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Engineering an Enantioselective Amine Oxidase for the Synthesis of Pharmaceutical Building Blocks and Alkaloid Natural Products

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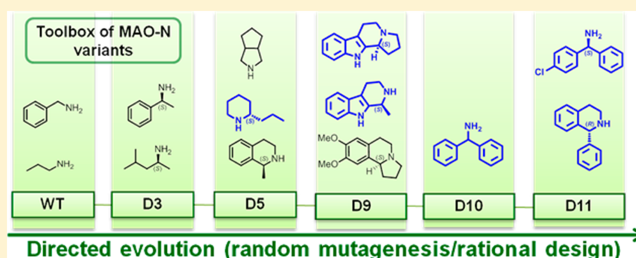
S *Supporting Information*

ABSTRACT: The development of cost-effective and sustainable catalytic methods for the production of enantiomerically pure chiral amines is a key challenge facing the pharmaceutical and fine chemical industries. This challenge is highlighted by the estimate that 40−45% of drug candidates contain a chiral amine, fueling a demand for broadly applicable synthetic methods that deliver target structures in high yield and enantiomeric excess. Herein we describe the development and application of a "toolbox" of monoamine oxidase variants from *Aspergillus niger* (MAO-N) which display remarkable substrate scope and tolerance for sterically demanding motifs, including a new variant, which exhibits high activity and enantioselectivity toward substrates containing the aminodiphenylmethane (benzhydrylamine) template. By combining rational structure-guided engineering with high-throughput screening, it has been possible to expand the substrate scope of MAO-N to accommodate amine substrates containing bulky aryl substituents. These engineered MAO-N biocatalysts have been applied in deracemization reactions for the efficient asymmetric synthesis of the generic active pharmaceutical ingredients Solifenacin and Levocetirizine as well as the natural products (*R*)-coniine, (*R*)-eleagnine, and (*R*)-leptaflorine. We also report a novel MAO-N mediated asymmetric oxidative Pictet−Spengler approach to the synthesis of (*R*)-harmicine.



INTRODUCTION

Many natural products and pharmaceutical drugs contain chiral amine functional groups, which in many cases, are the basis for the exquisite biological activity that these compounds exhibit.[1−4] The increasing demand to access chiral amines in optically pure form has stimulated the development of methodology for the asymmetric synthesis of key structural motifs[5] including those based upon catalytic approaches.[6] However, in many cases these methods are only applicable to a small subset of chiral amines (e.g., 1°, 2°, or 3° amines). As a result, classical resolution of racemic final products or key intermediates is often the method of choice to produce enantiomerically pure compounds on an industrial scale.[5]

Unfortunately, the maximum theoretical yield of the desired enantiomer from this method is 50%, severely limiting the efficiency of this process, particularly in situations where the synthesis of the racemic product in question requires a multistep route. As a result, the development and application of biocatalysts for the synthesis of chiral amines has received significant attention.[7] Examples of representative classes of enzymes that are utilized for amine synthesis are shown in Figure 1, including transaminases[8] and ammonia lyases[9], which are now finding application in the industrial synthesis of chiral amines.

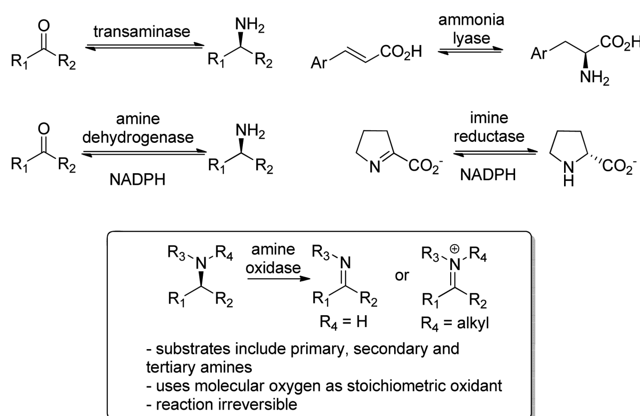


Figure 1. Examples of enzymes employed for the biocatalytic production of chiral amines.

