Directed Evolution of an Amine Oxidase for the Preparative Deracemisation of Cyclic Secondary Amines

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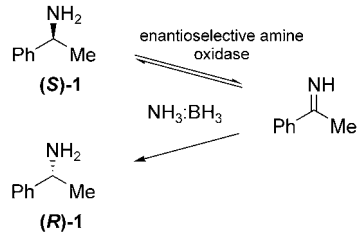
Enantiomerically pure primary and secondary amines are widely used as chiral auxiliaries and resolving agents and are also valuable intermediates for the synthesis of pharmaceuticals and agrochemicals.[1] Although enantiomerically pure amines are traditionally prepared by classical resolution of the corresponding racemate, alternative approaches have been developed based upon i) asymmetric reduction of imines,[2] ii) hydroamination of alkenes[3] and iii) lipase-catalysed kinetic resolution of racemic amines.[4] However, secondary amines, many of which have pronounced biological activity,[5] are poor substrates for lipases compared to the corresponding primary amines, with only a few documented examples in the literature.[6] Hence the use of lipase resolution does not offer a general route to this class of chiral molecule. Moreover, to date it has generally not been possible to achieve the in situ racemisation of amines to effect a dynamic kinetic resolution process due to the relatively harsh conditions required to racemise amines.[7]

Against this backdrop, we sought to extend our chemo-enzymatic deracemisation method to encompass chiral secondary amines. Based upon our earlier work with α-amino acids,[8] we recently reported the deracemisation of α-methylbenzyl amine (α-MBA, **1**) in a one-pot procedure by the combined use of an enantioselective amine oxidase and ammonia borane as the reducing agent (Scheme 1).[9]

In order to identify an enzyme with appropriate activity and enantioselectivity towards α-methylbenzylamine, the amine oxidase from *Aspergillus niger* (MAO-N) was subjected to directed evolution,[10] with (*S*)-**1** as the probe substrate, by random mutagenesis and selection employing a high-throughput agar-plate-based colorimetric screen. This approach led to the identification of an important amino acid substitution (Asn336Ser) that resulted in a variant enzyme possessing significantly enhanced activity (ca. 50-fold) and greater enantioselectivity towards **1** than the wild-type enzyme.[9] Subsequently, we showed that this variant was also characterised by broad substrate specificity, being able to oxidize a wide range of chiral primary amines with high enantioselectivity.[11] However, although this variant showed some activity towards chiral secondary amines (relative activity of 1-methyltetrahydroisoquinoline (MTQ, **2**) ≈15% of α-MBA), the rates of oxidation were too low to permit efficient preparative deracemisation reactions. Our goal therefore was to evolve a "secondary amine oxidase" for preparative-scale deracemisation reactions to complement the existing "primary amine oxidase".



Scheme 1. *Deracemisation of α-methylbenzylamine (**1**) with an amine oxidase in combination with ammonia borane.*

The MAO-N gene used as the starting point for further directed evolution contained four amino acid substitutions compared to the wild-type. In addition to the Asn336Ser mutation, which is important for catalytic activity/enantioselectivity, mutations were Arg259Lys and Arg260Lys (improved expression) and Met348Lys (improved activity). This gene was subjected to random mutagenesis, by using the *E. coli* XL1-Red mutator strain (mutation frequency ca. 1–2 base changes per gene), followed by transformation and screening of the library (ca. 20000 clones) against (*R/S*)-**2** as the substrate, as previously described.[9] A number of clones (ca. 10) showed greater activity than the parent, with one in particular appearing to be significantly more active. Purification of this variant amine oxidase showed that it possessed a k_{cat} value about 5.5-fold higher than the parent towards (*S*)-**2** (Table 1) and also a higher K_M value (0.31 vs. 0.06 mM). The new variant displayed very high (*S*)-selectivity ($E > 100$; calculated from ratio of k_{cat}/K_M for (*S*) vs. (*R*) enantiomer) towards MTQ and, upon sequencing, was found to possess an additional point mutation (Ile246Met) compared to the parent.

Table 1. *Kinetic parameters of Asn336Ser and Asn336Ser/Ile246Met variants towards selected amines.*

Amine	Asn336Ser			Asn336Ser/Ile246 Met		
	k_{cat} [min^{-1}]	K_M [mM]	k_{cat}/K_M [min^{-1} mM^{-1}]	k_{cat} [min^{-1}]	K_M [mM]	k_{cat}/K_M [min^{-1} mM^{-1}]
(*S*)-**2**	1.10	0.06	18.33	6.00	0.31	19.35
(*R*)-**2**	0.06	0.70	0.09	0.08	3.58	0.02
(*R/S*)-**1**	6.01	1.11	5.41	7.00	2.88	2.43
(*R/S*)-**4**	150.07	0.38	394.92	128.20	0.72	178.06
amylamine (**3**)	72.10	0.54	133.52	72.49	2.46	29.47

