative to wild-type (WT) enzyme, the activity and stereoselectivity of Asn336Ser variant increased 47-fold and 5.8-fold, respectively. Without further mutation, the Asn336Ser variant was tested for a panel of amine substrates with broad structural features. Variant Asn336Ser showed significantly wider substrate profile relative to WT MAO-N, particularly for primary amines, but also for a number of secondary amines. The new variant was used in the deracemization of *rac*-1-methyltetrahydroisoquinoline, producing high yield and *ee* value. To extend the chemoenzymatic deracemization method to encompass more types of amines, the MAO-N were then subjected to several rounds of directed evolution following a similar procedure as described above, identifying two property improved variants MAO-N-D3 (Asn336Ser/Met348Lys/Ile246Met) (Carr et al. 2005) and MAO-N-D5 (Asn336Ser/Met348Lys/Ile246Met/Thr384Asn/Asp385Ser) (Dunsmore et al. 2006). This D5 variant displayed good activity toward a wide range of tertiary amines (Fig. 2), particularly for pyrrolidine derivatives that are flanked by bulky aryl groups. Preparative scale deracemization of *Rac-N*-methyl-2-phenylpyrrolidine (**3**) was performed at 25 mM concentration using the D5 variant as oxidant, giving (*R*)-**3** in 75% isolated yield and 99% *ee* within 24 h (Fig. 3) (Dunsmore et al. 2006). As an illustration of the potential of variant D5 in organic synthetic chemistry, it was also applied for the desymmetrization of the bicyclic pyrrolidine **11** (Fig. 4), a building block for the synthesis of hepatitis C virus protease inhibitor telaprevir (**14**) (Köhler et al. 2010). The reaction reached more than 98% conversion within 7 h, giving 77% isolated yield and 94% ee, and recrystallization of the trimer of **13** improved the *ee* to at least 98% (Köhler et al. 2010).

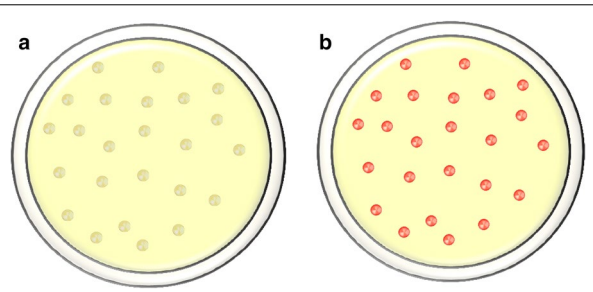


Fig. 1 Colorimetric plate-based screening assay for amine oxidase activity by capture of the hydrogen peroxide produced using 3,3′-diaminobenzidine with peroxidase. **a** Colonies with *D*-α-methylbenzylamine as screening substrate. **b** Identical clones with the *L*-enantiomer as substrate

Although impressive advances have been achieved in the directed evolution of MAO-N, the present "toolbox" of MAO-N did not accept bulky amines such as