A particularly significant example involved the extensive protein engineering of an (*R*)-selective transaminase for synthesis of the antidiabetic compound Sitagliptin.[8] New emerging biocatalysts for the asymmetric synthesis of chiral

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amines include engineered amino acid dehydrogenases, which are able to use ketones as substrates.[10]

Monoamine oxidases catalyze the conversion of amines to imines and represent a particularly attractive class of enzyme for chiral amine synthesis. The oxidation reaction is irreversible, avoiding the problem of controlling the reaction equilibrium position, which is associated with a number of alternative classes of enzymes. Additionally, these enzymes use molecular oxygen as the stoichiometric oxidant. We have previously reported the development of variants of monoamine oxidase from *Aspergillus niger* (MAO-N), which are able to selectively oxidize a range of chiral amines. When coupled with a nonselective chemical reducing agent, the MAO-N variants developed in our laboratory have been shown to mediate the deracemization of simple chiral primary, secondary, and tertiary amines.[11−13] Although impressive advances have been made in the generation of nonenzyme-based catalysts for the oxidation of amines to imines,[14] to date there have been no reports of enantioselective catalysts that are generally synthetically applicable.

We now demonstrate that the substrate range of MAO-N can be significantly increased, in particular, by the development of a new D11 variant, which possesses high activity and stereoselectivity toward substrates containing the sterically demanding aminodiphenylmethane (benzhydrylamine) motif. The application of this new variant is highlighted by the asymmetric synthesis of two important drug molecules, Levocetirizine and Solefenacin, which are currently prepared industrially using classical resolution. Other MAO-N variants are shown to display complementary substrate specificity enabling new important classes of alkaloid natural products to be efficiently deracemized. Finally, the application of MAO-N in a cascade sequence leading to the efficient formation of (*R*)-harmicine is also described. Overall, the results presented highlight the potential of MAO-N as a highly tunable biocatalyst for the production of a diverse range of alkaloid natural products and drug molecules in enantiomerically pure form.

RESULTS AND DISCUSSION

Engineering of MAO-N for the Enantioselective Oxidation of Substituted Benzhydrylamines. Substituted benzhydrylamines[15,16] and benzhydrylalcohols are sterically demanding structural motifs, which are present in a number of active pharmaceutical ingredient (API)s. As a result, development of catalytic methods to access these structures as single enantiomers represents an important challenge. For example, the asymmetric reduction of nonsymmetrical diarylketones to the corresponding diphenylmethanols using ketoreductases has been described.[17] Reduction of (4-chlorophenyl)(phenyl)-methanone proceeded in a moderate 64% e.e., highlighting the challenges associated with the chiral recognition of this particular substrate. The development of MAO-N variants with good activity and selectivity toward the benzhydrylamine template represented a significant challenge because previously reported MAO-N variants were found to display no activity toward the parent aminodiphenyl methane **3** or toward derivatives such as 4-chlorobenzhydrylamine **4** and 1-phenyl-tetrahydroisoquinoline **6**, motifs that are present in the commercially available drugs Levocetirizine **5** and Solefenacin **7**, respectively. Generation of activity toward substituted benzhydrylamines presented an additional challenge of enantioselectivity due to the difficulty in differentiating the two structurally related aryl substituents (Figure 2).

