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Identification of Novel Bacterial Members of the Imine Reductase Enzyme Family that Perform Reductive Amination

Scott P. France,^[a] Roger M. Howard,^[b,c] Jeremy Steflik,^[b] Nicholas J. Weise,^[a] Juan Mangas-Sanchez,^[a] Sarah L. Montgomery,^[a] Robert Crook,^[c] Rajesh Kumar^{*,[b]} and Nicholas J. Turner^{*,[a]}

Abstract: Reductive amination of carbonyl compounds constitutes one of the most efficient ways to rapidly construct chiral and achiral amine frameworks. Imine reductase (IRED) biocatalysts represent a versatile family of enzymes for amine synthesis *via* NADPH mediated imine reduction. The reductive aminases (RedAms) are a sub-family of IREDs which have recently been shown to catalyse imine formation as well as imine reduction. Herein, a diverse library of novel enzymes were expressed and screened as cell-free lysates for their ability to facilitate reductive amination, in order to expand the known suite of biocatalysts for this transformation and to identify more enzymes with potential industrial applications. A range of ketones and amines were examined and enzymes were identified that were capable of accepting benzylamine, pyrrolidine, ammonia and aniline. Amine equivalents as low as 2.5 were employed to afford up to >99% conversion and for chiral products, up to >98% e.e. could be achieved. Preparative-scale reactions were conducted with low amine equivalents (1.5 or 2.0) of methylamine, allylamine and pyrrolidine, achieving up to >99% conversion and 76% isolated yield.

Secondary and tertiary amines are motifs present in many natural products, active pharmaceutical ingredients (APIs) and agrochemicals. Unsurprisingly, the synthesis of these frameworks has been the focus of considerable research efforts. One of the most powerful ways to construct these architectures is *via* reductive amination of a carbonyl compound with an amine because it can enable the coupling of two smaller fragments directly with concomitant formation of a stereogenic centre. A number of advances have recently been made in the field of catalytic asymmetric reductive amination processes^[1–5] although there are still considerable challenges such as the chemoselectivity of the catalyst for imine functionality, the disfavoured equilibrium of imine formation in water and the enantioselectivity of the reaction.^[6] Alternatively, preformation of the imine followed by asymmetric transition metal-catalysed hydrogenation is an option,^[7–12] although it is a two-step process and brings with it additional safety considerations around the use of hydrogen. Furthermore, achiral secondary and tertiary amine motifs are also present in many bioactive compounds and their synthesis by reductive amination is often advantageous over *N*-alkylation which requires toxic alkylating agents and can easily lead to over-addition products.

As an alternative to traditional chemical methods, biocatalysis is becoming an increasingly desirable option for industry due to the benefits of sustainable catalyst supply, milder reaction conditions and the enantioselectivity offered by enzymes. One of the more recent additions to the suite of biocatalysts available for chiral amine synthesis are imine reductases (IREDs) that catalyse the asymmetric reduction of prochiral imines using the cofactor NADPH (Figure 1a).^[13–16] It has also been shown that, in addition to reduction of preformed imines, certain enzymes of this class can also enable reductive amination between a carbonyl and amine substrate.^[17–22] Furthermore, we have recently identified enzymes from a sub-group of the IRED enzyme family that catalyse imine formation as well as imine reduction. These reductive aminases (RedAms) from fungi are able to utilise stoichiometric or near-stoichiometric ketone:amine equivalents to achieve high conversions (Figure 1b).^[23] Other enzyme classes that catalyse reductive amination include amino acid dehydrogenases (AADHs) and the engineered amine dehydrogenases (AmDHs)^[24,25] as well as *N*-methyl-amino acid dehydrogenases (NMAADHs),^[26] although, to date, the latter are limited to ammonia or methylamine as the amine coupling partner.^[27] Opine dehydrogenases (OpDHs) catalyse reductive amination between an amino acid and keto acid and have been engineered to broaden their substrate scope and application by Codexis.^[28]

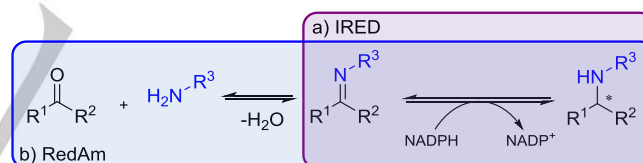


Figure 1: Imine reductase (IRED) and reductive aminase (RedAm) biocatalysts for amine synthesis. a) IRED-catalysed reduction of preformed imines. b) RedAm-catalysed imine formation and subsequent reduction.

