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Title: Photometric characterization of the reductive amination scope of the imine reductases from Streptomyces tsukubaensis and Streptomyces ipomoeae

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Photometric characterization of the reductive amination scope of the imine reductases from *Streptomyces tsukubaensis* and *Streptomyces ipomoeae*

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Abstract: Imine reductases (IREDs) emerged as promising enzymes for the asymmetric synthesis of secondary and tertiary amines starting from carbonyl substrate. Screening the substrate specificity of the reductive amination reaction is usually performed by time-consuming GC analytics. We found two highly active IREDs in our enzyme collection, IR-20 from *Streptomyces tsukubaensis* and IR-Sip from *Streptomyces ipomoeae* that allowed a comprehensive substrate screening with a photometric NADPH assay. We screened 39 carbonyl substrates combined with 17 amines as nucleophiles. Activity data from 663 combinations provide a clear picture about substrate specificity and capabilities in the reductive amination of these enzymes. Besides aliphatic aldehydes, the IREDs accepted various cyclic (C4-C8) and acyclic ketones, preferentially with methylamine. IR-Sip also accepts a range of primary and secondary amines as nucleophiles. In biocatalytic reactions, IR-Sip converted (*R*)-3-methylcyclohexanone with dimethylamine or pyrrolidine with high diastereoselectivity (>94-96%de). The nucleophile acceptor spectrum depends on the employed carbonyl substrate. Conversion of well-accepted substrates can also be detected when crude lysates are employed as enzyme source.

Enantiopure amines are used as important building blocks and fine chemicals in the pharmaceutical industry.[1] Within the collection of chemical[1a, 2] and enzymatic methods, imine reductase (IRED)-catalyzed reductive amination[1c, 3] emerged as a promising tool to approach secondary amines (Scheme 1). Compared to already established enzymatic routes[1b, 4] to primary amines, e.g. with lipases and transaminases, there are fewer biocatalytic options that give access to secondary and tertiary amines: (i) engineered monoamine oxidase variants[1b] allow the deracemization of amine racemates, and Pictet-Spenglerases give an entry into the indol and benzylisoquinoline alkaloid scaffolds by catalyzing a ring closure reaction. Interestingly, also 1,10-disubstituted (spiro) tetrahydroiso-quinolines bearing a nitrogen substituted quaternary carbon center have been prepared by this route recently.[5] IREDs offer a NAD(P)H-dependent[6] reductive approach: reduction of cyclic imine substrates (referred to as imine reduction) was described first, followed by the discovery of IRED's capability to reduce acyclic imines formed from the respective ketone and amine *in situ* (referred to as reductive amination of ketones).[3]

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

Especially reductive amination has certain advantages: (i) it is a one-step reaction, the starting materials are readily available and the reaction is very modular. The combinations of structurally different ketone and amine substrates result in a manifold of possible products, provided enzymes with the desired substrate specificities are available. Several IREDs were characterized towards their ability for reductive amination recently, and a range of model amines were synthesized with high yields and enantiomeric/diastereomeric excesses.[7] Also pharmaceutically relevant compounds such as (*R*)-rasagiline are accessible, albeit with lower efficiency.[8] Interestingly, IREDs also accept secondary amines as nucleophiles, resulting in the formation of tertiary amines.[8] These new findings highlight the potential of imine reductases for organic synthesis. However, there are the following limitations of IREDs: (i) several enzymes show only moderate enatioselectivities, (ii) a high amino donor excess is necessary, and (iii) the reaction in asymmetric reductive amination relatively slow. This is in contrast to cyclic imine reduction, which is a comparable fast reaction: Substrate specificity, catalytic constants and biochemical properties such as pH- and temperature optimum can be easily determined using photometric NADPH assays.[7b, 9]

Scheme 1. IRED-catalyzed reductive amination of the model ketone cyclohexanone and possible side reactions that might interfere with a photometric assay. The consumption of the cofactor NADPH can be followed photometrically at 340 nm. Whether the imine formation takes place in solution or in the active site of the enzyme is still not known. The following site reactions are possible: a) Reduction of cyclohexanone to the alcohol, b) deamination of substrate amine, c) autooxidation of NADPH, d) deamination of the product amine. All reactions are equilibrium reactions. For reasons of clarity, arrows indicate only the forward reaction.

