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# A reductive aminase from *Aspergillus oryzae*

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## **Abstract**

Reductive amination is one of the most important methods for the synthesis of chiral amines. Here we report the discovery of an NADP(H)-dependent reductive aminase from *Aspergillus oryzae* (*AspRedAm*, Uniprot code Q2TW47) which can catalyse the reductive coupling of a broad set of carbonyl compounds with a variety of primary and secondary amines with up to >98% conversion and with up to >98% enantiomeric excess. In cases where both carbonyl and amine show high reactivity, it is possible to employ a 1:1 ratio of the substrates, forming amine products with up to 94% conversion. Steady-state kinetic studies establish that the enzyme is capable of catalysing imine formation as well as reduction. Crystal structures of *AspRedAm* in complex with NADP(H) and also with both NADP(H) and the pharmaceutical ingredient (*R*)-rasagiline are reported. We also demonstrate preparative scale reductive aminations with wild-type and Q240A variant biocatalysts displaying total turnover numbers of up to 32,000 and space time yields up to 3.73 g L<sup>-1</sup> d<sup>-1</sup>.

An analysis of drugs approved by the FDA in recent years reveals that *ca.* 40% of new chemical entities (NCEs) contain one or more chiral amine building blocks.<sup>1</sup> This sustained prevalence of chiral amines in

24 APIs has driven the development of new and efficient catalytic methods for chiral amine synthesis that  
25 have broad application.<sup>2-6</sup> In this context, the reductive amination of ketones is one of the most  
26 powerful and frequently employed reactions for amine synthesis, enabling a wide range of ketones to be  
27 coupled to primary and secondary amines.<sup>7-11</sup>

28 In view of the fact that the products are often chiral, there is an increasing desire to develop asymmetric  
29 variants of this reaction, particularly utilising chemo- or biocatalysis. Specifically, transition metal-  
30 catalysed reductive amination and enantioselective enamide reduction approaches<sup>8</sup> to chiral amines  
31 have received considerable attention as well as biocatalytic routes employing transaminases,<sup>6,12-14</sup>  
32 ammonia lyases<sup>15-17</sup> or monoamine oxidases.<sup>18-20</sup>

33 In addition, a number of distinct enzyme families have previously been reported to catalyse the  
34 reductive amination of ketones. The NADPH-dependent octopine dehydrogenases (OctDHs) catalyse the  
35 coupling of  $\alpha$ -amino acids with  $\alpha$ -keto acids and have been the focus of recent attempts to broaden  
36 their substrate range using protein engineering.<sup>21</sup> Amino acid dehydrogenases (AADHs) perform  
37 aminations of  $\alpha$ -keto acids with ammonia by first catalysing formation of  $\alpha$ -imino acids followed by  
38 NADPH-dependent reduction to yield  $\alpha$ -amino acids. Although AADHs have been engineered to accept  
39 simple unfunctionalised ketones, they typically show strict specificity for ammonia as the amine  
40 nucleophile.<sup>22,23</sup> The related *N*-methyl-amino acid dehydrogenases (NMAADHs) use methylamine to  
41 generate the corresponding *N*-methyl-amino acids.<sup>24</sup> Recently, reductive amination has also been  
42 demonstrated using imine reductases (IREDs).<sup>25-28</sup> However, the reactions have involved the use of large  
43 quantities of IRED enzyme, and ratios of amine to ketone ranging from *ca.* 50:1<sup>26</sup> to 12.5:1<sup>27</sup> in order to  
44 achieve the conversions. This low reactivity of IREDs for the catalysis of reductive amination is almost  
45 certainly due to the fact that their principal role is to catalyse the reduction of preformed cyclic imines.<sup>29</sup>  
46 For example, we<sup>30-34</sup> and others<sup>25,26,35,36</sup> have shown that IREDs catalyse the asymmetric reduction of a  
47 wide range of 5-, 6-, and 7-membered imines with good conversions and high enantioselectivity.

48 Importantly, from a mechanistic viewpoint, OctDHs, AADHs and NMAADHs have been shown to catalyse  
49 imine formation, whereas the IREDs described so far have not.<sup>25-27</sup> Thus, one important goal is to  
50 identify an enzyme scaffold which can combine (i) high activity for imine formation from ketone and  
51 amine; (ii) high enantioselectivity for imine reduction and (iii) broad substrate tolerance with respect to  
52 both amines and ketones. Herein we report our efforts to find and develop an enzyme that possesses  
53 these properties through the discovery and investigation of a reductive aminase (RedAm) (Figure 1).

## 54 **Results and Discussion**

### 55 **Identification of AspRedAm**

56 A reductive aminase from *Aspergillus oryzae* (AspRedAm), the first IRED homolog from a eukaryotic  
57 source, was initially identified based upon its sequence similarity to known IREDs including those from  
58 *Amycolatopsis orientalis* (AoIRED)<sup>34</sup> and *Streptomyces* sp.<sup>37-39</sup> Those IREDs have been shown to possess  
59 high activity for imine reduction but modest to poor activity for reductive amination. Following cloning  
60 and expression of the gene encoding AspRedAm in *E. coli*, the purified enzyme was revealed to have  
61 remarkable potency as a catalyst of reductive amination. The characterisation of AspRedAm using  
62 biotransformations, kinetic and structural studies suggests it is representative of a subclass of IREDs that  
63 have evolved to possess a particular capability for reductive amination reactions.

### 64 **Investigation of substrate specificity of AspRedAm**

65 The relative specific activity of AspRedAm towards a representative library of carbonyl acceptors **1-32**  
66 was determined using propargylamine **a** and methylamine **g** as substrates, with the amine and NADPH  
67 concentrations maintained at saturation (Supplementary Section 7.1, Table 10). In order to assess the  
68 amine substrate scope, the relative specific activities of AspRedAm with cyclohexanone **1** and 4-phenyl-  
69 2-butanone **17** were also measured towards amines **a-s** (Supplementary Section 7.2, Table 11).  
70 AspRedAm exhibited higher specific activity for **1** with **a** (6.68 U mg<sup>-1</sup>) and with allylamine **c** (7.68 U mg<sup>-1</sup>)

71 compared to **g** (2.23 U mg<sup>-1</sup>), highlighting the contribution of the amine partner to the catalytic rates. A  
72 clear preference for cyclic ketones was observed (e.g. **1** and **4**) and C5 or C6 linear ketones and  
73 aldehydes (e.g. hexanal **3**, 2,5-hexanedione **5**, 2-hexanone **6**) were transformed faster than C4 carbonyl  
74 compounds (e.g. 2-butanone **26**). The screening of amine nucleophiles revealed a greater activity of  
75 *AspRedAm* towards primary amines, especially unsaturated aliphatic amines (**a** and **c**). Excellent activity  
76 was observed with cyclopropylamine **b**, however the activity was significantly lower when  
77 isopropylamine **n** was used as a nucleophile. In the presence of reactive carbonyl acceptors (e.g. **1**),  
78 amination with various amines, including *N*-methylprop-1-yn-1-amine **j**, pyrrolidine **l**, piperidine **p**,  
79 ammonia **k** and hydroxylamine **q**, proceeded with activities of up to 0.7 U mg<sup>-1</sup>. However, with a less  
80 reactive carbonyl acceptor (e.g. **17**), lower rates were observed with these reacting partners, although  
81 primary amines were tolerated.