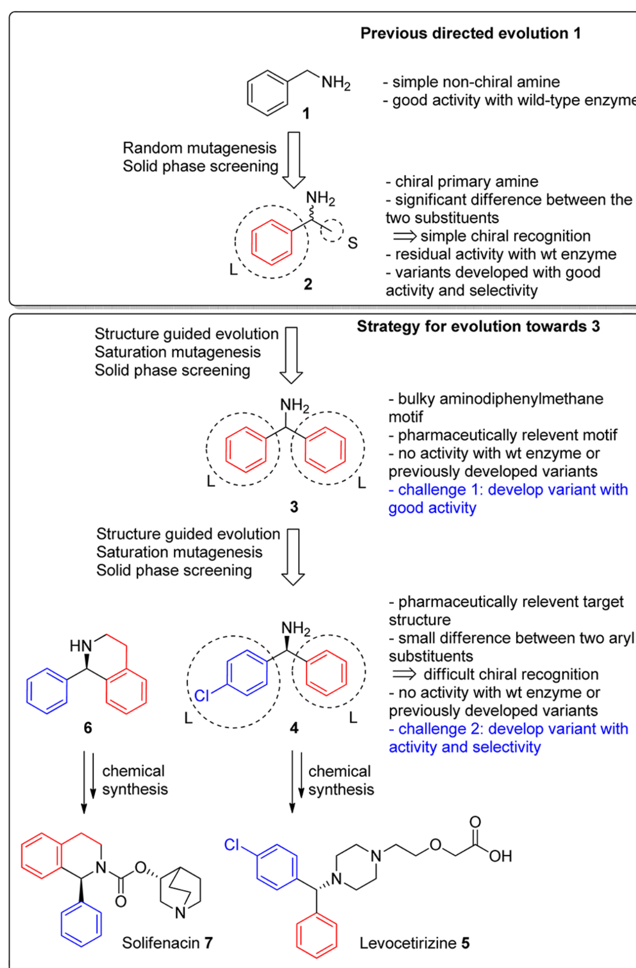
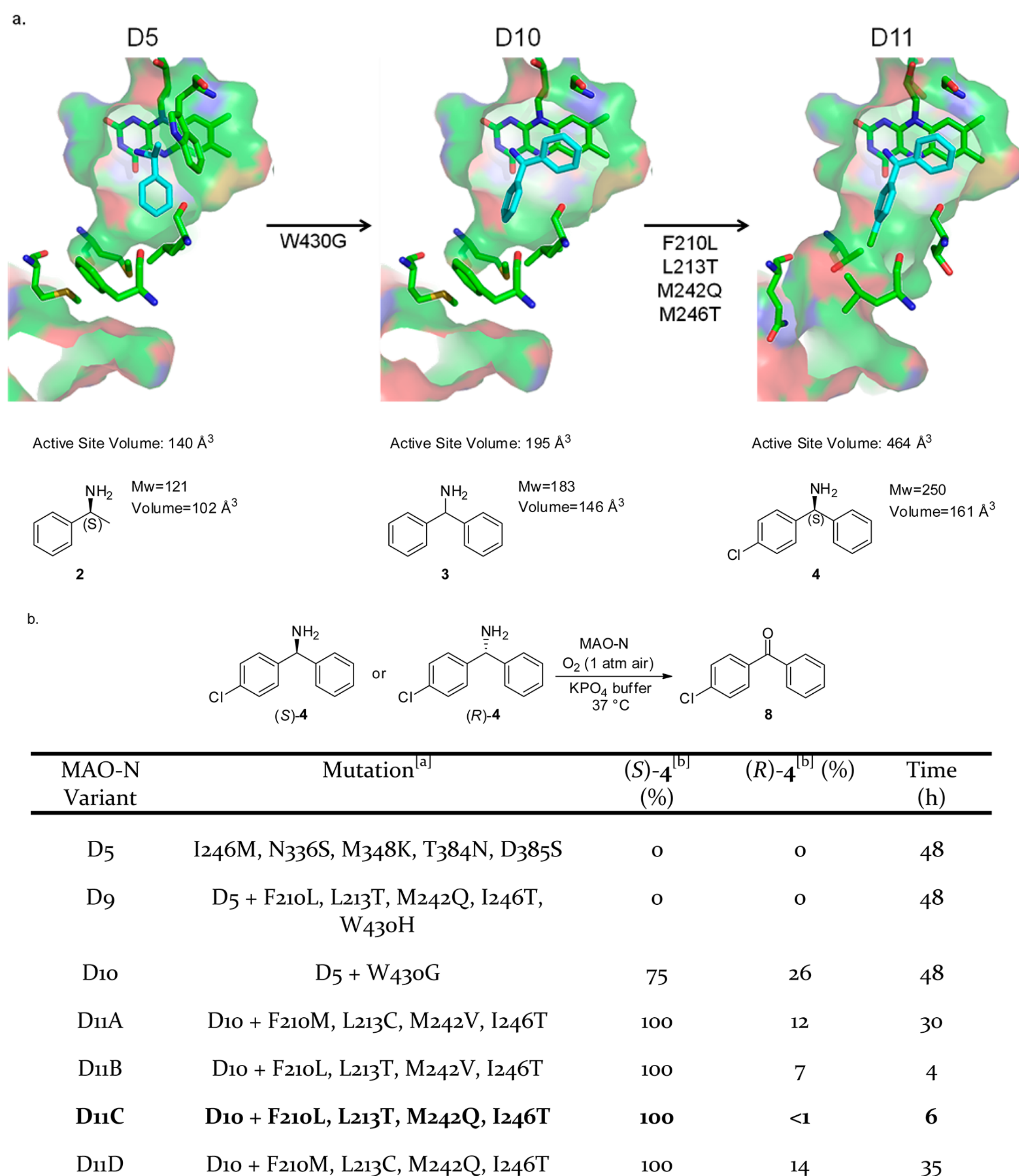