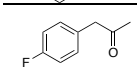
On the contrary, characterization in regard to asymmetric amination is time intensive until now and was performed by GC/HPLC in previous studies. This hinders to

get a clear picture about catalytic capabilities of IREDs. Also the application of protein engineering to improve activity would benefit if a fast photometric screening assay is available. However, we were surprised that we could detect a significant NADPH consumption in a standard photometric activity assay for the reductive methylamination of cyclohexanone (Scheme 1) with few enzymes of the IRED panel. This prompted us to investigate the substrate spectrum of selected IREDs in a systematic manner (during the revision of our manuscript, Aleku et al. published the identification of IRED homologues of fungal origin. These enzymes were also characterized by a photometric screening and revealed to be highly efficient enzymes for the reductive amination).[10]

Our combinatorial screening includes aldehydes, ketones, and keto acids in combination with ammonia, primary and secondary amines as nucleophiles. We choose to investigate the substrate scope of two promising imine reductases from *Streptomyces tsukubaensis* (IR-20)[11] and *Streptomyces ipomoeae* (IR-Sip).[12] These enzymes were identified as efficient IREDs regarding the reductive amination and showed a large substrate scope in our previous experiments. Compared to our earlier unsuccessful attemps to detect reductive amination photometrically,[12] we observed a significant decrease of the absorbance at 340 nm when we incubated purified IR-20 and IR-Sip with NADPH, cyclohexanone, and methylamine, which are the best substrates previously published. This indicates NADPH consumption at a rate of 0.44 µmol $min^{-1}mg^{-1}$ for IR-Sip and 0.55 µmol $min^{-1}mg^{-1}$ for IR-20. However, NADPH consumption does not guarantee that the product is formed in an equivalent amount. Possible side reactions that would simulate IRED activity (Scheme 1) are (i) autooxidation of NADPH by molecular oxygen or oxidation of NADPH catalyzed by enzyme impurities, (ii) IRED-catalyzed reduction of the ketone to the alcohol, and (iii) non-enzymatic side reactions of the substrates (or other trace impurities) leading to a change of the absorbance. To monitor these unwanted processes, we performed three negative controls where (a) enzyme solution, (b) ketone, or (c) amine substrate was replaced by enzyme storage buffer, DMSO, or CHES-buffer (pH 9.5), respectively. We observed a signal intensity of approx. 1-2 % in all controls (see Table S1 and Figure S1 in the Supporting Information for exemplary graphs of the NADPH depletion). This means that activities below 15 mU/mg can hardly be detected. The reaction is reversible: oxidation of the C–N bond leads to deamination via the imine intermediate, leading to NADPH formation. As secondary or tertiary amines contain two or three carbon-nitrogen bonds that might be cleaved by IREDs, deamination could yield additional amine and ketone compounds that differ from the original substrates. This would give rise to different side products (see Scheme S1 in the Supporting Information for an exemplary collection of 18 possible side products that might arise in a single reductive amination reaction, if the enzyme would show a broadly relaxed substrate specificity).

Using an excess of $NADP^+$, neither the oxidation of methylamine nor the conversion of *N*-methylcyclohexane to cyclohexaneamine could be detected (using GC and the purpald assay for formaldehyde detection). The reverse reaction (deamination of *N*-methylcyclohexane to cyclohexanone) was neglectible (< 0.2 % of the reductive amination rate). From these results and previous works showing that reaction rates of IRED-catalysed oxidations are usually much slower compared to reduction, we conclude that deamination should hardly affect the assay.

Table 1. Activities of IR-Sip in the reductive amination of certain carbonyl substrates with selected amines. Activities are shown in mU/mg and were determined by the photometric NADPH assay. Selected combinations were confirmed by GC-MS analytics (conversions of biocatalysis reactions are given in brackets.)

Substrate pair	Specific activity (mU/mg purified IRED)					
	H_2N	N H	N H	N H	N H	N H
	223	40	34	202	24	66
	291	91	93	412	0	103
	308	95 (>40%)	114	322 (>40%)	12	103
	64	0	0	*9*	0	0
	35	0	0	22	0	0
	66	0	0	0	0	0
	163	43	33	162	0	36
	173	*7*	0	41	0	0
	444 (>95%)	16 (34%)	*8*	66 (>95%)	0	*6*
	438 (>95%, 94%de	35 (16%, 96%de	*27*	169 (>95%, 96%de	0	36
	49	0	0	15	0	0
	146 (>80%)	0	0	28	0	0
	35 (72%)	0	0	*8*	0	0