82  
83 By combining the data of relative specific activities towards the carbonyl acceptors and amine partners  
84 (Supplementary Section 7, Table 10 and Table 11) we generated a reactivity chart to act as a predictive  
85 tool for the likely outcome of reductive amination between specific ketones and amines (Figure 2). The  
86 chart was constructed by combining the average relative activities of representative carbonyl  
87 compounds, measured with two amine nucleophiles (**a** and **g**), and plotting this against the average  
88 relative specific activities of amine nucleophiles measured with two ketones (**1** and **17**). The carbonyl  
89 compounds and amines were arranged in Groups I-IV and Groups A-C respectively based on their  
90 average relative specific activity value. For ketone-amine combinations that have high relative specific  
91 activities for both reacting partners (*i.e.* Groups I and II vs. Group A, Figure 2) it is likely that high-yielding  
92 reductive aminations can be achieved with *AspRedAm* with near stoichiometric equivalents of ketone  
93 and amine. Increasing the amine equivalents can improve conversions for substrates that have less  
94 favourable specific activities (*i.e.* Groups III and IV vs. Group B and C, Figure 2).

95  
96  
97 Using the reactivity chart as a guide, a series of biotransformations was performed with a range of  
98 carbonyl and amine combinations (Table 1). *AspRedAm* reactions with both cyclic and acyclic ketones  
99 afforded products in moderate to excellent conversion and enantioselectivity. In several cases,  
100 equimolar concentrations of ketone and amine gave high conversions (Table 1, products **1a**, **1b**, **1c**, **1m**),  
101 which is indicative of genuine *AspRedAm*-catalysed reductive amination processes. Ammonia **k** and  
102 secondary amines **l** and **p** were also accepted as reacting partners when coupled with particularly active  
103 carbonyls (Table 1, products **1k**, **9k**, **19p**, **19p**). In the *AspRedAm*-catalysed reaction between  
104 benzaldehyde **19** and **k**, the initial product of reductive amination was benzylamine **m** which acts as an  
105 amine reacting partner for a second reductive amination with the ketone substrate, resulting in product  
106 **19m**. Reductive amination of ethyl levulinate **10** afforded *N*-alkylpyrrolidinones (**10a-b**) as products  
107 following spontaneous cyclisation. Interestingly, *AspRedAm* could also distinguish to some extent  
108 between (*R*)- and (*S*)-*sec*-butylamine **t** as the amine coupling partner with (*S*)-**t** giving higher conversion.  
109 Furthermore, *AspRedAm* was able to directly produce the active pharmaceutical ingredient (API) (*R*)-  
110 rasagiline **29a** starting from 1-indanone **29** and **a** in 64% conversion and 95% *e.e.*

111

## 112 ***AspRedAm versus IREDs***

113 As an IRED homolog, purified *AspRedAm* displayed broad substrate scope in the reduction of cyclic and  
114 preformed imines and iminium ions, allowing access to secondary and tertiary amines. For example,  
115 dihydroisoquinoline derivative **45** was transformed to the natural product salsolidine **46** with >99%  
116 conversion and >99% *e.e.* (Supplementary Section 8). *AspRedAm* was also able to act in the reverse,  
117 oxidative direction and exhibited activity in the dehydrogenation of amines to yield imines. The highest  
118 activity was found for 1-methyl-tetrahydroquinoline **34** and acyclic amines were also found to be  
119 transformed (Supplementary Section 7, Table 12). This reactivity was exploited in the efficient kinetic  
120 resolution of rasagiline *rac*-**29a** to give the (*S*)-enantiomer in 49% conversion and 99% *e.e.* Interestingly,  
121 the enzyme displayed regioselectivity in the deamination as only indanone **29, a** and (*S*)-**29a** were  
122 detected after a 24-hour biotransformation of *rac*-**29a**.

123 To further investigate the unusual catalytic features of *AspRedAm*, we compared its reductive amination  
124 activity to those of the IRED from *Streptomyces* sp. GF3587 (*R*-IRED)<sup>31,38</sup> and the *Amycolatopsis orientalis*  
125 IRED (*Ao*IRED).<sup>34</sup> For enzymes only capable of reducing preformed imines, we anticipated that reductive  
126 amination activity with aldehydes would be highly dependent on pH, as it has been reported that  
127 spontaneous imine formation between benzaldehyde and methylamine in aqueous solution is  
128 insignificant at pH 7.6 (4%) but considerable at pH 9.0 (87%).<sup>26</sup> Conversely, for ketones, spontaneous  
129 imine formation is negligible at both pHs and, therefore, reductive amination activity is less likely to be  
130 pH dependent.

131 Initial rate measurements of the selected IREDs were performed at pH 7.0 and 9.0 using **1** and **3** with **c**  
132 (Supplementary Section 12). *AspRedAm* displayed much higher specific activities than *R*-IRED and  
133 *Ao*IRED for the reductive amination of both **1** and **3** regardless of pH. In the reductive amination of **3**, an  
134 approximate 20-fold improvement in specific activity was observed for *R*-IRED and *Ao*IRED when the pH

135 was increased from 7.0 to 9.0. This correlates with the difference in the imine concentration in aqueous  
136 media at different pHs that was previously reported and further suggests that these IREDs rely on  
137 preformed imine in solution which they are then able to reduce.<sup>26</sup> Remarkably, the specific activity of  
138 *AspRedAm* only increased 1.3-fold, showing that the spontaneous imine formation in solution is not  
139 essential for this enzyme. For the reductive amination of **1**, there was no significant change in activity  
140 from pH 7.0 to 9.0 with *AspRedAm*, *AolRED* or *R-IRED*. The high specific activity of *AspRedAm* at pH 7.0  
141 and pH 9.0 for reactions with both **1** and **3** is indicative of the role of *AspRedAm* in catalysing both the  
142 formation of imine and its subsequent reduction. The differences between *AspRedAm* and other IREDs  
143 are further highlighted by sequence comparison and structure studies, reported herein.