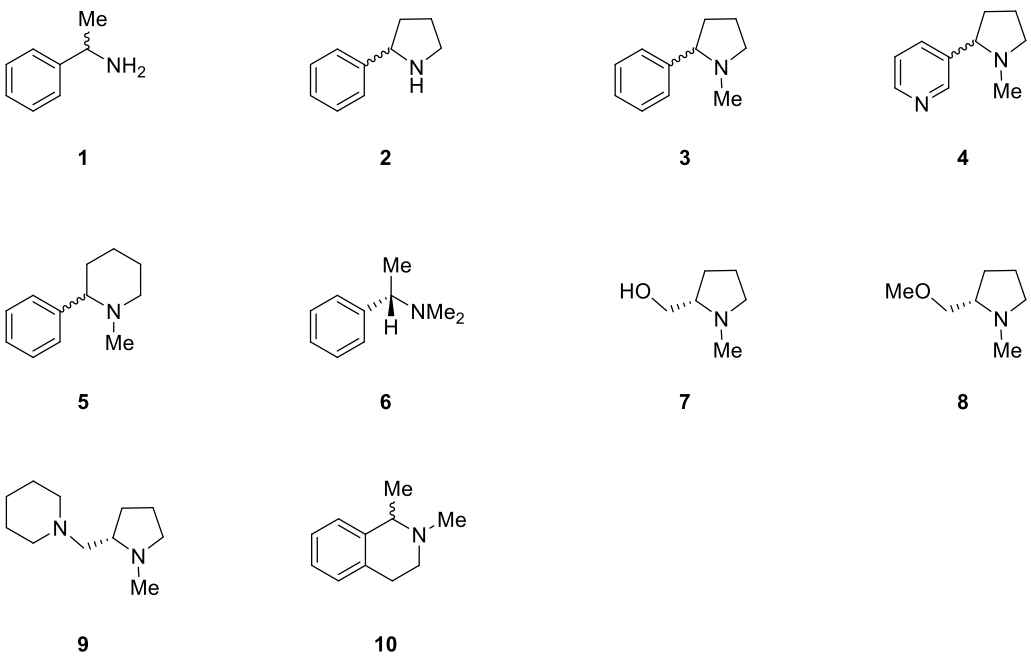


Fig. 2 Structurally diverse amines were subjected to the Turner's deracemization method using variant MAO-N-5

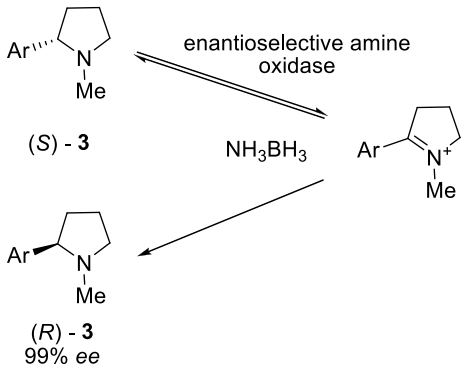


Fig. 3 Deracemization of racemic *N*-methyl-2-phenylpyrrolidine **3** to (*R*)-*N*-methyl-2-phenylpyrrolidine by employing a sequence of enantioselective oxidations using variant MAO-N-5 coupled with the non-selective reducing agent NH_3BH_3

4-chlorobenzhydrylamine (**15**) and 1-phenyltetrahydroisoquinoline (**16**) (Fig. 5), motifs that are present in the commercially available drugs Levocetirizine (**17**) and Solifenacin (**18**) (Fig. 5), respectively (Hermanns et al. 2002; Nguyen et al. 2011). To improve the "toolbox" of MAO-N further to catalyze these bulky amines, the D5 variant of MAO-N was subjected to further directed evolution. Based on the results of docking *α*-methylbenzylamine into the MAO-N D5 active center, it became clear that increasing the volume of binding pocket would allow the accommodation of the bulky substrates with two aryl substituents. To reach this goal, two residues (Ala429 and Trp430) were targeted for saturation mutagenesis (Ghislieri et al. 2013). By screening the two saturation mutagenesis libraries, variant MAO-N D10 with a new single amino acid substitution Trp430Gly was identified, which was active toward amine **15** for the first time, although only to a moderate extent. In an attempt to improve the activity further, attention was then turned to the active site channel, (previous) mutations in this region resulting in a panel of mutants MAO-N D9 (A-D) (Table 1) which showed significantly enhanced activity to the bulky amine crispine A relative to MAO-N D5. A series of mutants MAO-N D11 (A-D) (Table 1) were created by the combination of essential mutations in MAO-N D9 (A-D) with MAO-N D10, and their activity and stereoselectivity towards amine **15** were measured by monitoring the conversion of the corresponding enantiomer of **15** using HPLC, which proved MAO-N D11C to be the best mutant. Consistent with the activity tests, the computed volume of the binding pocket in D5, D10, and D11C clearly increase from 140 $Å^3$ (D5) to 195 $Å^3$ (D10), and further to 464 $Å^3$ (D11C). The D11C

