Heterogeneous & Homogeneous & Bio- & Nano-
CHEMCATCHEM
CATALYSIS

Accepted Article

Title: Efficient biocatalytic reductive aminations by extending the imine reductase toolbox

Authors: Gheorghe-Doru Roiban, Marcelo Kern, Zhi Liu, Julia Hyslop, Pei Lyn Tey, Matthew S. Levine, Lydia S. Jordan, Kristin Brown, Timin Hadi, Leigh Anne F Ihnken, and Murray J.B. Brown

To be cited as: *ChemCatChem* 10.1002/cctc.201701379

Link to VoR: http://dx.doi.org/10.1002/cctc.201701379

A Journal of
ChemPubSoc Europe

WILEY-VCH www.chemcatchem.org

10.1002/cctc.201701379

WILEY-VCH

COMMUNICATION

Efficient biocatalytic reductive aminations by extending the imine reductase toolbox

Gheorghe-Doru Roiban,*[a] Marcelo Kern,[a] Zhi Liu,[b] Julia Hyslop,[a] Pei Lyn Tey,[a] Matthew S. Levine,[b] Lydia S. Jordan,[b] Kristin K. Brown,[c] Timin Hadi,[b] Leigh Anne F. Ihnken,[b] and Murray J.B. Brown[a]

Abstract: Chiral secondary and tertiary amines are ubiquitous in pharmaceutical, fine, and specialty chemicals, but their synthesis typically suffers from significant sustainability and selectivity challenges. Biocatalytic alternatives, such as enzyme catalyzed reductive amination, offer several advantages over traditional chemistry, but industrial applicability has not yet been demonstrated. Herein, we report the use of cell lysates expressing imine reductases operating at 1:1 stoichiometry for a variety of amines and carbonyls. A collection of biocatalysts with diversity in coverage of small molecules and direct industrial applicability is presented.

The majority of marketed drugs either contain amine functional groups or are derived from amine intermediates, with up to 40% of active pharmaceutical ingredients (APIs) containing a chiral amine moiety.[1] Hence, the large-scale production of secondary and tertiary amines as structural motifs commonly found in APIs is of great interest to the pharmaceutical industry.[2] There are many established chemical methods for the preparation of amines, including nucleophilic alkylation, hydrogenation of imines, enamines, and enamides, enantioselective hydroamination, nucleophilic carbanion or radical addition to imino compounds, as well as classical Mannich and Strecker reactions. These methods tend to use precious or heavy metal catalysts, high pressures and/or temperatures, organic solvents, stoichiometric hydride reductants, or potentially genotoxic alkyl halides.[2-3] Recently, biocatalytic approaches have emerged as an attractive alternative, offering more sustainable processes under ambient aqueous conditions with high chemo-, regio-, and stereoselectivity with minimal hazardous waste. Biocatalysis also offers more flexibility since the adoption of directed evolution allows for rapid optimization of an enzyme to meet the requirements of process chemistry.[4]