144

#### 145 ***A Kinetic Model for AspRedAm Activity***

146 *AspRedAm*-catalysed reductive amination of ketones follows the Michaelis–Menten model based on  
147 initial rate studies. For a selected substrate panel, *AspRedAm* exhibited high activity in many cases; for  
148 example, the  $k_{\text{cat}}$  for *AspRedAm*-catalysed reductive amination of **1** and **c** was  $5 \text{ s}^{-1}$  (Supplementary  
149 Section 6.2). In order to further probe the mechanism of *AspRedAm*-catalysed reductive amination we  
150 carried out detailed steady-state kinetic studies using **1** and **g** as substrates (Supplementary Section 6).  
151 We simultaneously varied the concentration of **1** and **g** while NADPH was held at saturation; the  
152 resulting double-reciprocal plots ( $1/v_i$  versus  $1/[\mathbf{1}]$ ) yielded patterns of lines that intersected to the left  
153 of the  $1/v$  axis. When **g** was held at saturation and the NADPH concentration varied at different fixed  
154 concentrations of **1**, a similar intersecting pattern of lines was obtained. The intersecting lines were also  
155 obtained when **1** was held at a constant level, and NADPH was varied at fixed concentration of **g**. These  
156 patterns are consistent with a sequential mechanism and rule out a ping-pong mechanism for  
157 *AspRedAm* activity.



158 To investigate the order of substrate addition and product release, product inhibition studies were  
159 conducted in the forward and reverse directions (Supplementary Section 6.4). In the forward direction,  
160 inhibition by  $\text{NADP}^+$  is linearly competitive with respect to NADPH, uncompetitive with respect to **1** and  
161 non-competitive with respect to **g**. In the reverse reaction, NADPH behaves as a linear competitive and  
162 non-competitive inhibitor with respect to  $\text{NADP}^+$  and **1g** respectively. This inhibition pattern indicates  
163 that NADPH is the first substrate to bind while  $\text{NADP}^+$  is the last product released in the forward  
164 reaction.<sup>40,41</sup> Inhibition by **1g** was non-competitive with respect to NADPH, **1** and **g**. This pattern is  
165 consistent with **1g** being the first product to be released in the forward direction.<sup>40,41</sup> In the reverse  
166 direction, **g** behaves as a non-competitive inhibitor with respect to  $\text{NADP}^+$  and **1g** indicating that **g** is the  
167 first substrate to be released in the oxidation of **1g** and the last substrate to bind in the forward  
168 direction. Inhibition by **1** was uncompetitive with respect to  $\text{NADP}^+$  and **1g** in the forward direction, as  
169 would be expected of the substrate binding second in the sequence.

170 The kinetic behaviour observed when the concentrations of two substrates were simultaneously varied  
171 alongside the patterns of inhibition obtained from the product inhibition studies showed that  
172 *AspRedAm*-catalysed reductive coupling of **1** and **g** to form **1g** follows an ordered sequential Ter Bi  
173 mechanism. The cofactor NADPH, the ketone **1** and the amine **g** are added to the enzyme in that  
174 sequence followed by the release of product **1g** and  $\text{NADP}^+$  (Figure 3). The *AspRedAm*-catalysed  
175 reductive amination follows the kinetic model displayed by *N*-methyl-L-amino acid dehydrogenase from  
176 *Pseudomonas putida* with the same order of binding of substrates.<sup>24</sup> Other enzymes that catalyse imine  
177 formation also operate via a Ter Bi mechanism such as number of  $\alpha$ -keto dehydrogenases<sup>42-46</sup> and  
178 opine dehydrogenases (OpDHs)<sup>41,47</sup>, however, the order of ketone and amine binding can be different.

179

180

181

182 ***Crystal Structure of AspRedAm and mutagenesis studies***

183 The exceptional properties of *AspRedAm* prompted us to examine its structure using X-ray  
184 crystallography, and to compare it with IREDs that are not capable of catalysing equimolar reductive  
185 amination reactions. Co-crystallisation of *AspRedAm* with **29**, amine **a** and NADPH resulted in a ternary  
186 complex, in which both NADP(H) and the product, (*R*)-**29a**, were found in the active site. The crystals  
187 were in the *P1* space group, and four dimers were found in the asymmetric unit. *AspRedAm* possesses  
188 the canonical IRED fold, in which two monomers, each made up of an *N*-terminal Rossman domain and a  
189 *C*-terminal helical bundle connected by a long inter-domain  $\alpha$ -helix, associate to form a functional dimer  
190 in which the active site forms at the interface between the *N*- and *C*-terminal domains of different  
191 monomers (Figure 4A). In contrast to other IRED structures however, the ternary complex of *AspRedAm*  
192 is significantly more compact, with a relative movement between domains closing the active site over  
193 the NADP(H) and the product ligand to form a much smaller active site than has been observed in ‘open’  
194 forms of IREDs previously.<sup>31–34,48,49</sup>

195  
196 The ligand was bound within a hydrophobic pocket previously identified in the IRED from *AolRED*<sup>34</sup>  
197 adjacent to the (*Si*)-face of the nicotinamide ring of NAD(P)H. The ligand is somewhat mobile in the eight  
198 active sites in the asymmetric unit, but the nitrogen atom of the amine is 3.2–4.9 Å (4.5 Å in the case  
199 shown in Figure 4B) from the phenolic hydroxyl of Y177, suggesting a role in either proton donation or  
200 product anchoring by this residue. Mutation of Y177 to alanine resulted in a mutant Y177A with about a  
201 30-fold decrease in reductive aminase activity compared to the wild-type enzyme (Figure 4C). The ligand  
202 conformation in Figure 4B also positions the electrophilic carbon of the amine product at between  
203 approximately 3.4 and 4.2 Å from C4 of the nicotinamide ring of NAD(P)H (3.8 Å in the case shown in  
204 Figure 4B), an ideal distance for hydride delivery/acceptance. It was also interesting that mutation of  
205 D169, which has been thought to have a role in catalysis in some IREDs,<sup>33</sup> resulted in variants D169A and

206 D169N of significantly reduced reductive aminase activity (Figure 4C). Both mutants showed a *ca.* 200-  
207 fold decrease in reductive amination activity compared to the wild-type enzyme. Other residues of  
208 possible significance are N93, which hydrogen bonds to D169, Q240 and M239 at the front of the picture  
209 in Figure 4B that are brought nearly into contact with the ligand upon closure of the active site, and  
210 W210 at the back of the picture, which helps to complete the hydrophobic pocket.