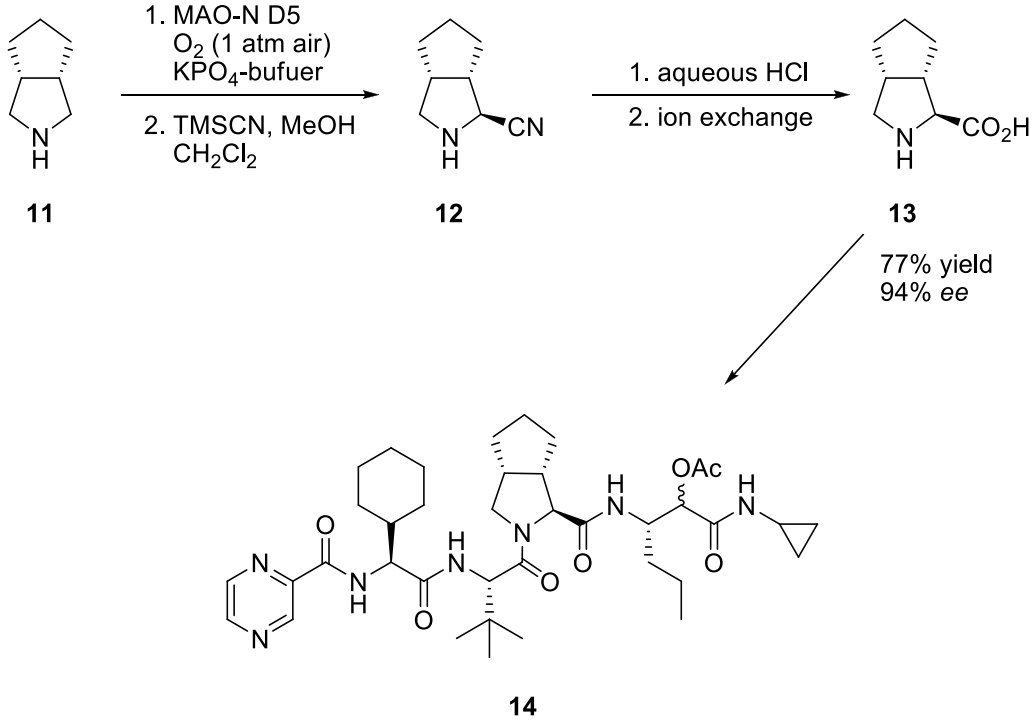


Fig. 4 Conversion of the symmetrical bicyclic pyrrolidine **11** to an *L*-proline analog **13** via initial MAO-N catalyzed desymmetrization followed by diastereoselective addition of cyanide and subsequent hydrolysis

Table 1 MAO-N variants obtained by directed evolution and respective oxidation rate towards (*S*)-15 and (*R*)-15

MAO-N variant	Mutation	(*S*)-15 (%)	(*R*)-15 (%)	Time (h)
MAO-N D5	I246M/N336S/M348K/T384N/D385S	0	0	48
MAO-N D9A	D5+ F210M/L213C/M242V/I246T	0	0	48
MAO-N D9B	D5+ F210L/L213T/M242V/I246T	0	0	48
MAO-N D9C	D5+ F210L/L213T/M242Q/I246T	0	0	48
MAO-N D9D	D5+ F210M/L213C/M242Q/I246T	0	0	48
MAO-N D10	D5+ W430G	75	26	48
MAO-N D11A	D10+ F210M/L213C/M242V/I246T	100	12	30
MAO-N D11B	D10+ F210L/L213T/M242V/I246T	100	7	4
MAO-N D11C	D10+ F210L/L213T/M242Q/I246T	100	< 1	6
MAO-N D11D	D10+ F210M/L213C/M242Q/I246T	100	14	35

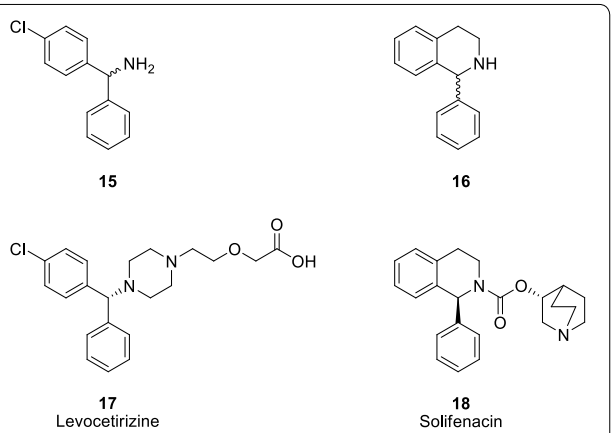


Fig. 5 Substituted benzhydrylamines **15** and **16** as important structural motifs in commercial pharmaceuticals Levocetirizine **17** and Solifenacin **18**, respectively

variant was applied for the deracemization of *rac*-**15** on a 0.5 g scale, and (*R*)-**15** with 97% *ee* was obtained within 48 h with 45% isolated yield. The compromised yield was shown to be due to partial hydrolysis of the unstable intermediate imine. A similar procedure was also applied for the deracemization of *rac*-**16** on a 1 g scale, 98% *ee,* and 90% isolated yield being obtained within 24 h (Ghislieri et al. 2013).