Established biocatalytic processes for the preparation of amines have focused on chiral primary amines, notably hydrolytic resolution of racemic amides, desymmetrisations with amine oxidases and chemical reductants, and direct installation via transaminases.[1] There are also emerging opportunities with amine dehydrogenases, ammonia lyases, and aminomutases, with all giving access to primary amines.[5] Direct biocatalytic routes to secondary and tertiary amines have been reported using imine reductases (IREDs).[6] Initially, cyclic imine reduction was reported,[7] followed by subsequent formal intermolecular reductive amination.[8] These activities were restricted to small primary aliphatic amines, required a large excess of amine nucleophile and high biocatalyst loading, making them less attractive for industrial applications. Recently, Aleku *et al.* reported an IRED homolog from *Aspergillus oryzae* (AspRedAm) capable of reductive amination at 1:1 stoichiometry for a subset of amines and carbonyls, using purified protein preparations.[9] The purified protein approach allows for activity enrichment and reduction in background carbonyl reduction from endogenous host enzymes, but is an undesirable manufacturing strategy due to the extra cost and complexity associated with the purification process. Maugeri and Rother reported successful reductive amination with lyophilized whole cells expressing IREDs,[10] a good first step toward industrial application, but with limited demonstrated substrate scope. Herein, we report a collection of enzymes catalyzing reductive amination at 1:1 stoichiometry, as cell lysates, with diverse coverage of small molecule space. We view the members of this IRED panel as potential evolution starting points on the path of delivering manufacturing-relevant enzymes for industrial biocatalytic reductive amination. The panel of 85 enzymes we tested includes AspRedAm,[9] several other known IRED sequences from the literature[7c, 7e, 9, 11] and IRED database,[12] and novel putative IREDs chosen based on homology to known, active sequences (see the Supporting Information, Table S1, Figures S1-2). The proteins were expressed in 96-well plate format (see the Supporting Information, Table S2, Figure S3) and used in screening reactions as clarified cell lysate. Initial screening with selected IREDs with arylamines indicated a pH optimum of 6-7 and a slight preference for potassium phosphate buffer (see the Supporting Information, Figure S3), in contrast to previously reported pH optima of 9-9.5 for enzymatic reductive amination with aliphatic amines.[8b-d] Subsequent reactions were all performed in 100 mM potassium phosphate pH 7 except where indicated.

[a] Dr. G.-D. Roiban, Dr. M. Kern, J. Hyslop, P. Lyn Tey, Dr. M. J. B. Brown
Advanced Manufacturing Technologies
GlaxoSmithKline
Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, United Kingdom
E-mail: gheorghe.d.roiban@gsk.com

[b] Z. Liu, M. S. Levine, L. S. Jordan, Dr. T. Hadi, Dr. L. A. F. Ihnken
Advanced Manufacturing Technologies
GlaxoSmithKline
709 Swedeland Road, King of Prussia, Pennsylvania 19406, United States

[b] Dr. K. K. Brown
Molecular Design, Computational and Modeling Sciences
GlaxoSmithKline
1250 S. Collegeville Road, Collegeville, Pennsylvania 19426, United States

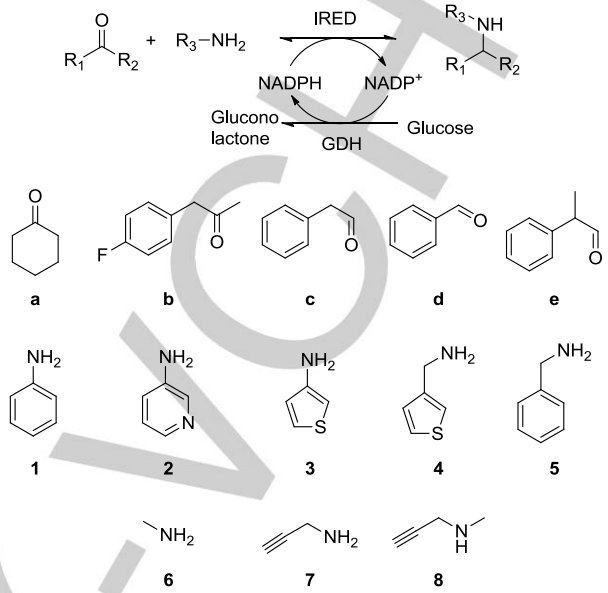
Supporting information for this article is given via a link at the end of the document.