Figure 2. Substituted benzhydrylamines are important structural motifs, which are present in a number of APIs including Levocetirizine and Solifenacin. The development of biocatalysts that are able to act with a high degree of enantioselectivity is particularly challenging due to the similar natures of the two aryl substituents.

The D5 variant of MAO-N[13] was selected for further rational engineering using the previously reported crystal structure to guide selection of residues for mutation.[18] We reasoned that increasing the volume of the active site pocket would allow the binding of larger substrates containing two aryl substituents. Modeling of α-methylbenzylamine **2** into the MAO-N D5 active site (Figure 3a) highlighted two residues (A429 and W430) as potential targets for site directed mutagenesis with the specific aim of increasing the size of the small cavity within the active site pocket to incorporate an additional phenyl group associated with the aminodiphenylmethane motif. Saturation mutagenesis was performed at amino acid positions A429 and W430, and the resulting libraries of variants were screened against aminodiphenylmethane **3** using our previously reported solid phase assay.[11]

After screening, a variant of MAO-N (D10) was identified with the single amino acid substitution W430G, which displayed activity toward aminodiphenylmethane **3**. There was no change to the amino acid at position 429 (A429) in this variant. A homology model of the MAO-N D10 variant is shown in Figure 3a. This structure clearly demonstrates how the single mutation leads to an increase in volume of the small cavity within the active site, allowing the bulky substrate



MAO-N Variant	Mutation[a]	(*S*)-**4**[b] (%)	(*R*)-**4**[b] (%)	Time (h)
D5	I246M, N336S, M348K, T384N, D385S	0	0	48
D9	D5 + F210L, L213T, M242Q, I246T, W430H	0	0	48
D10	D5 + W430G	75	26	48
D11A	D10 + F210M, L213C, M242V, I246T	100	12	30
D11B	D10 + F210L, L213T, M242V, I246T	100	7	4
D11C	**D10 + F210L, L213T, M242Q, I246T**	**100**	**<1**	**6**
D11D	D10 + F210M, L213C, M242Q, I246T	100	14	35

Figure 3. (a) Modeling (*S*)-**2** into the active site of the MAO-N D5 variant that highlights A429 and W430 as targets for site-directed mutagenesis. After screening, a new MAO-N variant (D10) was identified with activity toward **3**. Further modifications in the active site entrance channel led to the development of a MAO-N variant (D11) with excellent activity and selectivity toward 4-chlorobenzhydrylamine. (b) Oxidation of (*R*)-**4** and (*S*)-**4** carried out with variants of MAO-N. Previously reported MAO-N variants displayed no activity toward either enantiomer. The D10 and D11 variants all displayed activity toward **4** with varying degrees of selectivity. The MAO-N D11C variant displays a high degree of selectivity toward (*S*)-**4**, making it a promising candidate for production of the desired (*R*)-enantiomer. [a]Mutations relative to wild-type MAO-N. [b]Conversion was calculated by high-performance liquid chromatography (HPLC).

aminodiphenylmethane **3** to be accommodated. This D10 variant of MAO-N was screened against 4-chlorobenzhydrylamine **4** and was shown to display moderate activity and enantioselectivity toward this substrate (Figure 3b). In an attempt to improve the activity further, our attention turned toward modification of amino acid residues in the active site channel. We have previously described mutations in this region of the enzyme during the development of the MAO-N D9 panel of variants, which displayed significantly enhanced activity toward the bulky tertiary amine crispine A when compared with MAO-N D5.[19] The amino acid substitutions (in the active site channel) from MAO-N D9 (A-D) were combined with the W430G modification of MAO-N D10 to create the MAO-N D11A-D library of variants.

