Directed Evolution

Directed Evolution of an Amine Oxidase Possessing both Broad Substrate Specificity and High Enantioselectivity**

*Reuben Carr, Marina Alexeeva, Alexis Enright, Tom S. C. Eve, Michael J. Dawson, and Nicholas J. Turner**

Enantiomerically pure chiral amines are of increasing value in organic synthesis, especially as resolving agents,[1] chiral auxiliaries/chiral bases,[2] and catalysts for asymmetric synthesis.[3] In addition, chiral amines often possess pronounced biological activity in their own right and hence are in demand as intermediates for agrochemicals and pharmaceuticals.[4] Current methods for the preparation of enantiomerically pure chiral amines are largely based upon the resolution of racemates, either by recrystallization of diastereomeric salts[5] or by enzyme-catalyzed kinetic resolution of racemic substrates using lipases and acylases.[6] To develop more efficient methods, attention is turning towards asymmetric approaches or their equivalent, for example, the asymmetric hydrogenation of imines[7] or the conversion of ketones into amines by using transaminases.[8] Attempts to develop dynamic kinetic resolutions, which employ enzymes in combination with transition-metal catalysts, have unfortunately been hampered by the harsh conditions required to racemize amines.[9]

Recently we reported a novel catalytic method for the preparation of optically active chiral amines by deracemization of the corresponding racemic mixture (Figure 1).[10] The deracemization approach relies upon coupling an enantioselective amine oxidase with a nonselective reducing agent to effect stereoinversion of the *S* to *R* enantiomer via the intermediate achiral imine.

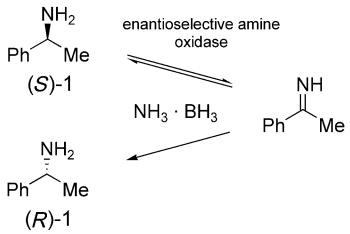

Figure 1. Deracemization of α-methylbenzylamine using an enantioselective amine oxidase in combination with ammonia·borane as the reducing agent.

The *S* enantiomer selective amine oxidase used for the deracemization of (*R/S*)-α-methylbenzyl amine was identified from a library of variants of the wild-type enzyme, from *Aspergillus niger*, by using a high-throughput colorimetric screen to guide selection.[10] The library of variants was generated by randomly mutating the plasmid harboring the amine oxidase gene by using the *E. coli* XL1-Red mutator strain. Using (*S*)-α-methylbenzylamine as the target substrate we were able to identify a variant (Asn336Ser) that possessed significantly improved catalytic activity (47 fold) and enantioselectivity (sixfold) towards this particular substrate compared to the wild type enzyme. To explore the opportunities for using this variant amine oxidase to deracemize other racemic chiral amines we decided to undertake a more detailed study of its substrate specificity. Herein we show that the Asn336Ser variant possesses broad substrate specificity and high enantioselectivity towards a wide range of chiral amines.

Prior to carrying out further studies with the Asn336Ser amine oxidase, an additional mutation was introduced into the sequence (Met348Lys) that resulted in a variant enzyme (hereafter referred to as Asn336Ser) with higher specific activity and expression levels although its substrate specificity appeared unchanged (data not shown). Incorporation of an *N*-terminal histidine tag into the amine oxidase allowed facile purification of both the wild-type and Asn336Ser variant in one step, by a nickel-affinity column, to yield protein of $>90\,\%$ purity as evidenced by gel electrophoresis (Figure 2, see Experimental Section). Solutions of the amine oxidases prepared in this manner were used for all the subsequent substrate specificity studies.