10.1002/cctc.201701379

COMMUNICATION

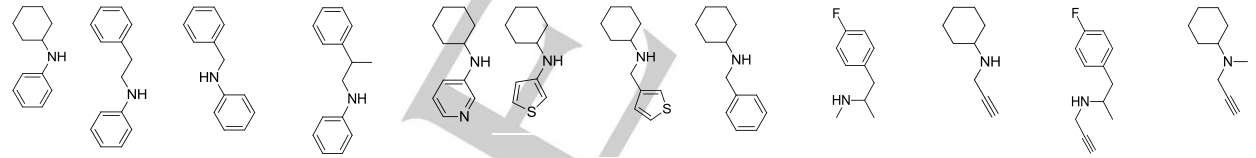
The reductive amination activity of each enzyme was tested across a number of carbonyl and amine pairs at 1:1 stoichiometry (Scheme 1). Results for selected enzymes are shown in Table 1 (see Supporting Information for full data set, Table S3). High conversions (>60%) were seen for several enzymes for arylamines **1** and **3** with cyclohexanone **a** and for aniline **1** plus phenylacetaldehyde **c** or benzaldehyde **d**. This represents an important extension of the substrate scope of IREDs as arylamines have not previously been reported as good enzymatic reductive amination substrates. The compounds in Scheme 1 were chosen as model substrates as they are representative of pharmacologically active moieties. *N*-Benzylaniline is used in the manufacture of Antazolin, Bepridil, and Efonidipine[13] and substituted *N*-phenethylanilines have been reported as potent thrombin inhibitors.[14]

Moderate (10-60%) to high conversions were also observed with aliphatic amines, including formation of *N*-(thiophen-3-ylmethyl)cyclohexanamine **4a**, the core of a potent melanin-concentrating hormone receptor antagonist[15] whose chemical synthesis is reported as tedious and low yielding.[16] Moderate conversions to amphetamine analogues **6b**, and **7b** were observed after reductive amination of *p*-fluorophenylacetone **b** with methylamine **6**, and propargylamine **7**. Both IR-01 and IR-13 showed moderate conversion with *N*-methylpropargylamine **8** with cyclohexanone **a**, the first report of IRED-catalyzed tertiary amine formation using equimolar reactants. No conversion was observed from any of the top seven enzymes tested for reaction of cyclohexanone **a** with equimolar amounts of isopropylamine, cyclohexylamine, *n*-propylamine, or *N*-methyl propan-1-amine.



Scheme 1. Biocatalytic reductive amination.

Table 1. Conversion and selectivity for chosen enzymatic reductive amination reactions.



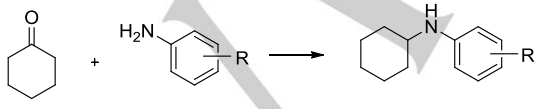
	1a[a]	**1c**[a]	**1d**[a, d]	**1e**[a]		**2a**[a]	**3a**[b]	**4a**[a]	**5a**[a]	**6b**[c]		**7a**[a]	**7b**[c]		**8a**[a]
IRED	conv	conv	conv	conv	ee[e]	conv	conv	conv	conv	conv	ee[f]	conv	conv	ee	conv
IR-01	93	>99	99	54	26 (-)	19	62	97	74	26	90 (+)	68	60	83 (*R*)	17
IR-10	99	99	96	32	62 (+)	12	53	19	20	4	37 (+)	28	15	22 (*R*)	4
IR-13	96	>99	99	46	41 (-)	10	61	85	59	14	61 (+)	55	27	34 (*S*)	14
IR-22	56	95	89	30	56 (-)	1	60	43	12	0	(nd)[g]	63	0	(nd)[g]	7
IR-24	78	91	81	18	>99 (-)	11	48	81	54	0	(nd)[g]	15	0	(nd)[g]	<2
IR-49	64	94	88	3	(nd)[g]	0	36	15	<2	12	83 (+)	39	9	97 (*R*)	<2
IR-59	0	6	3	0	(nd)[g]	0	3	11	-	12	91 (+)	-	62	98 (*R*)	-
IR-69	0	0	2	7	(nd)[g]	0	13	30	-	0	(nd)[g]	-	47	95 (*R*)	-
IR-72	0	0	2	0	(nd)[g]	0	6	13	-	0	(nd)[g]	-	40	99 (*R*)	-
IR-75	3	0	2	0	(nd)[g]	0	15	82	-	4	83 (-)	-	15	99 (*S*)	-