211 The characterisation of the active site of *AspRedAm* provided a basis for searching the sequence  
212 databases for other enzymes of similar properties, and also to compare the enzyme against IREDs  
213 reported previously, which have not displayed equimolar reductive aminase activity. A number of other  
214 sequences from filamentous fungi, including *Aspergillus terreus* (*AtRedAm*) and *Ajellomyces dermatitidis*  
215 (*AdRedAm*) were identified that each contained residues equivalent to N93, D169, Y177, W210, M239  
216 and Q240 in *AspRedAm*. The genes encoding *AtRedAm* and *AdRedAm* were cloned and expressed in *E.*  
217 *coli* and, following purification of the enzymes, we were able to confirm asymmetric reductive amination  
218 using a 1:1 ratio of amines **a**, **c** and **g** and ketone **1** as a property of these enzymes (Supplementary  
219 Section 11.3, Table 17). A phylogenetic tree that compares these fungal RedAms with sequences of  
220 enzymes for which non-equimolar reductive amination reactions have been reported<sup>26,27,48</sup> shows that  
221 fungal RedAms form a distinct sub-group (Supplementary Section 11.1, Figure 67). Analysis of the  
222 sequences of these enzymes reveals that while one or two bacterial IREDs may feature some of the  
223 active site residues of RedAms, none of the bacterial homologs is likely to contain all of them within the  
224 active site (Supplementary Section 11.2, Table 16). IR\_9 and IR\_23, described by Wetzl and co-  
225 workers<sup>27,35</sup> are most similar, containing five and four out of the six residues respectively, but each has a  
226 threonine residue in the place of asparagine in positions equivalent to 93 in RedAms. A direct  
227 comparison of *AspRedAm* with IR\_23 shows that the former catalysed the formation of amine **1g** with  
228 84% conversion at a ketone:amine ratio of 1:2; IR\_23 was reported to catalyse this transformation with  
229 80% conversion, but only at a ketone:amine ratio of 1:12.5.<sup>27</sup> Whilst we cannot conclude that these six

230 residues uniquely describe the requirements of a RedAm active site, their identification should prove a  
231 useful guide to the identification of further RedAm enzymes in the sequence databases.

232 The structure of *AspRedAm* suggested that W210 and Q240 may be good target residues to mutate in  
233 order to alter substrate specificity. Indeed, the W210A variant displayed a dramatic selectivity switch to  
234 yield the antipodal (*S*)-amine products upon the reductive amination of **17** with a variety of amine  
235 nucleophiles (Table 2, entries 1-4, Supplementary Section 9.1, Figure 66). (*S*)-Selectivity was also  
236 observed when **a** was reductively coupled with 2-tetralone **9** (Table 2, entry 6), as well as in the coupling  
237 of **10** with **c** to form the *N*-substituted lactam **10c**. Variant W210S displayed similar stereoselective  
238 properties to W210A, with the (*S*)-amine products formed upon the reductive amination of a panel of  
239 substrates (Table 2). From the determination of the kinetic parameters, both W210A and W210S  
240 displayed similar activity profiles although W210A appeared to be slightly more active (Supplementary  
241 Section 9, Table 14). Interestingly, the Q240A variant displayed significant improvements in (*R*)-  
242 selectivity for most substrates compared to the wild-type enzyme. For example, the enantioselectivity in  
243 the reductive amination of **17** with **c** was greatly improved (94% *e.e.*) compared to the wild-type (30%  
244 *e.e.* Table 2, entry 1). The Q240A variant was also capable of coupling **k** to **17** to yield the primary chiral  
245 amine **17k** in excellent *e.e.* (>98%). The significant improvement in the (*R*)-selectivity of *AspRedAm*  
246 Q240A also permitted the successful synthesis of (*R*)-**29a** in >98% conversion with >98% *e.e.* using this  
247 mutant.

#### 248 249 ***Preparative-Scale Reductive Aminations using AspRedAm***

250 To test the synthetic applicability of *AspRedAm*, a series of preparative-scale reactions were performed.  
251 Taking **1** and **g** as model substrates, certain process parameters were investigated on an analytical-scale  
252 prior to implementing the reaction on a larger scale. The concentration of ketone, the number of amine  
253 equivalents and the enzyme loading were investigated (Supplementary Section 13, Table 18).

254 Interestingly, excellent conversion (>97%) could be achieved using 50 mM **1**, 2 amine equivalents and  
255 0.1 mg mL<sup>-1</sup> *AspRedAm* and so these conditions were employed for the 100 mg scale synthesis of **1g**,  
256 which was isolated as a hydrochloride salt, in 75% yield. A variety of other reductive amination products  
257 **1a**, **6g**, **10a** and **17g** were successfully recovered with either wild-type *AspRedAm* or the Q240A variant  
258 on a preparative scale to afford products in good to excellent isolated yields of 70%, 70%, 48% and 78%  
259 respectively after hydrochloride salt formation or column chromatography (Supplementary Section 13).  
260 These reactions compare favourably with other preparative biocatalytic processes<sup>50,51</sup> with total  
261 turnover numbers (TTNs) up to 32,000, turnover frequencies (TOFs) up to 300 min<sup>-1</sup> and space time  
262 yields (STYs) up to 3.73 g L<sup>-1</sup> d<sup>-1</sup>.

263

## 264 **Conclusion**

265 In summary, we report the discovery and characterization of a reductive aminase from *Aspergillus*  
266 *oryzae* (*AspRedAm*) which has been shown to possess remarkably high activity for the reductive  
267 amination of ketones and amines, often with high stereoselectivity and in some cases with  
268 ketone:amine ratios as low as 1:1. By examining the relative activities of a broad range of different  
269 amines and ketones it has been possible to construct a predictive reactivity chart in which the likely  
270 outcome of a reductive amination reaction can be appraised. We also present detailed kinetic studies, to  
271 support the order of substrate binding and product release, together with an X-ray crystal structure of a  
272 ternary complex of *AspRedAm* which has been used to inform mutagenesis studies and has allowed us  
273 to identify key active-site residues that may be involved in ligand binding and catalysis. The  
274 demonstrated activity in the reductive amination of aldehydes between pH 7.0 and 9.0 provides further  
275 evidence that *AspRedAm* catalyses imine formation. Finally we have illustrated the synthetic potential of  
276 *AspRedAm* through the reductive amination of a number of ketone substrates and successfully  
277 demonstrated the preparative-scale synthesis of a selection of amine products. Taken together, these

278 results serve to highlight RedAms as an important sub-group of IREDs that possess unique and attractive  
279 properties for the biocatalytic preparation of industrially important amines.

280

281

## 282 **EXPERIMENTAL SECTION**

### 283 **General**

284 For full details of synthetic procedures and characterisation data, see Supplementary Information.

### 285 **Gene synthesis, cloning, expression and protein purification**

286 The codon-optimized gene sequence encoding *AspRedAm* (GenBank accession number, KY327363) was  
287 sub-cloned into pET28a-(+) vector form pET 28a-His-*AspRedAm* plasmid (Figure S2). Site-directed  
288 mutagenesis for the creation of *AspRedAm* variants were performed using primers as listed in the  
289 Supplementary Information (Section 3.2). Cultivation was performed in 500 mL 2x YT broth medium  
290 with kanamycin ( $30 \mu\text{g mL}^{-1}$ ). Cultures were initially incubated at  $37^\circ\text{C}$  with shaking at 250 rpm. At an  
291 optical density ( $\text{OD}_{600\text{nm}}$ ) of between 0.6 and 0.8, isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was  
292 added to a final concentration of 0.5 mM to induce the expression of *AspRedAm*. Incubation was  
293 continued at  $20^\circ\text{C}$  and 250 rpm for 18 h. Cells were then harvested by centrifugation and resuspended  
294 in sodium phosphate buffer (100 mM, pH 7.5). Cells were disrupted by ultrasonication at  $0^\circ\text{C}$ . The  
295 enzyme was purified from the clarified lysate by Ni-affinity chromatography. To further purify the  
296 protein for crystallisation, size exclusion chromatography (SEC) was performed in Tris-HCl buffer (50  
297 mM, pH 8.0) containing 500 mM NaCl. The protein concentration was determined using the Bradford  
298 assay against BSA as a concentration standard. Further details and general information on strains and  
299 plasmids, and details of gene design and cloning protocols can be found in the Supplementary  
300 Information (Section 3).