The activity and stereoselectivity of the MAO-N variants toward **4** was then determined by monitoring the conversion of enantiopure (*R*)-**4** and (*S*)-**4** to the corresponding ketones by high-performance liquid chromatography (HPLC). Biotransformations were carried out using whole *Escherichia coli* cells expressing the relevant MAO-N variant in aqueous phosphate buffer (1 M, pH 7.7). The results of these transformations are illustrated in Figure 3b. As anticipated, the existing D5 and the D9 variants displayed no activity toward (*R*)-**4** or (*S*)-**4** after incubation for 48 h. Introduction of the W430G mutation to

the MAO-N D5 led to the generation of a variant of MAO-N (D10), which displayed significant activity but only moderate selectivity toward **4**.

All MAO-N D11 variants displayed high selectivity and reaction rates for (*S*)-**4** when compared with the MAO-N D10 variant. The most promising candidate for the deracemization of (±)-**4** for the production of the desired (*R*)-enantiomer was the MAO-N D11C variant, which was shown to mediate the conversion of 100% of (*S*)-**4** to the corresponding ketone in 6 h, with <1% of the corresponding (*R*)-enantiomer converted in the same reaction time.

We next examined the activity of the D11 variant toward a structurally related template based on 1-phenyltetrahydroisoquinoline **6**. Interestingly, the D11 variant showed high (*R*)-selectivity toward this substrate (see deracemization section), which is in contrast to the (*S*)-selectivity observed for all previously reported substrates for MAO-N. Modeling of (*S*)-**4** and (*R*)-**6** into the active site of MAO-N D11 highlights the preferred modes of binding of these two substrates (Figure 4).

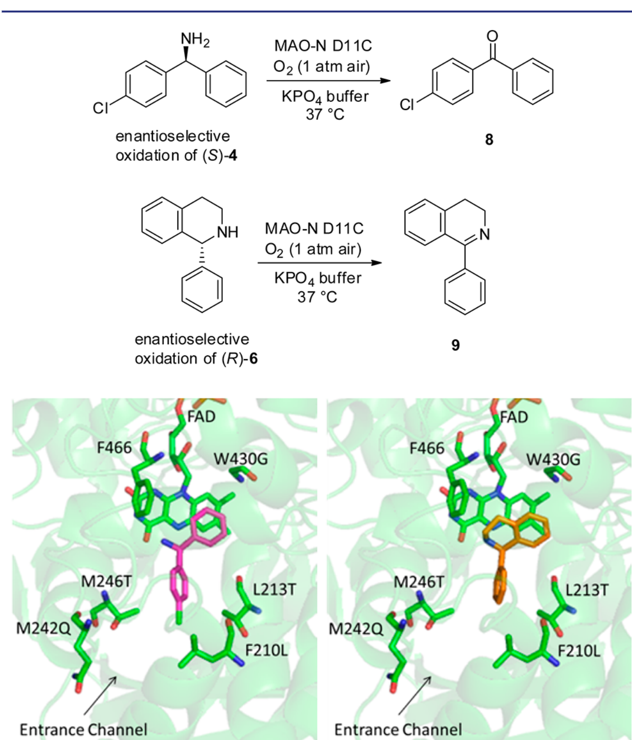


Figure 4. MAO-N D11C variant displaying excellent activity and enantioselectivity toward the oxidation of phenyltetrahydroisoquinoline **6**. Interestingly, this variant displays selectivity for the oxidation of the (*R*)-enantiomer of this substrate, in contrast with the (*S*)-selectivity observed for 4-chlorobenzhydrylamine **4**. Docking of (*S*)-**4** and (*R*)-**6** into the active site of MAO-N D11C provides an explanation for this observed switch in selectivity.