In light of the recent identification of fungal RedAms, we decided to investigate new bacterial IREDs as biocatalysts for reductive amination with particular emphasis on operating at near-stoichiometric amounts of ketone and amine reagents.

A group of 45 bacterial IREDs was selected using the Basic Local Alignment Search Tool (BLAST) which included 27 novel enzymes and 18 enzymes previously disclosed^[17,29–35] (see Supporting Information). The protein sequences of the known IREDs from *Pseudomonas putida* (3L6D),^[30] *Streptomyces* sp. GF3546 (BAM99301)^[33,34] and an undisclosed IRED of industrial interest, all of which have broad substrate specificity, were used as query sequences. Genes were synthesised, subcloned into pET28b, expressed in *E. coli* BL21 (DE3) and applied as cell-free lysates (see Supporting Information). Preliminary screening with cell-free lysate containing the reductive aminase from *Aspergillus oryzae* (AspRedAm)^[23] suggested successful reductive amination could be achieved by using a crude biocatalyst preparation (see Supporting Information).

[a] Mr S. P. France, Dr. N. J. Weise, Dr. J. Mangas-Sanchez, Miss S. L. Montgomery, Prof. N. J. Turner

School of Chemistry, University of Manchester, Manchester Institute of Biotechnology, 131 Princess Street, Manchester M1 7DN, UK. E-mail: nicholas.turner@manchester.ac.uk

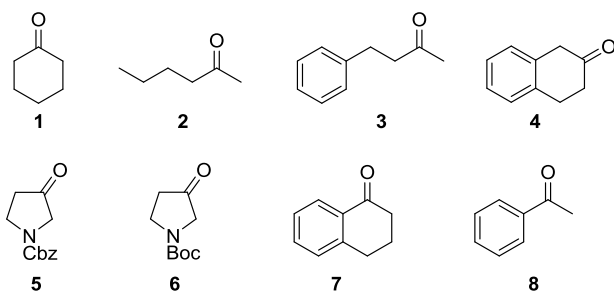
[b] Dr. R. M. Howard, Mr. J. Steflik, Dr. R. Kumar

Groton Laboratories, Pfizer Worldwide Research and Development, 445 Eastern Point Road, Groton, Connecticut, 06340, US. E-mail: Rajesh.Kumar3@pfizer.com

[c] Dr. R. M. Howard, Mr. R. Crook

Sandwich Laboratories, Pfizer Worldwide Research and Development, Discovery Park, Sandwich, Kent, CT13 9NJ, UK.

Ketone Substrates



Amine Substrates

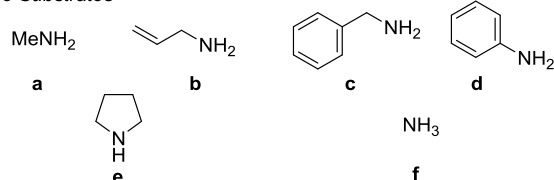


Figure 2: Reductive amination ketone and amine substrate panel for IRED screening.

A panel of model ketones **1-8** and amines **a-f** was selected to study the reductive amination activity of the novel enzymes. The IRED library was first screened with the panel of ketones **1-8** and methylamine **a**. For the purposes of initial testing, 50 equivalents of amine **a** were used to maximise the chances of identifying hits. However, with ketone substrates **1** and **2** that have recently been demonstrated to work well with near stoichiometric quantities of amine coupling partner,^[23] reactions were conducted at 2.5 amine equivalents. The results of biotransformations with a selection of the best performing enzymes are presented in Table 1 (for full screening results see the Supporting Information).

Ketone **1** was accepted by the majority of enzymes in the library with modest to high conversions achieved. Interestingly, in the conversion of the prochiral ketones **2-4**, a large proportion of the enzyme library afforded (*S*)-configured products. These examples constitute valuable additions to the toolbox of biocatalysts for reductive amination since they are enantiocomplementary to the previously described *AspRedAm*. The *N*-protected pyrrolidinone substrates **5** and **6** were generally more challenging substrates. However, interestingly IR48 afforded high conversion with the Cbz-protected analogue **5**. The products **5a** and **6a** are industrially relevant since the *N*-methylpyrrolidin-3-amine framework is found in a number of biologically active compounds described in the academic and patent literature.^[36-39] Substrates α -tetralone **7** and acetophenone **8** showed little to no conversion across the entire library of enzymes screened.