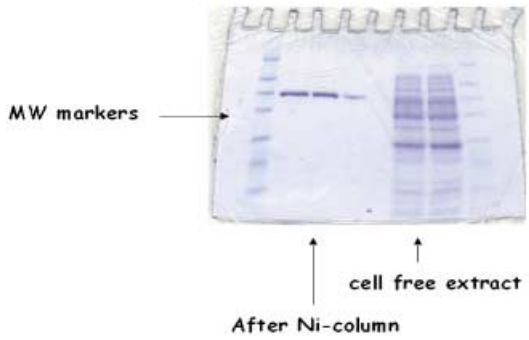

Figure 2. Polyacrylamide gel of amine oxidase enzyme after affinity purification on a nickel column.

A panel of amine substrates **1**–**51**, with broad structural features, was selected to characterize both the wild-type amine oxidase and Asn336Ser variant (Figure 3). Each substrate was screened individually, at 10 mM substrate concentration, against the partially purified wild-type and mutant amine oxidase in 96-well microtitre plate format using a UV/Vis plate-reader. The rate of oxidation was monitored by measuring hydrogen peroxide production by using a coupled enzyme assay.[11] For each substrate the k_{cat} and K_m values were calculated but for clarity only the relative activities are shown. These values have been calculated by

[*] Prof. Dr. N. J. Turner, R. Carr, M. Alexeeva, Dr. A. Enright, T. S. C. Eve
School of Chemistry
The University of Edinburgh
King's Buildings, West Mains Road, Edinburgh EH9 3JJ (UK)
Fax: (+44) 131 650 4717
E-mail: n.j.turner@ed.ac.uk
Dr. M. J. Dawson
GlaxoSmithKline R&D
Medicines Research Centre
Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY (UK)

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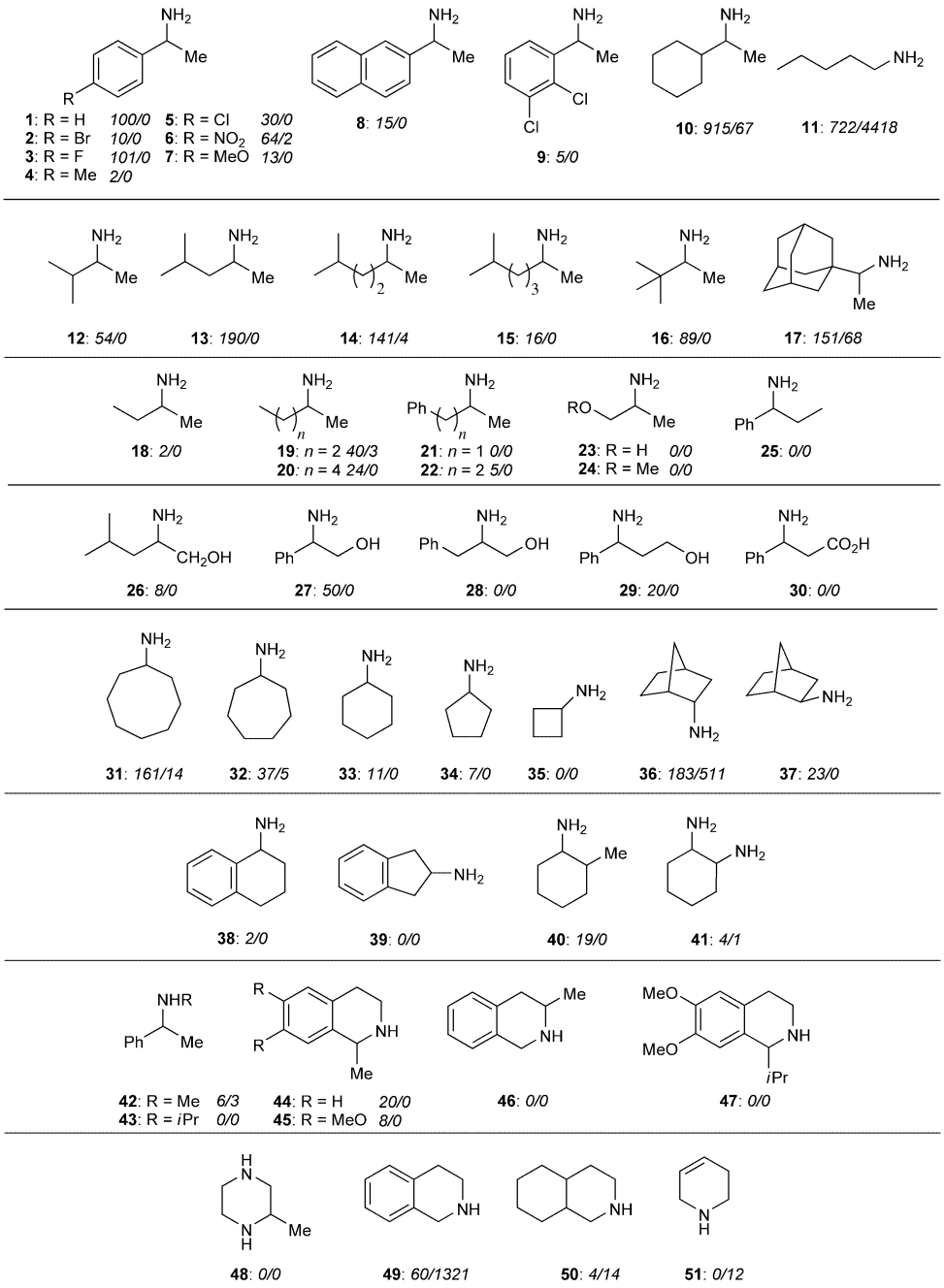