% Conversions at [a]4h, or [b]1h in 100 mM potassium phosphate pH 7 or [c]24h in 100 mM Tris pH 9; Reaction conditions: **a-d** (32 mM), **1-8** (35 mM, 1.1 equiv), GDH (0.31 mg/mL), glucose (63 mM), $NADP^+$ (1.5 mM), 30 °C, final volume 0.4 mL. [d]Approximately 2% conversion seen in background reaction. [e]Enantiomeric excess (%ee) of first eluting enantiomer by chiral HPLC. [f]%ee reported with optical rotation sign. [g]Not determined (nd) due to low or no conversion. Dash "-" indicates not tested.

Such levels of reductive amination using aqueous cell lysate, at neutral pH, and with low amine equivalents are unprecedented. Furthermore, there was <2% background carbonyl reduction to the corresponding alcohol, indicating relatively high IRED activity compared to endogenous host cell ketoreductase activity, in contrast to previous reports.[8b,10,17] Given the challenges of performing reductive aminations without large amine excess,[8] we were pleased to find a high number of active enzymes (69 out of 86, 80%) giving at least 5% conversion for at least one carbonyl plus amine combination using stoichiometric reactants with unoptimized conditions (see the Supporting Information, Table S3). Further, in most cases, at least one enzyme gave >50% conversion, often with several >90% conversion. This indicates the high quality of this panel of enzymes in comparison with other reported IRED collections.[18] For the reactions presented here, IR-01 proved to be the most active enzyme in many cases. However, other screening efforts have found IR-01 to be inactive while other IREDs show excellent activity, demonstrating that a diverse collection of biocatalysts is beneficial. Furthermore, excellent and complementary enantiospecificity is demonstrated for **6b** and **7b** providing access to both enantiomers. Reported chemical approaches to these compounds have been limited to wasteful resolutions[35-36] and recently reported IRED catalysed conversions have required large amine excesses and only provide a single enantiomer with good[9] or moderate[8d] ee. Additionally this panel demonstrates resolution of prochiral **e** with access to both enantiomers with moderate to excellent ee.

The substrate scope of the seven best-performing enzymes producing **1a** was further investigated at lower biocatalyst loading with a series of substituted anilines and cyclohexanone **a** (Table 2). Both steric and electronic effects seemed to play a role in enzyme activity. Generally, the tested IREDs were more active with *m*- and *p*-subsituted anilines than *o*-substitued, with bulky *o*-substituents like ethyl and isopropyl not tolerated at all. Anilines with electron-donating substituents were preferred over those with electron-withdrawing groups. Though the mechanism was not explicitly investigated here, this suggests amine nucleophilicity and therefore imine formation at least partially contributes to the rate-determining step of the overall transformation.

Table 2. Aryl amine substrate scope for enzymatic reductive amination.[a]



a

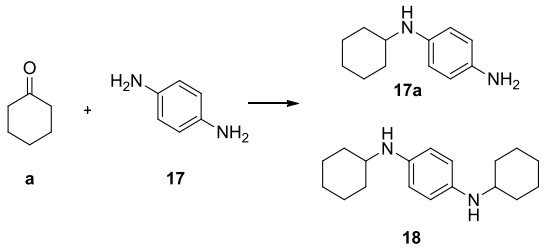
1, R=H	**1a**, R=H
9, R=2-OH	**9a**, R=2-OH
10, R=2-OMe	**10a**, R=2-OMe
11, R=3-OMe	**11a**, R=3-OMe
12, R=3-Me	**12a**, R=3-Me
13, R=4-OH	**13a**, R=4-OH
14, R=4-OMe	**14a**, R=4-OMe
15, R=4-Cl	**15a**, R=4-Cl
16, R=4-NO_2	**16a**, R=4-NO_2

	1a	**9a**	**10a**	**11a**	**12a**	**13a**	**14a**	**15a**	**16a**
IR-01	36	9	4	60	65	52	70	42	<2
IR-10	20	<2	<2	49	53	59	16	<2	<2
IR-13	25	8	<2	46	52	57	68	32	<2
IR-22	4	18	<2	3	4	16	6	<2	<2
IR-24	12	<2	9	53	55	71	69	34	<2
IR-49	3	<2	<2	4	<2	39	13	<2	<2

Percent conversions under same reaction conditions as for Table 1[a], 4h.