Attempts to dock (*S*)-**6** into the active site of the D11 variant failed to provide a reasonable productive binding mode for this substrate, providing an explanation for the observed switch in selectivity.

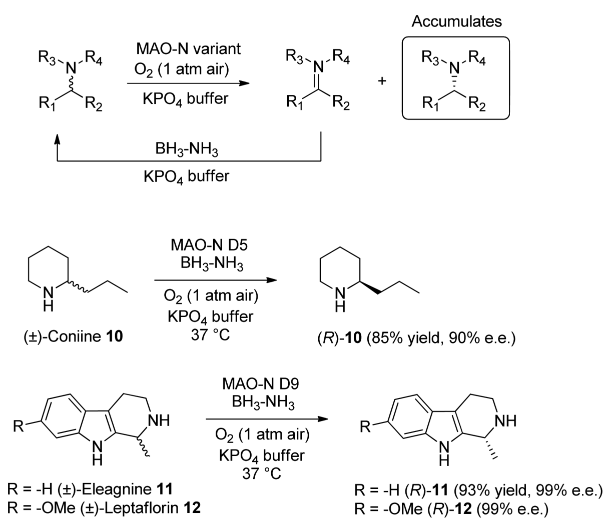
The crystal structure of this MAO-N D11C variant, refined to a resolution of 2.55 Å, was solved (see Supporting Information). This structure superimposes well with that of the D5 variant described previously (2VVM)[18] with few significant movements in the peptide backbone (the rmsd between one subunit of the two structures was 0.23 Å over 478 residues).

The anticipated increase in volume of the small cavity within the active site pocket as a result of the W430G modification was clearly visible in the maps for the D11C structure (Figure 3a). The modifications in the substrate access channel (F210L, L213T, M242Q, and I246T) result in a reduction in steric obstruction to the active site (Figure 3a) and have a significant effect on both activity and selectivity toward oxidation of **4**. Interestingly, the newly introduced Q242 residue forms new hydrogen bonds with D146. This interaction occurs as a result of rotation of the side chain of D146 in comparison to the D5 mutant in which D146 forms a salt bridge with R150. Q242 also forms a new H-bond with the phenolic hydroxyl of Y238, as does the newly positioned D146 residue. As a result of the new H-bonding interactions to Y238, this residue moves a distance of 2 Å out of the access channel, again relieving steric obstruction (see Figure S1 of the Supporting Information).

Application of the MAO-N Biocatalyst for the Deracemization of Alkaloids Natural Products and API Building Blocks. The piperidine alkaloid coniine (2-propylpiperidine) **10** is a potent neurotoxin, which is isolated from the plant *Conium maculatum* (poison hemlock). Although both enantiomers of coniine are natural products, their biological properties are not identical.[20] As a result, methods for the asymmetric synthesis of **10** are of interest, and a number of multistep strategies have been reported.[21,22] The previously reported MAO-N D5 variant (see Table S3 of the Supporting Information for sequences of all variants described in this manuscript) was able to mediate the efficient deracemization of commercially available (±)-coniine. Repeated cycles of selective oxidation of the (*S*)-enantiomer followed by nonselective chemical reduction of the resulting imine with BH_3-NH_3 leads to the accumulation of the (*R*)-enantiomer in 90% e.e. (Scheme 1). This represents the first reported example of the use of MAO-N for the oxidation of a 2-substituted piperidine, a common structural motif found in many alkaloid natural products.

The tetrahydro-*β*-carboline ring system is found in a large number of bioactive alkaloid natural products. Eleagnine **11** is an alkaloid isolated from *Chrysophyllum albidum*, which displays