Figure 3. Panel of amines used in the screening experiments. Numbers in italics beneath the structures refer to relative rates of oxidation for Asn336Ser mutant/wild-type enzyme compared to α-methylbenzylamine.

setting the activity of the Asn336Ser variant towards α-methylbenzylamine as 100% and reporting all other rates as relative values. In addition, for a number of the chiral racemic substrates which gave positive assay results, the individual *R* and *S* enantiomers were also examined to determine the enantioselectivity of the reaction.

The wild-type amine oxidase was found to be inactive towards most of the amines shown in Figure 3. Of the 51 substrates examined, only nine gave relative activities of over 5%. The wild-type enzyme is most active towards simple straight-chain amines (e.g. pentylamine (**11**)) and generally shows poor activity towards more sterically demanding branched amines. By comparison, however, the Asn336Ser variant amine oxidase showed a quite different substrate profile with significant activity (≧5%) towards more than half of the substrates examined (33 out of 51). Eight of the substrates tested (**3**, **10**, **11**, **13**, **14, 17**, **31**, and **36**) were more reactive than α-methylbenzylamine itself with 1-cyclohexylethylamine (**10**) reacting approximately nine-times faster. The Asn336Ser variant amine oxidase showed high reactivity towards certain classes of chiral amines, particularly substituted phenethylamines (**2**, **3**, **5**–**8**) and 1-alkylethylamines (**12**–**17**, **19, 20**). Secondary amines reacted more slowly (cf. **42** versus **1**) although 2-methyltetrahydroisoquinoline **44** was

oxidized with a relative activity of 20% and the dimethoxy derivative **45** with a relative activity of 8%. Other substrates of interest that showed good activity were 2-phenyl-2-aminoethanol (**27;** rel. activity = 50%), *endo*-1-amino-norbornane (**36**; 183%), and 3-amino-3-phenylpropanol (**29**; 20%).

The enantioselectivity of the Asn336Ser variant towards 11 selected chiral amine substrates was then examined and the results are shown in Figure 4 and Table 1. The enantio-

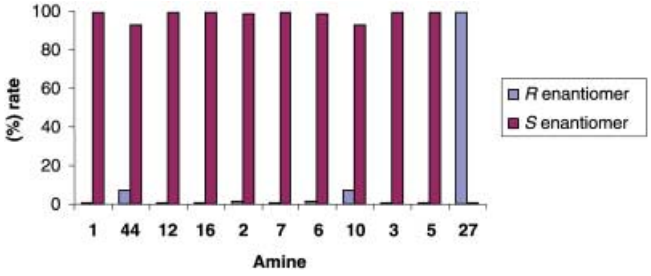

Figure 4. Graph showing relative rate of oxidation of individual *S* and *R* enantiomers by Asn336Ser amine oxidase. The rate of each substrate has been normalized to 100%.