The synthetic utility of these IREDs was further demonstrated by exploration of their regioselectivity. Selective monoalkylation of a symmetric diamine is chemically challenging, frequently requires the use of route extending protecting groups, and employs catalytic nitro reduction in the case of aromatic amines.[20] We incubated the top seven IREDs with benzene-1,4-diamine **17** with either 1 or 2 equiv of cyclohexanone **a** and determined the mono:di alkylated product ratio (Table 3). Remarkably, IR-01and IR-10 provided almost complete selectivity for mono-alkylated product **17a** even in the presence of 2 equiv of cyclohexanone **a**. Complementary preference for the disubstituted product **18** was observed for IR-13 and IR-24, even in the presence of 1 equiv of cyclohexanone **a**.

Table 3. Regioselective reductive amination.[a]



a **17** **17a** **18**

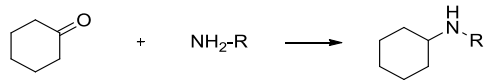
IRED	% conversion of **a**[b]	Product ratio **17a**:**18**
IR-01	88 (97)	100:0 (99:1)
IR-10	78 (88)	99:1 (97:3)
IR-13	81 (98)	27:73 (6:94)
IR-22	32 (43)	88:12 (84:16)
IR-24	76 (89)	21:79 (10:90)
IR-49	71 (96)	94:6 (69:31)

[a]Reaction conditions same as for Table 1[a]. [b]Values shown in parentheses are for reactions run at 2 equiv of cyclohexanone **a**.

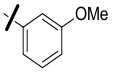
Industrial applicability was examined with a subset of enzymes in 400 mg preparative reactions with cyclohexanone **a** plus a selection of amines (Table 4 and Chart S1). Reaction at neutral pH with 1 equiv of amine gave isolated yields in the range of 20-50% from unoptimized reaction and isolation conditions, with automated purification emphasizing purity over yield. We also demonstrated successful preparative scale reactions using stoichiometric reactants for several other carbonyl and amine combinations (Chart S1).

These results demonstrate that IRED catalyzed reductive amination at low stoichiometric excess is far more common than previously reported. Aleku *et al*.[9] described six key residues related to reductive aminase activity, which are fully conserved in only three of the 85 IREDs reported in this work. This includes the previously reported fungal enzymes AspRedAm[9] (IR-72) and AdRedAm[9] (IR-59), and bacterially derived IR-69 from *Streptomyces* sp. PRh5. None of these were in the top seven performing enzymes, but did give activity for at least one carbonyl-amine combination.

Table 4. Regioselective reductive amination.[a]



a

R	IRED	Product	Isolated yield [b] (%)
	IR-49	**1a**	37
	IR-22	**7a**	23
	IR-10	**11a**	46
	IR-13	12a	51

[a]Reaction conditions: ketone/aldehyde (11 mM), amine (11 mM), GDH (0.08 mg/mL w/w), glucose (22 mM), $NADP^+$ (0.5 mM), 100 mM potassium phosphate buffer pH 7, 30 °C, final volume 400 mL, 4h. [b]Isolated yields after mass directed auto purification (MDAP).

We have shown extended IRED amine substrate scope to include a range of anilines and heteroaromatic amines, which were previously reported as incompatible with biocatalytic reductive amination.[9] Importantly, these results were obtained with cell lysates without significant side reactions, allowing for economical scale up. The recently reported work with whole cells expressing IREDs also has large scale potential, but the biocatalyst loading was ~10-30x higher than in this study and appreciable ketone reduction was observed.[10] Finally, we demonstrated that this panel of enzymes can perform regio- and stereoselective transformations in an efficient manner, even at low amine stoichiometry. With selectivity, high activity, crude preparation, low loadings, and low substrate stoichiometry demonstrated, members of this collection of enzymes exhibit all the characteristics of a potential industrial biocatalyst. These enzymes are actively being used at GSK for project-direct applications.