Interestingly, this new variant was found to have k_{cat} values towards α-MBA and amylamine (**3**) comparable to those of the parent. However, the new variant showed evidence for en-

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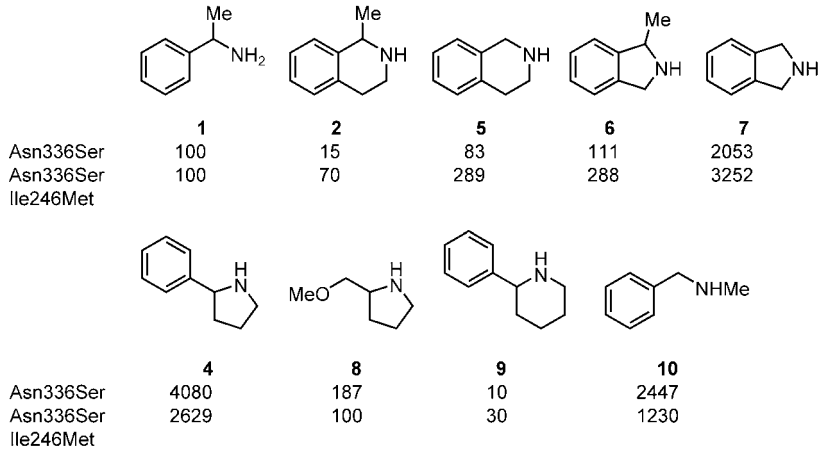
hanced activity towards chiral secondary amines (i.e., **2**, **5**, **6**, **7** and **9**) as shown in Scheme 2, with 2-phenylpyrrolidine (**4**) showing the highest k_{cat} (Table 1). Although in some cases the activity was reduced (**8** and **10**), in general, the initial aim of using a model secondary amine substrate (**2**) to select for a variant enzyme with improved activity towards a wider range of chiral secondary amines had been realised.



Scheme 2. *Relative activities of various amines towards Asn336Ser and Asn336Ser/Ile246Met variants.*

To further probe the importance of Ile246 as a key residue in controlling substrate specificity, we screened a second library in which position 246 was fully randomized. By using the Quikchange kit (Stratagene), a library of 220 colonies was screened, with (*R/S*)-**2** as substrate, to ensure a 99% probability of all possible 32 NNS codons being represented ($p = 1 - e^{-N/V}$ where p = % probability, N = sample size required, V = total number of combinations). Eight colonies showed distinctly greater activity than the rest and, when picked and sequenced, to our surprise all contained methionine at position 246 (statistically expected 6.9 clones). Thus although the use of the mutator strain places constraints on the range of amino acids that can be introduced at specific sites, in the present example the optimal amino acid was indeed found by this approach.

Prior to assessing the suitability of the Asn336Ser/Ile246Met variant for preparative-scale deracemisation reactions of secondary amines, we screened a variety of reducing agents for their ability to reduce 1-methyl-3,4-dihydroisoquinoline (MDQ) to MTQ. (Figure 1). Amine boranes were generally found to be highly reactive under aqueous conditions with the following order of activity: 4-dimethylaminopyridine borane (DMAP borane) > 2-(methylamino)pyridine borane > ammonia borane. Catalytic transfer hydrogenation (Pd/C ammonium formate) was also found to be effective. By using ammonia borane, it was found that as little as 1.0 equivalent (0.5 equiv for DMAP borane), relative to the substrate, could be used for complete reduction of MDQ to MTQ. For ease of use, ammonia borane was chosen for all subsequent preparative-scale reactions.

Initial small-scale deracemisation reactions of (*R/S*)-MTQ were carried out at 10 mM substrate concentration with 10 equiv of ammonia borane and washed whole cells (*E. coli*) expressing the Asn336Ser/Ile246Met variant amine oxidase. Complete deracemisation (*ee* = 99%) was achieved within 8 h. At 20 mM MTQ, on a preparative scale, the reaction was complete after 48 h and yielded (*R*)-MTQ in 71% isolated yield (*ee* = 99%). To complement the whole-cell approach, we also developed an immobilised form of the amine oxidase. The soluble fraction from the cell-free extract was subjected to purification by Ni-affinity chromatography to give the amine oxidase in > 80% purity. Treatment with Eupergit C resulted in immobilisation onto the resin. By using the immobilised amine oxidase with 20 mM MTQ, the deracemisation process was found to be slower (ca. 96 h) than with the whole-cell approach, but the isolated yield was considerably higher (yield = 95%; *ee* = 99%). Finally to demonstrate the generality of the approach and explore the effect of working at higher substrate concentration, we examined the preparative deracemisation of (*R/S*)-2-phenylpyrroldine, analogues of which are potent ligands for the nicotinic acetylcholine receptor.[12] With the immobilized enzyme, a substrate concentration of 100 mM (14.7 gL^{-1}) and ammonia borane (2.5 equiv), the deracemisation was complete within 48 h and, after work-up, yielded (*R*)-2-phenylpyrrolidine in 80% yield and 98% *ee*.