Relevant to this work is a study of the Reetz group involving the simultaneous manipulation of activity and stereoselectivity of MAO-N by focusing simultaneously on residues lining the entrance tunnel and the binding pocket (Li et al. 2017). In this case, 23 residues located in the entrance tunnel and the binding pocket of MAO-N were chosen and split into six groups for saturation mutagenesis using NDT codon degeneracy (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, and Gly) as building blocks (Fig. 6). The corresponding saturation mutagenesis libraries were then screened toward three model amines for identifying positive hits and critical ("hot") positions. Subsequently, further ISM was carried out based on the information obtained in the first round of directed evolution, leading to high active and stereoselective variants LG-I-D11 (W230R/W430C/C214L) and LG-J-B4 (W230I/T354S/W430R/M242R/Y365 V). The mutations induced in these variants lead to reversal of enantioselectivity of Turner-type deracemization in the synthesis of amines (*S*)- 1-methyl-1,2,3,4-tetrahydroisoquinoline (*S*-**19**), (*S*)-1-phenyl-1,2,3,4-tetrahydroisoquinoline (*S*-**20**), (*S*)-1-ethyl-1,2,3,4-tetrahydroisoquinoline (*S*-**21**), and (*S*)-1-isopropyl-1,2,3,4-tetrahydroisoquinoline (*S*-**22**) (Fig. 7). This is a significant result, because only the corresponding (*R*)-**19** were previously accessible using the earlier MAO-N variants. Molecular Docking and Molecular Dynamics Simulations indicate that it is the increased hydrophobicity of the entrance tunnel acting in concert with the altered shape of the binding pocket that results in the altered catalytic profile.

In another significant case, directed evolution for simultaneously improving activity and thermostability of MAO-N was achieved in a Merck/Codexis collaboration, which developed a chemoenzymatic method for the synthesis of a bicyclic[3.1.0]proline moiety "P2" (Fig. 8), the key structural feature in Boceprevir (Li et al. 2012). Since the crystal structure of MAO-N was not available at that time, error-prone PCR and homology model-guided mutagenesis were employed for the first round of directed evolution, and variant MAO-N 156 with two single mutations A289V and K348Q was obtained, which showed 2.4-fold higher activity in comparison with WT. Thereafter, two parallel strategies were chosen in the second round evolution: recombination of positive mutations with MAO-N 156; family shuffling of MAO-N 156 with the homolog from *A. oryzae*, leading to two positive hits (MAO-N 274 and MAO-N 291) with respective threefold and sixfold improvement in activity. Two further rounds