## 301 **Biotransformations**

302 Typical procedure for *AspRedAm*-catalysed reductive amination: a 500  $\mu\text{L}$  reaction mixture contained 30  
303 mM D-glucose, 0.4  $\text{mg mL}^{-1}$  GDH (Codexis, CDX-901), 1 mM  $\text{NADP}^+$ , 1  $\text{mg mL}^{-1}$  purified *AspRedAm*, 5 mM  
304 carbonyl compound, the appropriate ratio of amine nucleophile (in buffer adjusted to pH 9.0) and 2%  
305 (v/v) dimethylformamide or dimethylsulfoxide. The reaction volume was made up to 500  $\mu\text{L}$  with Tris-  
306 HCl buffer (100 mM, pH 9.0). Reactions were incubated at 25°C with shaking at 250 rpm for 24 h, after  
307 which they were quenched by the addition of 30  $\mu\text{L}$  of 10 M NaOH and extracted twice with 500  $\mu\text{L}$  *tert*-  
308 butyl methyl ether. The organic fractions were combined and dried over anhydrous  $\text{MgSO}_4$  and analysed  
309 by HPLC or GC-FID on a chiral stationary phase. For further details see the Supplementary Information  
310 (Section 4 & 5).

311 Preparative-scale reactions were run using 100 mM D-glucose, 0.5 mM  $\text{NADP}^+$ , 0.3  $\text{mg mL}^{-1}$  GDH, 50 mM  
312 or 10 mM ketone, 2, 5 or 20 equivalents of amine, 0.1 to 0.5  $\text{mg mL}^{-1}$  purified wild-type *AspRedAm* or 1.0  
313  $\text{mg mL}^{-1}$  *AspRedAm* Q240A variant in 100 mM pH 9.0 Tris buffer. Reactions were incubated at 20°C or  
314 30°C, 250 rpm for 24 h. The reaction was basified to pH 12 with 10 M NaOH solution and the product  
315 extracted into diethyl ether or dichloromethane with intermediate centrifugation (4°C, 2,831 rcf, 5 min)  
316 to improve the separation of phases. The organic layers were combined, dried over anhydrous  $\text{MgSO}_4$   
317 and the solvent carefully concentrated. The residue was dissolved in dry diethyl ether and acidified with  
318 a solution of 2 M HCl in diethyl ether or purified by column chromatography. Further details can be  
319 found in the Supplementary Information (Section 13).

## 320 **Kinetic Assays**

321 The reductive aminase activity was measured using a modified method to that previously reported.<sup>24,52</sup>  
322 For substrate specificity screening, a typical reaction mixture contained 15 mM carbonyl compound, 60  
323 mM amine nucleophile from buffer stock adjusted to pH 9.3, 0.3 mM NADPH, 1 % (v/v)  
324 dimethylsulfoxide and 5-100  $\mu\text{g}$  of purified *AspRedAm* in a total volume of 200  $\mu\text{L}$  (100 mM sodium

325 tetraborate, pH 9). Activity measurements were performed in triplicate at 340 nm ( $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ )  
326 or 370 nm ( $\epsilon = 2.216 \text{ mM}^{-1} \text{ cm}^{-1}$ ) using a Tecan infinite M200 microplate reader (Tecan Group,  
327 Switzerland).

328 Steady state kinetic measurements were performed with various concentrations of one substrate at  
329 different fixed concentrations of the second substrate while the third substrate was held at a constant  
330 level. Double reciprocal plots were obtained and line patterns were examined against rate equations  
331 describing sequential mechanisms. Product inhibition studies for the reductive amination of **1** and **g**, and  
332 the deamination of **1g** were performed with various concentrations of the one substrate and fixed  
333 saturating concentrations of the other substrates in the presence of the product (inhibitor). Double  
334 reciprocal plots obtained were examined and data were fitted into equation describing competitive,  
335 non-competitive and uncompetitive inhibition. The reaction was initiated by the addition of purified  
336 *AspRedAm* to the mixture. A unit of *AspRedAm* was equal to the amount of the pure enzyme required to  
337 consume 1  $\mu\text{mol}$  NADPH/ NADP<sup>+</sup> per min. Activity measurements were performed in triplicate and  
338 kinetic constants were determined through nonlinear regression based on Michaelis–Menten kinetics  
339 (QtiPlot software). For further details see Supplementary Information (Section 6).

#### 340 **Protein Crystallization**

341 Purified *AspRedAm* was subjected to crystallisation trials using a range of commercially-available screens  
342 in 96-well sitting-drop format in which each drop consisted of 150 nL protein and 150 nL of precipitant  
343 reservoir solution. Crystallization experiments gave two structures of *AspRedAm*: an NADP(H) complex  
344 and also a ternary complex with NADP(H) and (*R*)-**29a**. For further details see Supplementary  
345 Information (Section 10). Crystals that diffracted to a resolution of equal to, or better than, 3 Å  
346 resolution were retained for dataset collection at the Diamond Light Source synchrotron. The coordinate  
347 files and structure factors have been deposited in the Protein DataBank (PDB) with coordinate accession  
348 numbers 5g6r [*AspRedAm*-NADP(H)] and 5g6s [*AspRedAm*-NADP(H)-(*R*)-rasagiline complex].



349 ***Data availability***

350 The authors declare that the data supporting the findings of this study are available within the paper  
351 and its supplementary information files. Additionally, sequence data has been deposited in Genbank  
352 with the accession code KY327363 (<https://www.ncbi.nlm.nih.gov/nuccore/KY327363>) and the  
353 coordinate files and structure factors have been deposited in the Protein DataBank (PDB) with  
354 coordinate accession numbers 5g6r [*AspRedAm-NADP(H)*] and 5g6s [*AspRedAm-NADP(H)-(R)*-rasagiline  
355 complex].