Table 1: Enantioselectivity of Asn336Ser mutant towards selected chiral amines.[a]

Amine	*E*	Amine	*E*
1	199	**6**	86
44	13	**10**	13
12	142	**3**	275
16	96	**5**	110
2	84	**27**	270
7	184		

[a] The numbers reported refer to the enantiomeric ratio (*E*) for the individual substrates.

meric ratio *E*[12] for α-methylbenzylamine was very high ($E = 199$) and was in general maintained with most of the other chiral amines. Such *E* values translate to *ee* values of around 97→99%. Only 1-methyltetrahydroisoquinoline (**44**) and 1-cyclohexylethylamine (**10**) gave significantly lower values (both $E = 13$) although even these values would equate to an *ee* value of about 85%. Significantly, in all cases the Asn336Ser variant amine oxidase was found to be selective for the *S* enantiomer of the amine substrate (note that for **27** the *R* enantiomer is oxidized owing to the change in priority of the substituents).

For secondary amine substrates, for example, **42**, **44**, **45**, **49**, and **50** the possibility arose as to the regioselectivity of oxidation with respect to the amine functionality. Thus **44** could, in principle undergo oxidation to yield either imine **A** or **B**, or a mixture of both (Figure 5). To establish that the former pathway operated at least to some extent, we carried out the Asn336Ser amine oxidase catalyzed oxidation of (*S*)-**44** in the presence of the reducing agent ammonia·borane which we have previously shown to be effective for reduction of the intermediate imine.[10] Analysis of the chiral HPLC profile clearly showed that after 90 h significant production of (*R*)-**44** had occurred. The formation of (*R*)-**44** from (*S*)-**44** can only occur via the achiral imine **A** and not the chiral imine **B** (see Experimental Section).

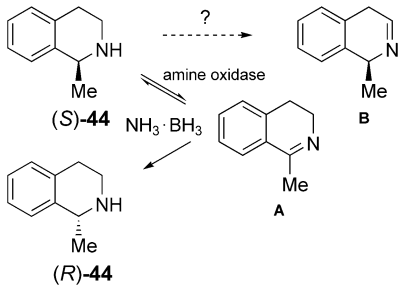

Figure 5. Stereoinversion of (*S*)-**44** to (*R*)-**44**, via imine **A**, by using Asn336Ser amine oxidase with ammonia borane.

In summary, we have shown that a directed evolution approach, based initially upon screening a library of mutant amine oxidases for activity against one enantiomer of a specific substrate ((*S*)-α-methylbenzyl amine), has lead to the identification of an enzyme possessing much broader substrate specificity whilst retaining high enantioselectivity. The Asn336Ser variant shows highest activity towards substrates containing a primary amine group flanked by a methyl group and a bulky alkyl/aryl group (Figure 6). In all cases so far examined the variant enzyme is selective for the *S* enantiomer

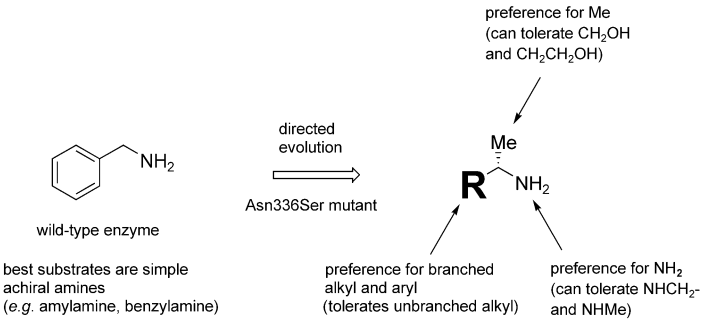

Figure 6. Comparison of substrate specificity of wild-type and Asn336Ser mutant amine oxidase.