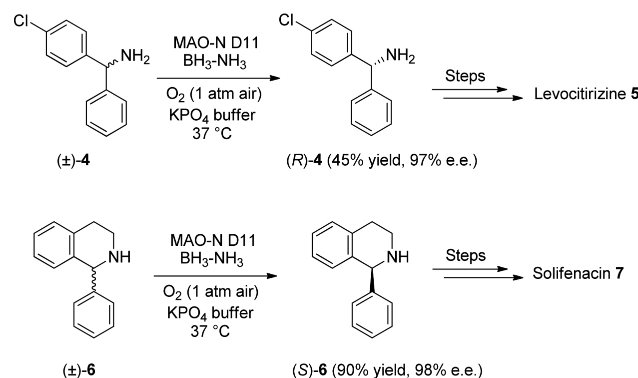
Scheme 1. Application of the MAO-N Variants for the Deracemization of Alkaloid Natural Products



potent analgesic, anti-inflammatory, and weak antioxidant properties.[23] The asymmetric synthesis of this structure has previously been described via transition metal catalyzed reduction of the corresponding imine.[24,25] Leptaflorin **12** has been isolated from *Peganum harmala* and from *Leptactina densiflora*, a stove shrub from central Africa, which is a known psychedelic.[26] Leptaflorin possesses an additional methoxy substituent at the 7-position of the indole ring when compared with eleagnine, a modification commonly found in indole alkaloid natural products. The D9 variant of MAO-N[19], when combined with BH_3-NH_3, was able to mediate the deracemization of both eleagnine and leptaflorin leading to the formation of the (*R*)-enantiomers of these natural products in >99% e.e.

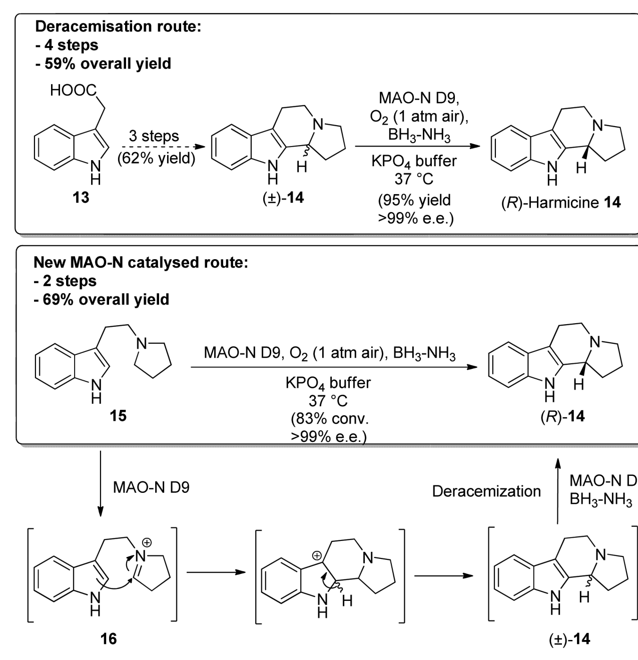
Deracemization of (±)-**4** on a 500 mg scale resulted in production of (*R*)-**4** in 97% e.e. after 48 h. The isolated yield of 45% is compromised by partial hydrolysis of the intermediate imine to the ketone during the deracemization process. Completion of the synthesis of Levocetirizine **5** from the amine (*R*)-**4** was carried out according to the previously described method.[27] The deracemization of the structurally related 1-phenyltetrahydroisoquinoline **6** proceeded in high yield and e.e. After incubation for 24 h, complete deracemization was observed as judged by chiral HPLC leading to the production of (*S*)-**6** in 98% enantiomeric excess. (*S*)-**6** is a key chiral intermediate in the synthesis of Solifenacin **7**, a competitive muscarinic acetylcholine receptor antagonist used in the treatment of overactive bladder.[28] Using the MAO-N D11 variant, the deracemization of (±)-**6** was carried out on a preparative scale (1 g, 15 mM), leading to the formation of (*S*)-**6** in 90% isolated yield and 98% enantiomeric excess after 48 h. Conversion of this product to Solifenacin **7** was carried out in two steps, according to the previously reported procedure (Scheme 2).[28]

Scheme 2. Application of the MAO-N D11 Variant for the Deracemization of API Building Blocks