356 ***Author Contributions***

357 N.J.T. and G.G. initiated the study and directed the project. G.A.A., M.S. and F.L. cloned and expressed  
358 the enzymes. G.A.A. performed the kinetics and mutagenesis studies. G.A.A., S.P.F., J.M.S., S.L.M. and  
359 M.S. performed biotransformations. H.M. obtained crystal structures. S.P.F., J.M.S., S.L.M., G.A.A. and  
360 S.H. chemically synthesised substrates and product standards.

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374

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496

497



498 Figure Captions:

499

500 **Figure 1. Examples of biocatalytic routes to chiral amines *via* monoamine oxidase catalysed resolution, or**  
501 **asymmetric synthesis catalysed by ammonia lyases, transaminases, amine dehydrogenases and imine**  
502 **reductases (IREDs). This work describes the reductive aminase from *Aspergillus oryzae* (*AspRedAm*) that is**  
503 **capable of performing imine formation as well as reduction to afford a wide variety of chiral amines.**

504 **Figure 2. Reactivity chart for *AspRedAm*-catalysed reactions based on specific activities of a panel of carbonyl**  
505 **compounds and amine reacting partners. a) Chart displaying relative activity of amine/carbonyl pairs in**  
506 **reductive amination reactions. Compounds presented in the plot area are representative examples of products**  
507 **obtained in biotransformations. Conversions of >50% were achieved in all cases when the recommended**  
508 **amine:ketone ratios were used. Framed structures correspond to scaled-up biotransformations with isolated**  
509 **products. b) Carbonyl acceptors and amine nucleophiles arranged in Groups based on their average relative**  
510 **specific activity value. c) Legend for the reactivity chart with specific activity ranking and recommended ratio of**  
511 **amine to carbonyl compound for reductive amination.**

512

513 **Figure 3. Reductive amination of 1 with g and kinetic model for *AspRedAm* showing sequential cofactor and**  
514 **substrate binding followed by product and cofactor release based on steady-state kinetic studies. Following**  
515 **binding of the nicotinamide cofactor (*i*), ketone is bound (*ii*), followed by the amine (*iii*), followed by enzyme-**  
516 **catalysed imine formation and NADPH-mediated reduction. The amine product is then released (*iv*) prior to**  
517 **NADP<sup>+</sup> (*v*).**

518

519 **Figure 4. Structural and mutagenesis data of *AspRedAm* highlighting essential catalytic residues. a) Dimeric**  
520 **structure of *AspRedAm* in complex with NADP(H) and (*R*)-29a dimer in which the active site is at the interface**  
521 **between the Rossmann fold of one monomer and the C-terminal bundle of its neighbour; b) Active site of**  
522 ***AspRedAm* at dimer interface. Electron density represents the  $2F_o-F_c$  (blue) and  $F_o-F_c$  (omit, green) maps, the**  
523 **latter obtained prior to refinement of the ligand, and contoured at levels of 1.0 and  $2.5\sigma$  respectively. Distances**

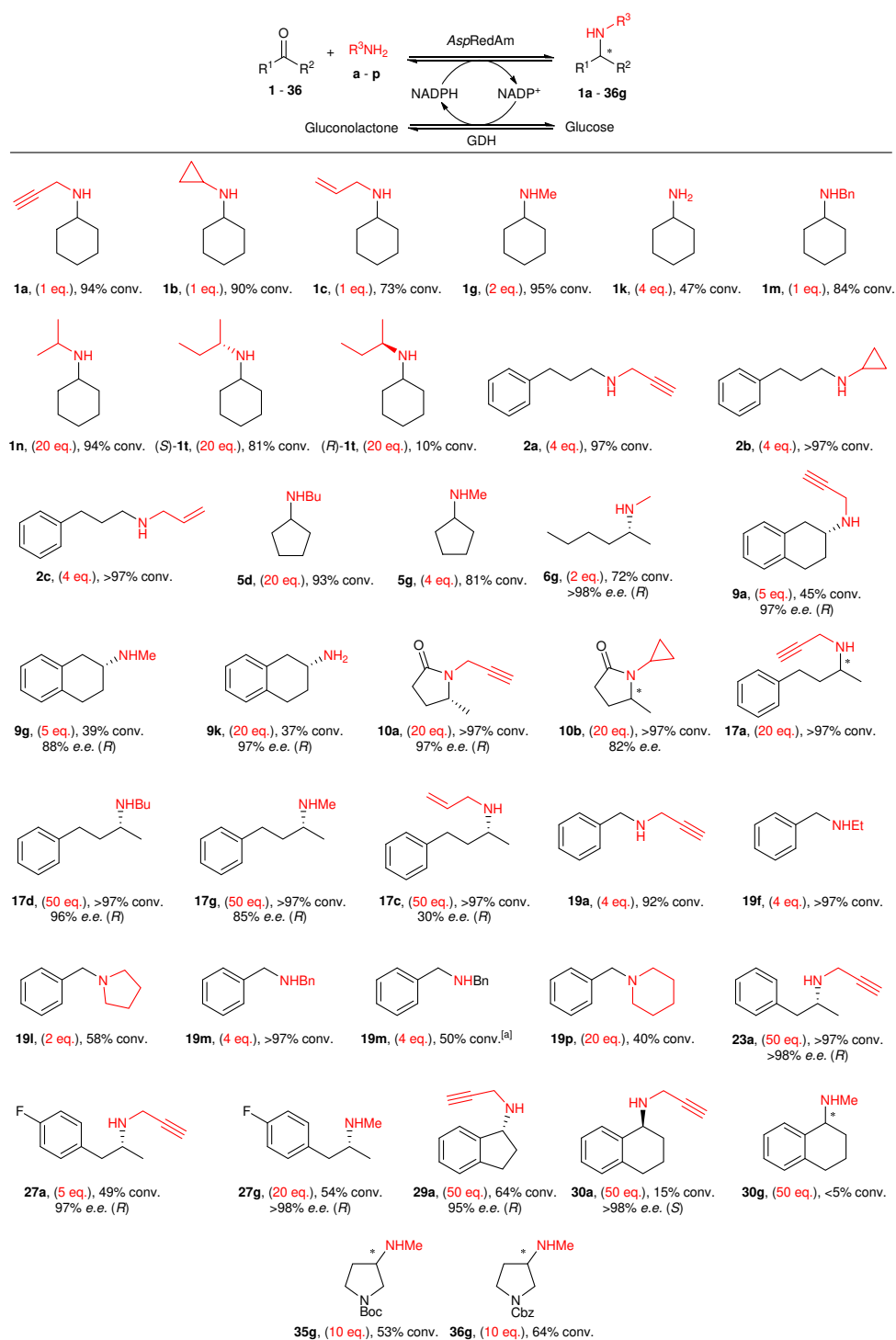
524 are shown in Ångstroms. c) Kinetic data of *AspRedAm* wild-type and mutants D169A, D169N and Y177A.

525 Mutation at D169 and Y177 resulted in a marked decrease in activity suggesting essential roles for these

526 residues in catalysis.