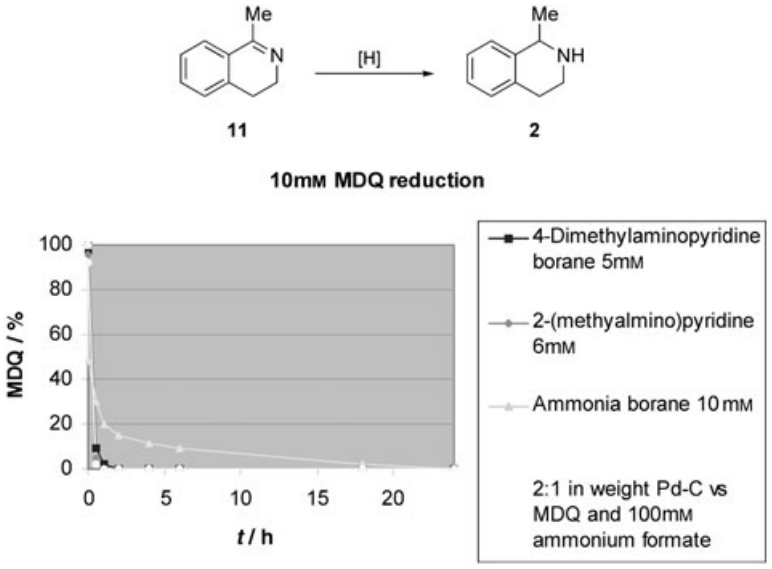


Figure 1. *Reduction of MDQ **11** to MTQ **2** by using various reducing agents.*

In order to gain insights into the possible significance of the mutation at Ile246, we generated an homology model of the active site. Although there is no X-ray structure available for monoamine oxidase N (MAO-N) from *A. niger*, the related enzyme monoamine oxidase B (MAO-B) from human liver has been crystallized, and the structure has been solved to a resolution of 2.5 Å.[13] Figure 2 shows a picture of the suicide inhibitor pargyline covalently bound at the active site of MAO-B, together with the key amino acid residues that line the active site (black). We carried out a WU-Blast2 sequence alignment (EMBL-EBI) between MAO-N and MAO-B which revealed 24% sequence identity and 43% sequence similarity between the two proteins. The corresponding residues from MAO-N are shown in grey, and it is interesting to note that i) the active sites of both enzymes contain a high proportion of aromatic residues and ii) there is considerable homology, and often identity, between the two enzymes (e.g. Tyr60/Trp94, Tyr435/Phe466, Tyr188/Trp230, Phe168/Phe210, Tyr365/Tyr326, Leu171/Leu213, Cys172/Cys214, Tyr398/Trp430). Ile246 (MAO-N) maps onto Gln206 (MAO-B), the latter appearing to make intimate contact with the substrate. It is also perhaps significant that these two amino acids are quite different in nature and steric demand, and might therefore represent important residues in the respective proteins for controlling substrate specificity.

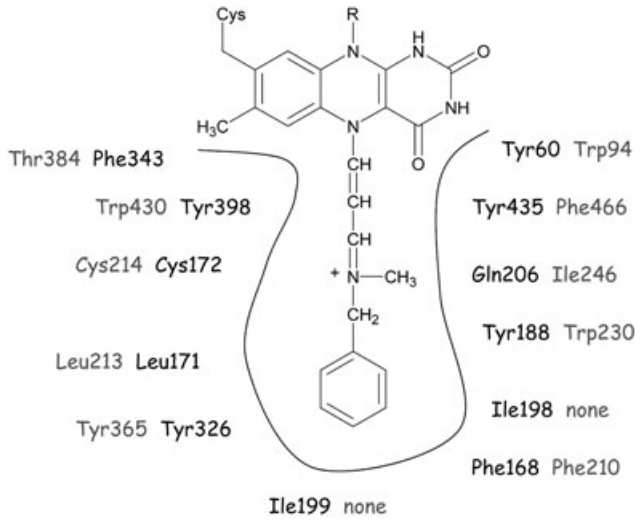


Figure 2. *Model of the active site of MAO-B showing the residues that make contact with the inhibitor. The corresponding residues of MAO-N are shown alongside.*

In summary we have developed a practical procedure for deracemisation of cyclic secondary amines by a further round of directed evolution of the monoamine oxidase from *A. niger*. By using a representative secondary amine as the substrate for screening the library, we were able to identify a variant enzyme that possessed general activity towards a range of structurally related, but different, secondary amines. Furthermore the key mutation Ile246Met appears to be important in terms of controlling substrate specificity, and therefore constitutes a "hot spot" that can be explored for altering specificity towards other amine substrates of interest.

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Keywords: amine oxidase · amines · biocatalysis · deracemisation · directed evolution · enantioselectivity

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