Acknowledgements

This research was supported by the Innovative Medicines Initiative (IMI) joint undertaking project CHEM21 under grant agreement no. 115360, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies in kind contribution.

Keywords: Imine reductases • reductive amination • chiral amines • biocatalysis • chiral synthesis

[1] D. Ghislieri, N. J. Turner, *Top. Catal.* **2013**, *57*, 284-300.

[2] *Chiral Amine Synthesis: Methods, Developments and Applications*, (Ed T.C. Nugent), Wiley-VCH, Weinheim, **2010**.

[3] a) S. A. Lawrence, *Amines: synthesis, properties and applications*, Cambridge University Press, **2004**; b) *Stereoselective Formation of Amines, Vol. 343*, (Eds.: W. Li, X. Zhang) Springer, Berlin, Heidelberg, **2014**.

[4] a) J.-M. Choi, S.-S. Han, H.-S. Kim, *Biotechnol. Adv.* **2015**, *33*, 1443-1454; b) G. W. Huisman, S. J. Collier, *Curr. Opin. Chem. Biol.* **2013**, *17*, 284-292; c) R. Martínez, U. Schwaneberg, *Biological research* **2013**, *46*, 395-405; d) N. J. Turner, *Catalysis Science & Technology* **2012**, *2*, 1523-1523.

[5] a) M. J. Abrahamson, E. Vázquez-Figueroa, N. B. Woodall, J. C. Moore, A. S. Bommarius, *Angew. Chem. Int. Ed.* **2012**, *51*, 3969-3972; b) M. M. Heberling, B. Wu, S. Bartsch, D. B. Janssen, *Curr. Opin. Chem. Biol.* **2013**, *17*, 250-260.

[6] a) J. H. Schrittwieser, S. Velikogne, W. Kroutil, *Adv. Synth. Catal.* **2015**, *357*, 1655-1685; b) G. Grogan, N. J. Turner, *Chem. Eur. J.* **2016**, *22*, 1900-1907.

[7] a) K. Mitsukura, M. Suzuki, K. Tada, T. Yoshida, T. Nagasawa, *Org Biomol Chem* **2010**, *8*, 4533-4535; b) K. Mitsukura, M. Suzuki, S. Shinoda, T. Kuramoto, T. Yoshida, T. Nagasawa, *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1778-1782; c) M. Rodriguez-Mata, A. Frank, E. Wells F. Leipold, N. J. Turner, S. Hart, J. P. Turkenburg, G. Grogan, *ChemBioChem* **2013**, *14*, 1372-1379; d) M. Gand, H. Müller, R. Wardenga, M. Höhne, *J. Mol. Catal. B: Enzym.* **2014**, *110*, 126-132; e) P. N. Scheller, S. Fademrecht, S. Hofelzer, J. Pleiss, F. Leipold, N. J. Turner, B. M. Nestl, B. Hauer, *ChemBioChem* **2014**, *15*, 2201-2204.

[8] a) T. Huber, L. Schneider, A. Präg, S. Gerhardt, O. Einsle, M. Müller, *ChemCatChem* **2014**, *6*, 2248-2252; b) P. N. Scheller, M. Lenz, S. C. Hammer, B. Hauer, B. M. Nestl, *ChemCatChem* **2015**, *7*, 3239-3242; c) D. Wetzl, M. Gand, A. Ross, H. Müller, P. Matzel, S. P. Hanlon, M. Müller, B. Wirz, M. Höhne, H. Iding, *ChemCatChem* **2016**, *8*, 2023-2026; d) P. Matzel, M. Gand, M. Höhne, *Green Chem.* **2017**, *19*, 385-389.