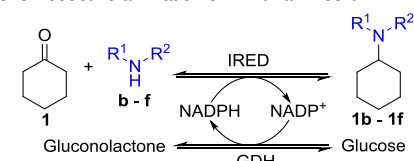
To explore the substrate scope of reductive amination with respect to the amine substrate, cyclohexanone **1** was chosen as the model compound and screened against a panel of amines **b-f**. This panel encompassed primary and secondary amines including ammonia **f** as well as bulkier amines and those with chemical handles for further synthesis. With larger and more expensive amine donors, it is clearly important that this component is added in a low number of equivalents relative to the ketone substrate to lower costs and reduce product contamination, thereby minimising downstream processing. However, when using ammonia **f**, for example, the volatility of the amine necessitates a higher loading but it is also inexpensive and can be easily removed during work up. Therefore, for the library screening presented in Table 2, 2.5 equivalents of amines **b-e** were used while 20 equivalents were added in the case of ammonia **f**.

Table 1: Biotransformation data for screening of a subset of the best performing enzymes for reductive amination of a panel of ketones **1-6** with methylamine **a**.

Enzyme	Apparent conversion to product/%, e.e./% (<i>R</i> or <i>S</i>)					
IR44	94	45, >98 (<i>S</i>)	>99, >98 (<i>S</i>)	19, >98 (<i>S</i>)	0	25
IR46	95	6	6	53, >98 (<i>R</i>)	0	0
IR47	98	19, 78 (<i>S</i>)	>99, 26 (<i>R</i>)	41, 18 (<i>S</i>)	58, 89 (<i>S</i>)	14
IR48	96	41, >98 (<i>S</i>)	>99, >98 (<i>S</i>)	22, >98 (<i>S</i>)	89, 96 (<i>S</i>)	38
IR50	98	46, >98 (<i>S</i>)	>99, >98 (<i>S</i>)	19, >98 (<i>S</i>)	23	4
IR51	95	4	1	44, >98 (<i>R</i>)	0	0
IR56	97	43, >98 (<i>S</i>)	>99, >98 (<i>S</i>)	12	0	4
IR58	98	7, 53 (<i>R</i>)	2	51, >98 (<i>S</i>)	0	0
IR59	13	7, >98 (<i>S</i>)	56, >98 (<i>S</i>)	1	0	0
IR61	79	32, >98 (<i>S</i>)	99, >98 (<i>S</i>)	2	27	16
IR62	31	1	1	66, 7 (<i>R</i>)	0	5
IR66	99	9, 50 (<i>R</i>)	8, 62 (<i>S</i>)	50, 51 (<i>S</i>)	2	0
IR67	70	2	0	10, >98 (<i>S</i>)	0	0
IR68	19	3	43, >98 (<i>S</i>)	3	2	0
IR69	18	0	0	10, >98 (<i>S</i>)	0	0
IR70	97	5	1	10, >98 (<i>S</i>)	1	23
IR77	98	43, >98 (<i>S</i>)	>99, >98 (<i>S</i>)	18, >98 (<i>S</i>)	0	0
IR91	22	0	30, 76 (<i>S</i>)	74, >98 (<i>S</i>)	22	85

Reaction conditions: Ketone (**1**, **2**: 10 mM; **3-6**: 5 mM), methylamine **a** (for reactions with **1**, **2**: 25 mM; for **3-6**: 250 mM), glucose (50 mM), GDH CDX-901 (0.3 mg/mL), NADP⁺ (0.4 mM), enzyme lysate in Tris buffer (100 mM, pH 9.0). Incubated at 25°C, 250 rpm for 24 h. For full screening results, see the Supporting Information. Conversion determined by GC-FID and e.e. by chiral phase GC-FID or HPLC.

Table 2: Biotransformation data for screening of a subset of the best performing enzymes for reductive amination of **1** with amines **b – f**.