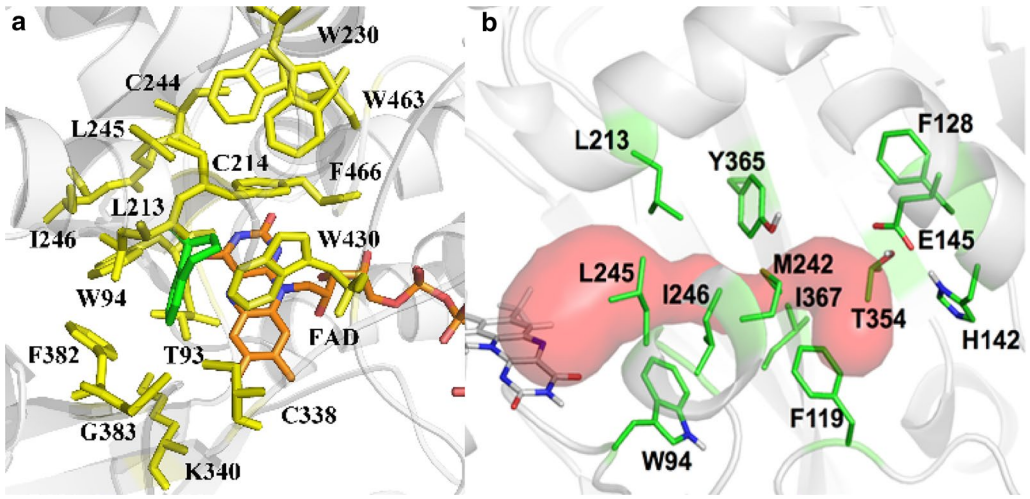


Fig. 6 MAO-N residues chosen for saturation mutagenesis, marked in the homology model, which was built using the crystal structure of MAO-N-D3 (PDB: 2VVL). A: Active site mutation sites (yellow), selected on the basis of induced fit docking of amine **19** (green). B: Residues surrounding the substrate access tunnel (red) likewise chosen for mutagenesis (shown in green)

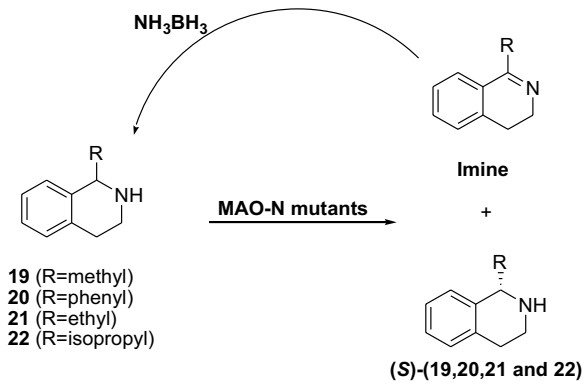


Fig. 7 Deracemization of racemates **19**, **20**, **21,** and **22** by employing a sequence of enantioselective oxidations with MAO-N mutants and non-selective reduction with NH_3BH_3

of directed evolution were conducted based on family shuffling and active site-targeted mutation. This finally provided the super variant MAO-N 401, which was successfully applied for large-scale production of (1*R*,2*S*,5*S*)-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carbonitrile (**26**) with correct absolute configuration and > 99% *ee* value in a chemoenzymatic process, thereby significantly decreasing the cost, while improving the sustainability for the production of Boceprevir (Li et al. 2012).

Directed evolution of monoamine oxidase from *Brevibacterium oxydans* IH-35A (CHAO)

The monoamine oxidase CHAO, a 50 kDa flavoprotein responsible for the oxidation of cyclohexylamine to cyclohexanone in *Brevibacterium oxydans* IH-35A, displays high activity towards a wide range of structurally

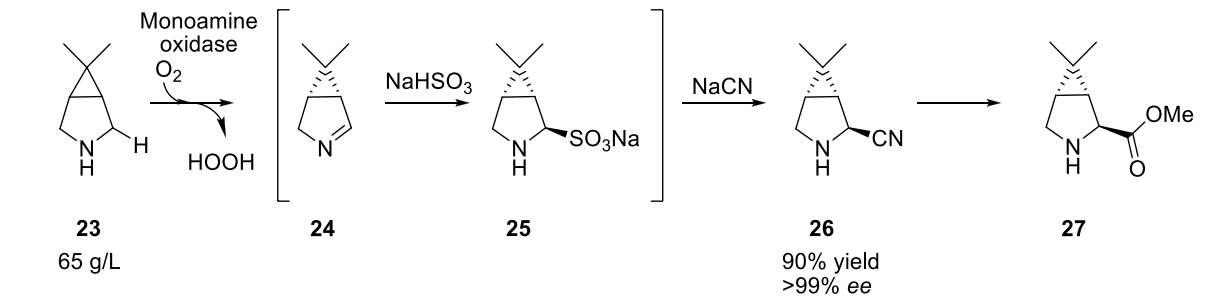


Fig. 8 The Merck/Codexis process for manufacturing a Boceprevir intermediate based on amine oxidase-catalyzed desymmetrization