[9] G. A. Aleku, S. P. France, H. Man, J. Mangas-Sanchez, S. L. Montgomery, M. Sharma, F. Leipold, S. Hussain, G. Grogan, N. J. Turner, *Nat Chem* **2017**, doi:10.1038/nchem.2782.

[10] Z. Maugeri, D. Rother, *J. Biotechnol.* **2017**. doi.org/10.1016/j.jbiotec.2017.05.015.

[11] a) S. Hussain, F. Leipold, H. Man, E. Wells, S. P. France, K. R. Mulholland, G. Grogan, N. J. Turner, *ChemCatChem* **2015**, *7*, 579-583; b) G. A. Aleku, H. Man, S. P. France, F. Leipold, S. Hussain, L. Toca-Gonzalez, R. Marchington, S. Hart, J. P. Turkenburg, G. Grogan, N. J. Turner, *ACS Catalysis* **2016**, *6*, 3880-3889.

[12] S. Fademrecht, P. N. Scheller, B. M. Nestl, B. Hauer, J. Pleiss, *Proteins* **2016**, *84*, 600-610.

[13] a) M. Sittig, *Pharmaceutical Manufacturing Encyclopedia*, William Andrew Pub., **2015**; b) I. Agranat, *Synthesis* **2009**, *2009*, 2116-2116.

[14] K. D. Kreutter, T. Lu, L. Lee, E. C. Giardino, S. Patel, H. Huang, G. Xu, M. Fitzgerald, B. J. Haertlein, V. Mohan, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2865-2870.

[15] F. Giordanetto, O. Karlsson, J. Lindberg, L.-O. Larsson, A. Linusson, E. Evertsson, D. G. Morgan, T. Inghardt, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4232-4241.

[16] N. Fleury-Brégeot, J. Raushel, D. L. Sandrock, S. D. Dreher, G. A. Molander, *Chem. Eur. J.* **2012**, *18*, 9564-9570.

[17] P. N. Scheller, B. M. Nestl, *Appl. Microbiol. Biotechnol.* **2016**, *100*, 10509-10520.

[18] a) D. Wetzl, M. Berrera, N. Sandon, D. Fishlock, M. Ebeling, M. Muller, S. Hanlon, B. Wirz, H. Iding, *Chembiochem* **2015**, *16*, 1749-1756; b) H. Li, Z.-J. Luan, G.-W. Zheng, J.-H. Xu, *Adv. Synth. Catal.* **2015**, *357*, 1692-1696.

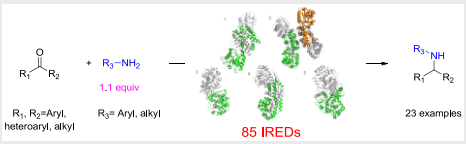
[19] a) Z. Nad, T. Kallay, M. Sziladi, T. Montay, Process for the preparation of propargyl ammonium-chloride. US Patent 5449828A, Sep 12, **1995**.; b) A. Plenevaux, S. L. Dewey, J. S. Fowler, M. Guillaume, A. P. Wolf, *J. Med. Chem.* **1990**, *33*, 2015-2019.

[20] P. G. Wuts, T. W. Greene, *Greene's protective groups in organic synthesis*, John Wiley & Sons, **2006**.

10.1002/cctc.201701379

WILEY-VCH

COMMUNICATION



Biocatalytical reductive amination made easy: A panel of IREDs give broad substrate coverage using stoichiometric reactants with excellent selectivity.

Gheorghe-Doru Roiban,[a] Marcelo Kern,[a] Zhi Liu,[b] Julia Hyslop,[a] Pei Lyn Tey,[a] Matthew S. Levine,[b] Lydia S. Jordan,[b] Kristin K. Brown,[c] Timin Hadi,[b] Leigh Anne F. Ihnken,[b] and Murray J.B. Brown[a]*

Page No. – Page No.

Efficient biocatalytic reductive aminations by extending the imine reductase toolbox