527

528 **Table 1. AspRedAm-catalysed reductive amination of carbonyl compounds.**

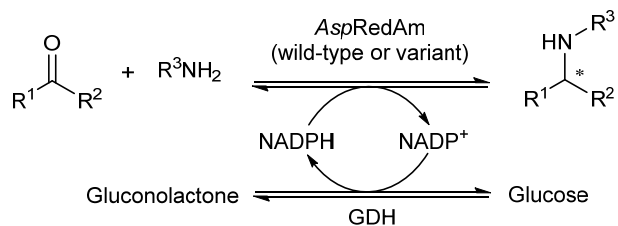


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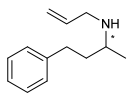
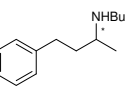
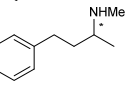
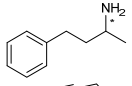
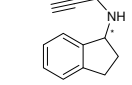
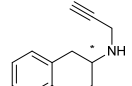
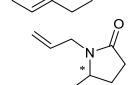
530 Conversions determined by HPLC or GC-FID analysis. Reaction conditions: ketone/aldehyde (5 mM), amine (1 to 50  
 531 eq.), AspRedAm (1 mg mL<sup>-1</sup>), NADP<sup>+</sup> (1 mM), GDH (0.2 mg mL<sup>-1</sup>), D-glucose (30 mM), Tris buffer (100 mM, pH 9.0),  
 532 25°C, 250 rpm, 24 h. [a] Only the product of double reductive amination was observed.

533

534 **Table 2. Comparison of stereochemical outcomes from biotransformations catalysed by *AspRedAm* variants**  
 535 **W210A and Q240A.**

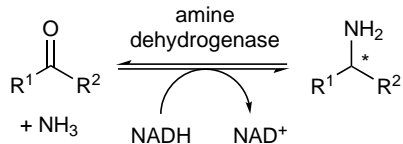
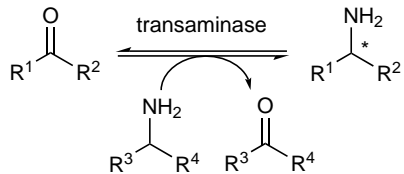
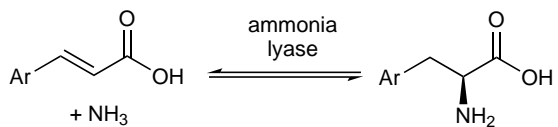
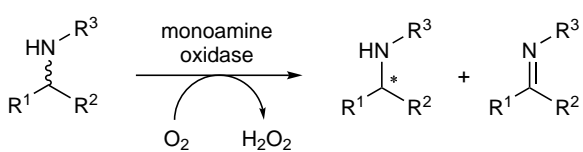


536

Entry	Ketone	Amine	Product	AspRedAm WT		AspRedAm Q240A		AspRedAm W210A	
				Conv. (%)	e.e. (%) (R or S)	Conv. (%)	e.e. (%) (R or S)	Conv. (%)	e.e. (%) (R or S)
1	17	c		>97	30 (R)	90	90 (R)	>97	94 (S)
2	17	d		>97	96 (R)	97	>98 (R)	>97	70 (S)
3	17	g		72	85 (R)	>97	>97 (R)	>97	90 (S)
4	17	k		0	n.a.	56	>98 (R)	0	n.a.
5	29	a		64	95(R)	>97	>98 (R)	65	31 (S)
6	9	a		>97	88 (R)	>97	>97 (R)	>97	80 (S)
7	10	c		>97	59 <sup>[a]</sup>	>97	85 <sup>[a]</sup>	>97	49 <sup>[a][b]</sup>

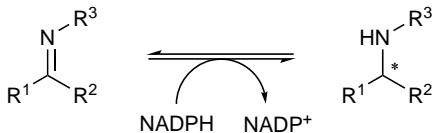
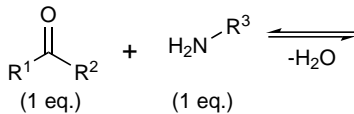
537 [a] Absolute configuration not assigned [b] gives opposite enantiomer to the wild-type enzyme. n.a. not  
 538 applicable. N.B. Reactions carried out with 20 amine eq except for entry 5 (50 eq.). *AspRedAm* variant Q240A  
 539 displayed improved (*R*)-selectivity compared to the wild-type enzyme whereas W210A mutant was (*S*)-selective for  
 540 investigated substrates.