Apparent conversion to product/%

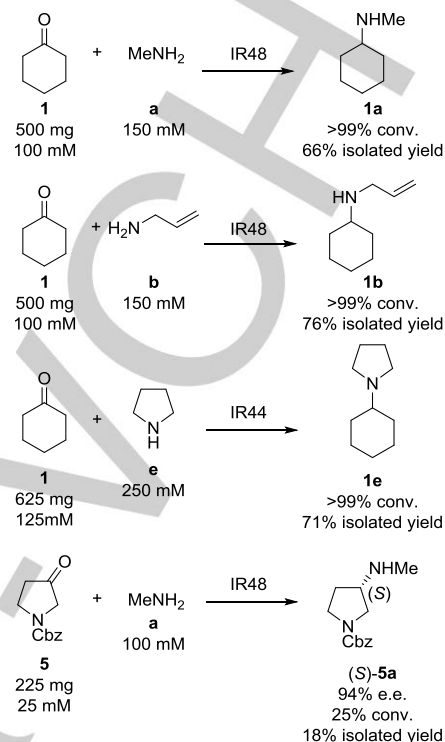
Enzyme	1b	1c	1d	1e	1f
IR44	92	79	11	99	0
IR46	11	1	0	15	0
IR47	72	66	30	83	5
IR48	83	79	9	91	0
IR50	80	76	20	99	0
IR51	15	4	0	13	0
IR56	87	81	16	99	0
IR58	74	1	0	78	10
IR59	24	61	1	14	0
IR61	65	78	10	83	0
IR62	35	31	0	2	0
IR66	84	2	1	79	20
IR67	62	32	0	1	0
IR68	10	23	5	7	0
IR69	46	43	0	4	0
IR70	78	1	0	55	2
IR77	84	78	23	97	0
IR91	30	63	1	8	0

Reaction conditions: **1** (10 mM), amine (**b – e**: 25 mM; **f**: 200 mM), glucose (50 mM), GDH CDX-901 (0.3 mg/mL), NADP⁺ (0.4 mM), enzyme lysate in Tris buffer (100 mM, pH 9.0). Incubated at 25°C, 250 rpm for 24 h. For full screening results, see the Supporting Information. Conversion determined by GC-FID.

Interestingly, the enzyme library performed well with allylamine **b** as well as the more bulky substrate benzylamine **c**. Furthermore, several enzymes were identified that gave high conversions with the secondary amine pyrrolidine **e** which must react via an iminium ion intermediate to afford the tertiary amine product **1e**. Interestingly, *AspRedAm* is unable to catalyse the formation of **1e**, further highlighting the utility of the enzymes described herein. Aniline **d** was also a substrate for several of the biocatalysts. Due to the lower nucleophilicity of arylamine systems compared to simple alkylamines, enzymes that are capable of utilising **d** in reductive aminations with ketones are of particular interest with some recent advances in this area made independently by Roiban *et al.*^[22] during the course of our investigations. Ammonia **f** did not give high conversions, despite 20 equivalents being employed, although IR66 afforded 20% conversion to the product **1f**.

Preparative-scale reactions were performed with **1** with 100 mM or 125 mM substrate loading and 1.5 or 2 equivalents of amines **a**, **b** and **e**, achieving high conversions and good isolated yields. All these reactions showed good initial rates of reaction, resulting in >60% conversion in the first 6 h before slowing down. These larger-scale reactions revealed that the use of the glucose/GDH cofactor recycling system resulted in a drop in reaction pH, and therefore titration with 2N sodium hydroxide solution was required to maintain a pH of 8 over the course of the reaction. To date, these represent the highest substrate

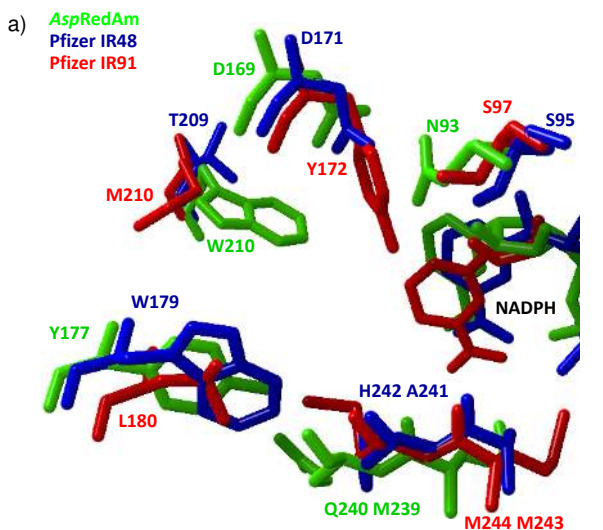
loadings for preparative reductive aminations with IREDs described in the literature. Intensification efforts for the synthesis of (*S*)-**5a** were limited by the solubility of substrate **5**.



Scheme 1: Preparative-scale reductive amination biotransformations. Reaction conditions: Enzyme lysate (10% v/v), glucose (1.1 eq.), NADP⁺ (0.5–0.6 mg/mL) and GDH (CDX901, 0.5–0.6 mg/mL) in Tris buffer (100 mM), 30°C, pH 9, 350 rpm for 24 h. Maintained at pH 8.0 with titration with 2N NaOH.