different primary amines (Leisch et al. 2011). However, deracemization of secondary amines via WT CHAO cannot be achieved due to low or no activity towards this kind of amines. To explore the potential of CHAO for the synthesis of optically pure secondary amines, for example 1,2,3,4-tetrahydroquinoline (THQ) derivatives, directed evolution was carried out for the first time in Dunming Zhu's group (Li et al. 2014b). In this attempt, 11 amino acid residues (F88, T198, L199, M226, Q233, Y321, F351, L353, F368, P422, and Y459) located in the active center of CHAO were targeted for saturation mutagenesis. Four single mutants (T198F, L199T, M226F, and Y459T), displaying more than 20-fold activity towards 2-methyl-THQ relative to WT CHAO, were identified. Subsequently, ISM were performed on the four positive "hot" positions (T198, L199, M226, and Y459) for improving the activity further. This led to two new hits, namely T198F/L199S and T198F/L199S/M226F. The triple variant T198F/L199S/M226F was successfully applied for the deracemization of 2-methyl-1,2,3,4-tetrahydroquinoline on a preparative scale, giving 76% isolated yield and 98% *ee* of the (*R*)-enantiomer (Fig. 9).

To further expand the biocatalytic repertoire of CHAO, Yao et al. created a new library containing diverse mutants, and then assayed them towards a panel of 2-substituted THQs (Fig. 10). Several highly (*S*)-selective mutants with notably enhanced activity were identified. Significantly, variant L225A showed reversed selectivity (*R*-selectivity) in reactions of 1,2,3,4-tetrahydro-2-methylquinoline (**31**), 1,2,3,4-tetrahydro-2-isopropylquinoline (**32**), 2-cyclopropyl-1,2,3,4-tetrahydroquinoline (**33**), and 2-(2-Benzo[1,3]dioxol-5-yl-ethyl)-1,2,3,4-tetrahydroquinoline (**42**). Molecular dynamic simulations were conducted based on the models of variant L225A harboring (*R*)- and (*S*)-cyclopropyl-THQ, and computational results revealed

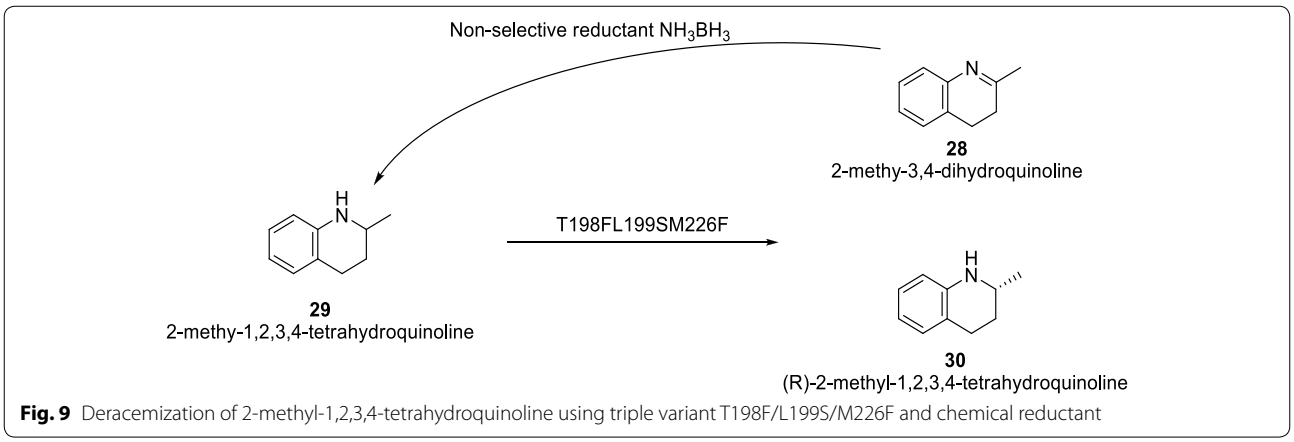


Fig. 9 Deracemization of 2-methyl-1,2,3,4-tetrahydroquinoline using triple variant T198F/L199S/M226F and chemical reductant