541

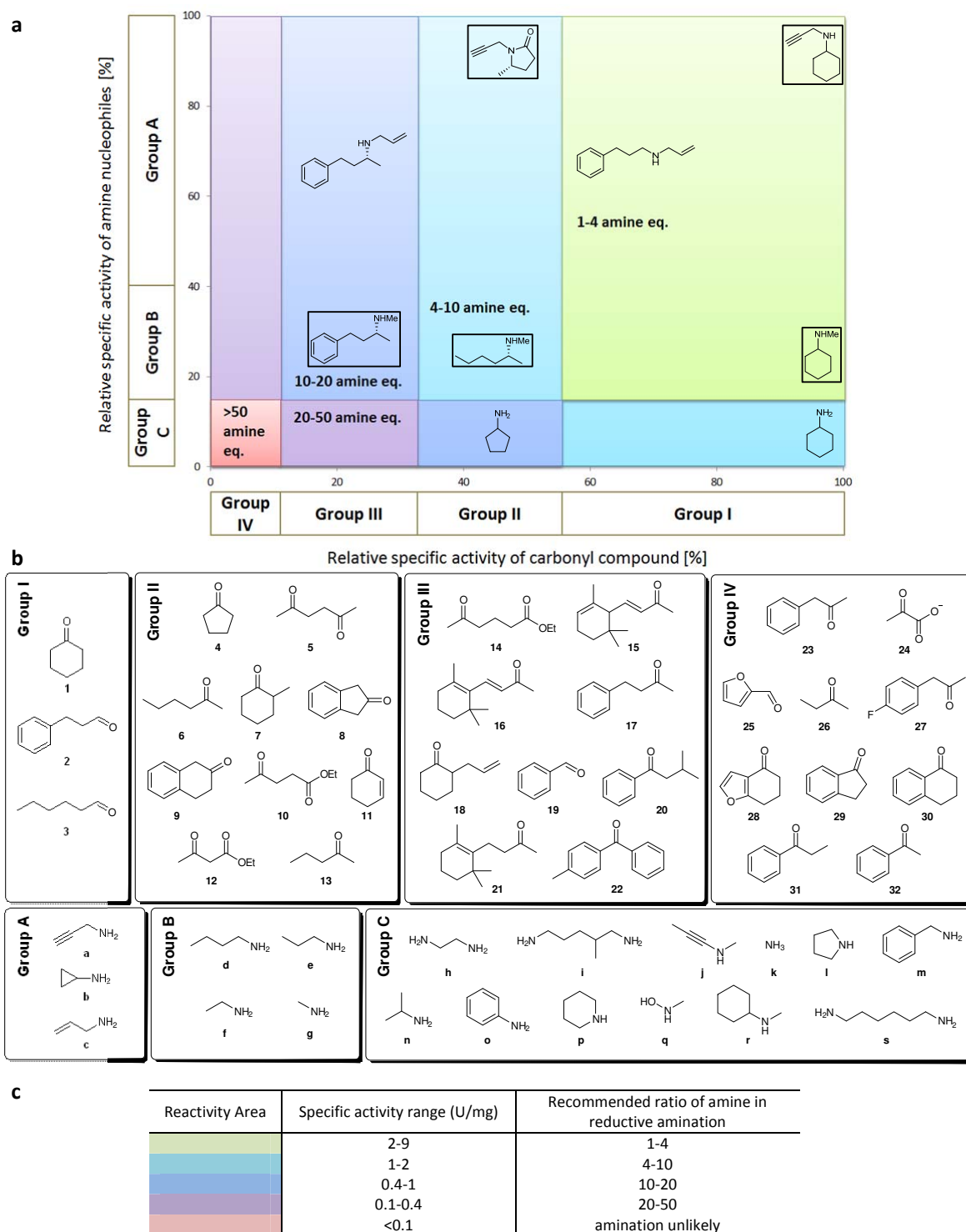


*this work*

**AspRedAm**

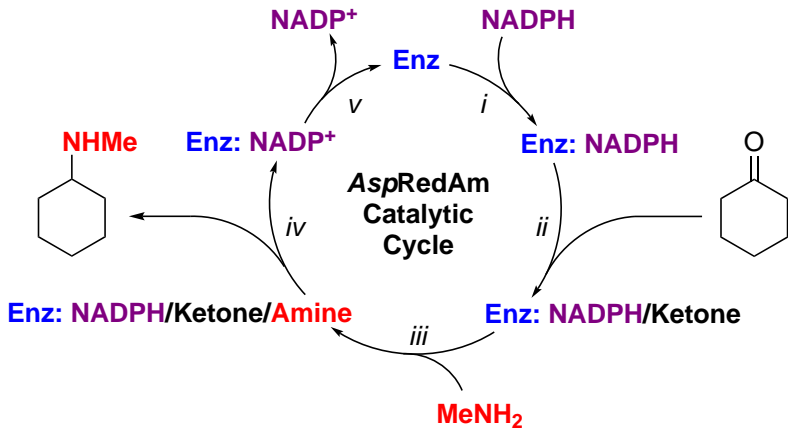
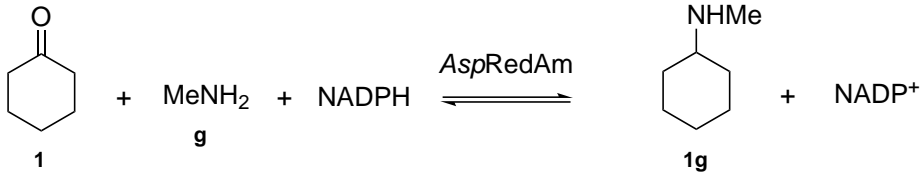


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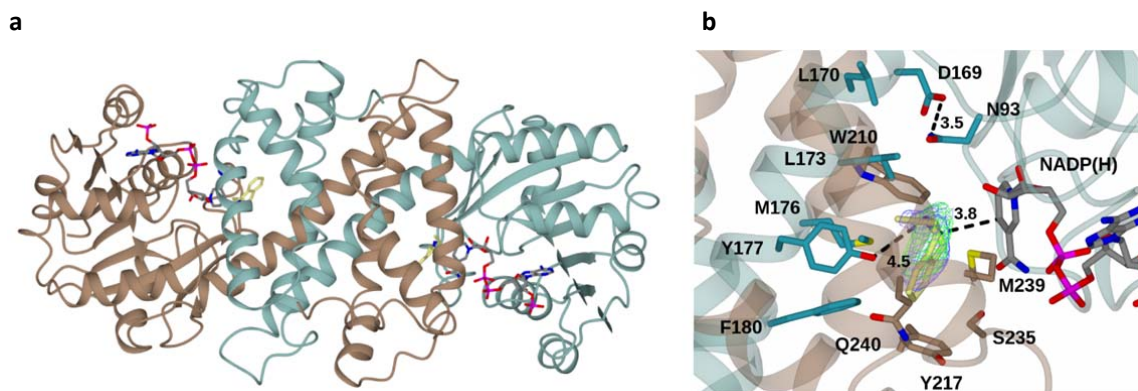


**Figure 2.** Reactivity chart for AspRedAm-catalysed reactions based on specific activities of a panel of carbonyl compounds and amine reacting partners. **a)** Chart displaying relative activity of amine/carbonyl pairs in reductive amination reactions. Compounds presented in the plot area are representative examples of products obtained in biotransformations. Conversions of >50% were achieved in all cases when the recommended amine:ketone ratios were used. Framed structures correspond to scaled-up biotransformations with isolated products. **b)** Carbonyl acceptors and amine nucleophiles arranged in Groups based on their average relative specific activity value. **c)** Legend for the reactivity chart with specific activity ranking and recommended ratio of amine to carbonyl compound for reductive amination.









**c**

Enzyme variants	$K_m$ (mM)	$k_{cat}$ ( $s^{-1}$ )	$k_{cat}/K_m$ ( $s^{-1} mM^{-1}$ )	$K_m$ (ketone)	$k_{cat}$ ( $s^{-1}$ )	$k_{cat}/K_m$ ( $s^{-1} mM^{-1}$ )
wild-type	0.352	3.243	9.213	1.901	1.470	0.733
D169A	1.101	0.016	0.014	2.700	0.008	0.003
D169N	0.320	0.009	0.028	2.080	0.007	0.003
Y177A	0.689	0.063	0.091	2.212	0.050	0.023

**Figure 4. Structural and mutagenesis data of *AspRedAm* highlighting essential catalytic residues.** a) Dimeric structure of *AspRedAm* in complex with NADP(H) and (*R*)-29a dimer in which the active site is at the interface between the Rossman fold of one monomer and the C-terminal bundle of its neighbour; b) Active site of *AspRedAm* at dimer interface. Electron density represents the  $2F_o - F_c$  (blue) and  $F_o - F_c$  (omit, green) maps, the latter obtained prior to refinement of the ligand, and contoured at levels of  $1.0$  and  $2.5\sigma$  respectively. Distances are shown in Ångstroms. c) Kinetic data of *AspRedAm* wild-type and mutants D169A, D169N and Y177A. Mutation at D169 and Y177 resulted in a marked decrease in activity suggesting essential roles for these residues in catalysis.