The 45 enzymes reported in this study were then ranked based on their conversions in reductive amination reactions across the entire substrate set. For the top performing enzymes, their active-site residues were compared to *AspRedAm* (Figure 3). Interestingly, several of the best performing enzymes cluster together in the cladogram (see Supporting Information) and based on sequence alignments, show a conserved active site aspartate at the position homologous to D169 in *AspRedAm*. However, at the homologous positions to *AspRedAm*'s N96, Y177 and Q240, there are different residues with serine, tryptophan and histidine being the most commonly found respectively. Homology models of IR48 from *Rhizobium sulae* and IR91 from *Kribbella flavida* DSM 17836 were created and their active sites overlaid with that of *AspRedAm* (Figure 3a). IR48 is representative of the best performing enzymes that have the conserved residues described above and IR91 is an enzyme that has some significantly different active site residues, yet still facilitates reductive amination. It should be noted that none of the highlighted residues is completely conserved across the three active-site architectures shown. There are also significant differences in residue functional equivalence, for example between the homologous Y177, W179 and L180 in *AspRedAm*, IR48 and IR91 respectively. This analysis serves to highlight that reductive amination catalysed by these enzymes may occur, mechanistically, in different ways compared to that of the recently discovered fungal RedAms, with different modes of

binding of substrates. However, further experiments are required to prove whether these enzymes are capable of catalysing imine formation, and thus be confidently classified as RedAms.



b)

IREDD	Selected active site residues compared to AspRedAm					
	N93	D169	Y177	W210	M239	Q240
IR48	S	D	W	W*	A	H
IR47	T	D	W	M	V	H
IR50	S	D	W	W	P	H
IR44	S	D	W	W	A	H
IR77	S	D	W	W	P	H
IR56	S	D	W	W	P	H
IR61	S	D	W	W	P	H
IR66	S	D	W	L	A	H
IR91	S	Y	L	M	M	M
IR58	S	D	W	L	A	H

Figure 3: a) Overlay of the active sites of IR48 and IR91 with that of the known reductive aminase AspRedAm. IR48 (blue), IR91 (red), AspRedAm (green). b) Comparison of the active site residues of the best performing enzymes with those of AspRedAm based on identification of homologous residues from sequence alignments. Residues in bold are well conserved. *The result of the structural alignment shown in a) highlights a T in the active site rather than W.

In summary, we have identified a new set of bacterial enzymes capable of performing reductive amination reactions. Several enzymes were found that gave high conversions at low amine equivalents with primary and secondary amines, as well as some with aniline which, until recently,^[22] had not been extensively demonstrated as a substrate for reductive aminations with RedAms or IREDDs. Preparative-scale biotransformations have shown the potential application of these enzymes, particularly for reactions with near stoichiometric quantities of ketone and amine. If these enzymes indeed catalyse imine formation as well as reduction, then the analysis of active-site residues suggests that this catalysis may be brought about in different ways relative to one another and to the fungal RedAms based on interesting differences in active-site residue functional equivalence. This work broadens the portfolio of IREDDs available as the field moves towards the industrial application of biocatalysts for reductive amination.

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Experimental Section

See supporting information.

Keywords: biocatalysis • imine reductase • reductive amination • amines • chirality

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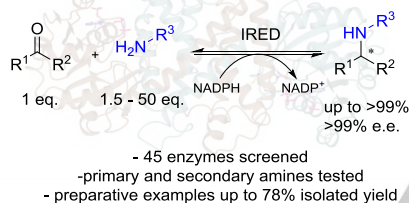
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Layout 1:

COMMUNICATION

A diverse group of novel imine reductases (IREDs) were expressed and screened as cell-free lysates for their ability to facilitate reductive amination. A range of ketones and amines were examined and enzymes identified that accepted benzylamine, pyrrolidine, ammonia and aniline. Amine equivalents as low as 2.5 afforded up to >99% conversion and up to >98% e.e. Preparative-scale reactions with low amine equivalents (1.5 or 2.0) of methylamine, allylamine and pyrrolidine, achieved up to >99% conversion and 76% isolated yield.



Scott P. France, Roger M. Howard,
Jeremy Steflik, Nicholas J. Weise, Juan
Mangas-Sanchez, Sarah L.
Montgomery, Robert Crook, Rajesh
Kumar* and Nicholas J. Turner*

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**Identification of Novel Bacterial
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Enzyme Family that Perform
Reductive Amination**