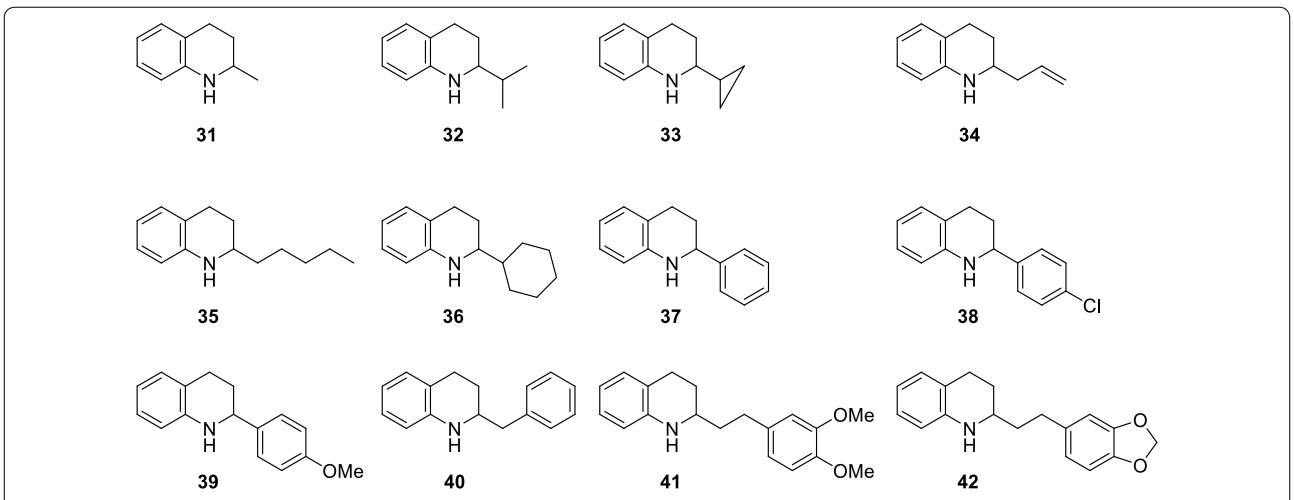


Fig. 10 2-Substituted THQs were tested as substrates in deracemization using CHAO mutants

the mechanism of enantioselectivity reversal (Yao et al. 2018).

Directed evolution of *R*-selective monoamine oxidases

Directed evolution of MAO-N and CHAO has provided diversified biocatalysts for the synthesis of chiral amines. However, until 2014, no enantio-complementary (*R*)-amine oxidases had been reported for general application. In this respect, Asano et al. evolved a flavin-dependent porcine kidney *D*-amino acid oxidase (pkDAO) into an *R*-stereoselective amine oxidase (Yasukawa et al. 2014). Based on the crystal structure of pkDAO, two residues Tyr228 and Arg283 at the active center were subjected to saturation mutagenesis. Screening the respective libraries towards the model amine *rac*-*α*-methylbenzylamine identified three hits, namely R283G, R283A, and R283C. These were then used as templates for ISM at residue Tyr 228, leading to three improved mutants (Y228L/R283G, Y228L/R283A and Y228L/R283C). Deracemization of *rac*-*α*-methylbenzylamine on a preparative scale was conducted using the best variant Y228L/R283G, producing 99% (*S*)-*α*-methylbenzylamine with 65% isolated yield. Crystal structure analysis revealed that the mutations introduced in the directed evolution induced critical changes at the active center, which in turn render the *α*-hydrogen atom of (*R*)-*α*-methylbenzylamine to point towards the N5 atom of FAD, while in the case of (*S*)-*α*-methylbenzylamine, the N5 atom of FAD remains remote, therefore, leading to high *R*-stereoselectivity (Yasukawa et al. 2014). For further expanding the substrate specificity of pkDAO towards bulky amines, such as amine **15**, variants R283G and Y228L/R283G were chosen as templates for saturation mutagenesis at residues Leu51, Ile215, and Ile230, which could play a critical role in accommodating the bulky substrate (Yasukawa et al. 2018). After high-throughput screening with colorimetric assay, four positive mutants, namely I230A/R283G, I230C/R283G, I230F/R283G, and Y228L/I230C/R283G, were identified. Among them, variant I230A/R283G showed highest catalytic efficiency towards (*S*)-**15**, and the deracemization of *rac*-**15** using this variant was also achieved successfully within 1 h, producing (*R*)-**15** in 98% *ee*. As expected, the crystal structure of variant I230A/R283G indicated that the mutations introduced in the variant provided extra space to accommodate the 4-Cl-phenyl ring of amine **15** (Yasukawa et al. 2018).