Harmicine **14** is an alkaloid-natural product isolated from the Malaysian plant *Kopsia griffithii*, which displays strong *anti-Leishmania* activity.[29] Currently there are relatively few synthetic methods available for the enantioselective synthesis of naturally occurring (*R*)-harmicine **14**, which include a diastereoselective Pictet−Spengler type cyclization using tryptophan derivatives[30] or a thiourea catalyzed asymmetric Pictet−Spengler reaction of an *N*-acyl iminium ion.[31] We have developed two approaches to the synthesis of (*R*)-harmicine using the D9 variant of MAO-N (Scheme 3). The first relies on a MAO-N mediated deracemization (>99% e.e.) of the racemate, which was prepared in three steps from indole-3-acetic acid.[32] We also recognized the potential for using MAO-N as a biocatalyst for the oxidative generation of reactive iminium ions, thereby generating a conceptually new approach to this class of alkaloid. Pyrrolidine **15** is available in one step from tryptamine and 1,4-butanediol in high yield.[33]

Scheme 3. Biocatalytic Oxidative Pictet−Spengler Approach to the Synthesis of (*R*)-Harmicine



Incubation of **15** with MAO-N D9 resulted in generation of the intermediate iminium ion **16**, which underwent a nonstereoselective cyclization to form harmicine as a mixture of enantiomers. The (*S*)-enantiomer was then further oxidized to the corresponding iminium ion, which upon reduction with BH_3-NH_3 and further rounds of oxidation/reduction, was fully converted to the desired (*R*)-enantiomer. The overall transformation from pyrrolidine **15** to (*R*)-harmicine occurs in >99% e.e. with an 83% conversion and formally represents an oxidative asymmetric Pictet−Spengler reaction under aqueous conditions using molecular oxygen as the stoichiometric oxidant (Scheme 3). This synthesis of (*R*)-harmicine represents the shortest route reported to date and demonstrates the potential of variants of MAO-N in the development of novel synthetic strategies to access target molecules.

■ CONCLUSIONS

Biocatalysis is increasingly being viewed as a sustainable, green, and cost-effective technology for the manufacture of a diverse range of chemical products including pharmaceuticals, agrochemicals, fine chemicals, and biofuels. Modern tools for enzyme discovery, combined with the development of increasingly sophisticated techniques for protein engineering, have greatly expanded the range of different enzymes that possess suitable properties for biocatalytic applications.[34,35] The increased range of available biocatalysts has led to their more widespread use in synthesis with the prospect of even greater impact in the future as chemists seek to incorporate enzymes in retrosynthetic design tools.[36] In the examples presented here, we have used a combination of directed evolution, rational design, and high-throughput screening methods to develop a

"toolbox" of MAO-N variants that display complementary substrate specificity, which are able to mediate the synthesis of enantiomerically pure chiral amines with diverse structural architectures.

Interestingly, in the case of MAO-N, it has been possible to derive all of the variants D3-D11 from the original wild-type sequence by systematic introduction of point mutations in and around the active site. Remarkably, all variants display a high degree of selectivity and hence broad substrate coverage has been achieved without compromising the optical purity of the amine products. The broad applicability of this technology has been demonstrated by synthesis of the APIs Levocetirizine and Solifenacin as well as a number of important classes of biologically active alkaloid-natural products including pyrrolidines, piperidines, isoquinolines, and β-carbolines. The application of the MAO-N variants is not limited to the deracemization of chiral amines as demonstrated by previous studies from our group regarding the desymmetrization of prochiral amines[37] and the development of a MAO-N mediated asymmetric oxidative Pictet−Spengler reaction described in the current study. This latter process in particular highlights the potential for future applications of the MAO-N variants in the design of novel synthetic strategies to access target structures.

An increasingly important challenge associated with biocatalysis remains the broader availability of enzymes to facilitate their use by the wider synthetic community. Research groups often lack the facilities or the expertise for protein expression. In order to make the technology described herein more broadly accessible, the MAO-N variants generated in our laboratory have recently been made available.[38] These enzymes have great potential to become the catalysts of choice in synthetic laboratories for the generation of imines and iminium ions from amines, a transformation that is often challenging using traditional chemical reagents. This point is perfectly highlighted by the recent application of MAO-N in an industrial manufacturing process developed by Codexis and Merck for the desymmetrization of a prochiral amine to produce a key intermediate in the synthesis of the Hepatitis C drug Boceprevir.[39]

■ ASSOCIATED CONTENT

Supporting Information

Information on enzyme crystal structure, experimental procedures and characterization is included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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