of the chiral amine substrate. Other groups have also reported the identification of highly enantioselective enzymes by screening against single enantiomer substrates.[13] Although the *A. niger* Asn336Ser amine oxidase is suitable at present for small-scale deracemization reactions,[10] we are continuing to evolve this enzyme to develop an enzyme that has the required characteristics (e.g. stability, activity, selectivity) to be used for large-scale applications.

The development of enzymes possessing broad substrate specificity combined with high enantioselectivity remains an important goal in biocatalysis. Previous studies have demonstrated that directed evolution can be used to alter the substrate specificity and enantioselectivity of enzymes and moreover such variant enzymes often possess broader specificity when compared with the wild-type enzyme.[14] The results described herein represent the most in-depth study to date of how the substrate specificity of an enzyme can be dramatically altered by a point-mutation. The ability to select for such enzymes, using appropriate high-throughput screens, is critical to success in this area and highlights the need for new methods to enable the detection of a wider range of enzyme activities than is currently possible.[15]

Experimental Section

Expression and purification of amine oxidase: BL21 star was transformed with the wild-type/mutant amine oxidase gene and plated onto LB (70 μg mL^{-1} ampicillin) petri dishes. A single colony was added to LB media (6 × 300 ml) containing ampicillin and grown at 30 °C for 24 h. The cells were spun and the cell pellet stored at −20 °C. Lysis of the cells was performed in 25 mM Tris/HCl pH 7.8, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 300 mM NaCl and the lysate centrifuged. The cell-free extract from a 1 g pellet was loaded onto a 1-mL Ni-*N*,*N*-bis(carboxymethyl) glycine (nitrilotriacetic acid (NTA)) column. Column wash (five column volumes); 25 mM Tris/HCl pH 7.8, 60 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, and 300 mM NaCl. Protein elution (the amine oxidase elutes in ≈2nd–7th 1 ml fractions); 25 mM Tris/HCl pH 7.8, 200 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, and 300 mM NaCl. The protein was desalted in 25 mM Tris/HCl pH 7.8, 1 mM threo-1,4-dimercapto-2,3-butanediol (dithiothreitol (DTT)), 1 mM PMSF, and 300 mM NaCl using a Pharmacia PD10 column. Samples were stored frozen at −80 °C and thawed prior to use.

*Stereoinversion of (*S*)-**44***: A solution (600 μL) containing 20 mM (*S*)-**44**, 400 mM $NH_3{\cdot}BH_3$, 25 mM Tris/HCl pH 7.8, 1 mM DTT, 1 mM PMSF, and 300 mM NaCl aqueous buffer was held at 30 °C with shaking for 2 h. A 100-μL aliquot was removed and analyzed by HPLC as a $t = 0$ sample. amine oxidase (0.215 mg) in 500 μL of 25 mM Tris/HCl pH 7.8, containing 1 mM DTT, 1 mM PMSF and 300 mM NaCl was added to the remaining 500 μL of the (*S*)-**44** solution. The mixture was shaken at 30 °C and after $t = 90$ h a 100 μL aliquot was removed and analyzed by HPLC. Some precipitation was observed over the course of the reaction.

HPLC sample preparation: An aliquot (100 μL) of the reaction mixture was extracted with hexane (2 × 150 μL). The combined hexane extracts were analyzed directly by HPLC: Chiracel OD-H column 46 cm, eluent hexane:ethanol 98:2 (v/v), flow rate = 0.5 ml min^{-1}, column temperature = 0 °C; retention times, imine **A** = 18.7 min, (*S*)-**44** = 22.4 min, (*R*)-**44** = 26.2 min.

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