Another *R*-selective amine oxidase was developed based on 6-hydroxy-*D*-nicotine (6-HDNO) in Turner's group. Wild-type 6-HDNO displayed relative narrow substrate scope, and hence, directed evolution again based on CASTing was performed with the aim to broaden its substrate spectrum (Heath et al. 2014). Two saturation mutagenesis libraries A (Leu373/Leu375) and B (Glu350/Glu352) were constructed using NNK code degeneracy, which were then screened with the colorimetric solid-phase assay. Although no positive variants was found in library A, three positive hits, namely E350L/E352D, E350 V/E352D, and E350L, were identified in library B. The E350L/E352D variant, showing significant improvement in substrate specificity, was used for the deracemization of several amines. As expected, in all cases, the variant exhibited *R*-stereoselectivity, the opposite enantioselectivity observed when using MAO-N (Heath et al. 2014).

Conclusion

Monoamine oxidases use molecular dioxygen as the stoichiometric oxidant to catalyze the irreversible oxidation of amines to imines. This feature avoids the problem of controlling the reaction equilibrium position, making them an attractive class of enzyme for chiral amine synthesis (Turner 2011). Relative to the reported methods for chiral amine preparation, such as classical resolution of the corresponding racemate (Siedlecka 2013), lipase-catalyzed kinetic resolution (Poulhès et al. 2011; Verho and Bäckvall 2015; Oláh et al. 2016; Gustafson et al. 2014), and asymmetric hydrogenation of imines (Liu and Du 2013; Ghattas et al. 2012; de Vries and Mršić 2011; Li et al. 2016; Echeverria et al. 2016; Lautens and Larin 2018), monoamine oxidases, once subjected to protein engineering, display widespread substrate specificity in Turner-type deracemization for the industrial synthesis of enantiomerically pure primary, secondary, and tertiary amines as well as chiral heterocyclic amines. In this deracemization process, the chemical reducing agent (typically the ammonia borane complex NH_3BH_3) is added in excess amount for reducing the imine non-selectively back to the amine. To avoid an excess of NH_3BH_3, imine reductase as an excellent alternative of chemical reducing agent was successfully applied for the synthesis of chiral amines in a new cascade reaction based on the combination of imine reductase with amine oxidase (Gand et al. 2014; Leipold et al. 2013; Mangas-Sanchez et al. 2017; Mitsukura et al. 2010, 2013). These developments open the door for further industrial preparations of chiral amines by deracemization processes (Heath et al. 2016).

Although original monoamine oxidases have suffered traditionally from distinct limitations; for example, low activity, narrow substrate scope, wrong stereoselectivity, and insufficient thermostability, with the help of directed evolution as summarized here, most problems have been solved, thereby creating a versatile "toolbox" of monoamine oxidases. CAST/ISM has proven to be a particularly viable mutagenesis strategy. With the advance of new protein engineering techniques, such as rational design (Choi et al. 2015; Otten et al. 2010) and machine

learning (Li et al. 2019), it can be hoped that new and useful mutants of various monoamine oxidases will be generated even more rapidly and efficiently than in the past. In some cases, the insolubility of certain substrates in aqueous medium causes practical problems, which need to be solved in the future with the aid of bioprocess engineering. The pharmaceutical industry and other fine chemical companies will certainly profit from such potential advances.

Abbreviations

MAOs: monoamine oxidases; MAO-N: monoamine oxidase from *Aspergillus niger*; CHAO: cyclohexylamine oxidase from *Brevibacterium oxydans* IH-35A; pkDAO: *D*-amino acid oxidase porcine kidney; 6-HDNO: 6-hydroxy-*D*-nicotine oxidase; epPCR: error-prone polymerase chain reaction; CAST: Combinatorial Active Site Saturation Test; ISM: iterative saturation mutagenesis; DKR: dynamic kinetic resolution; WT: wild type; THQ: 1,2,3,4-tetrahydroquinoline.

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Authors' contributions

GYL and JQD conceived and wrote this paper. BBL, YCQ, YJD, and JR were involved collecting related material and critical reading of this paper. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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