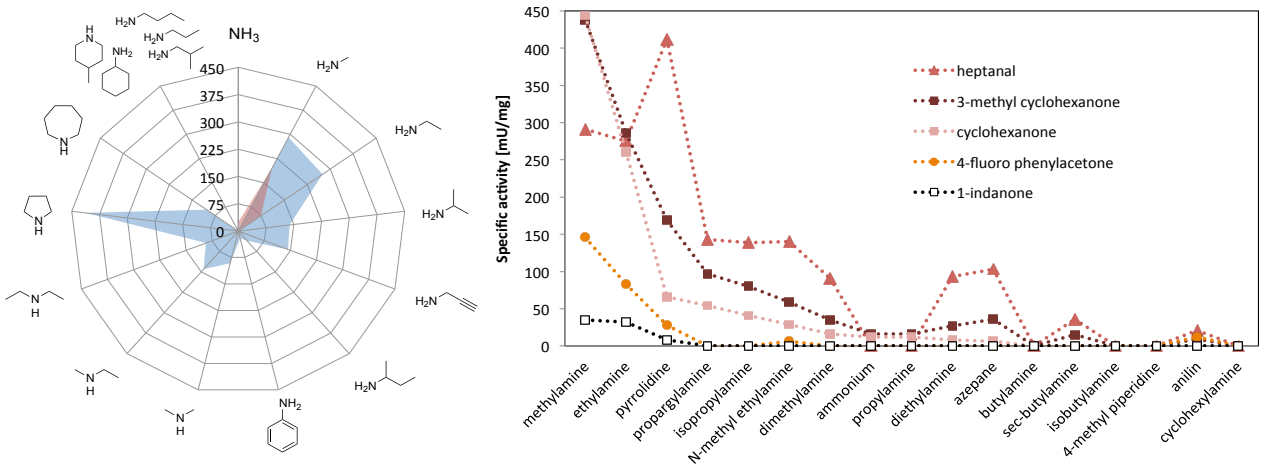


Figure 1. Activity plot for the conversion of amines (ammonia, primary and secondary amines) with heptanal (left) and different keto substrates with IR-Sip (right). Activities are shown in mU/mg and were determined by a photometric screening: 200 µl volume, 200 mM amine, 10 mM heptanal, 0.5 mM NADPH and 0.1-0.15 mg/ml IR-Sip or IR-20. The screeniong was performed for 10 min at 30°C and 340 nm

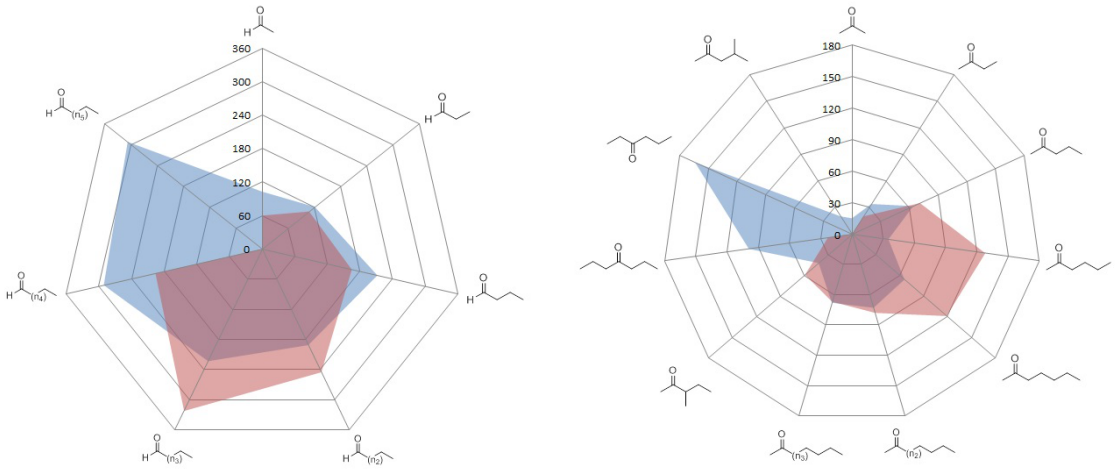


Figure 2. Activity plot for the conversion of aliphatic aldehydes (left) and aliphatic ketones (right) catalyzed by imine reductase IR-Sip (blue) and IR-20 (red) with methylamine as co-substrate. Activities are shown in mU/mg and were determined by photometric measurements.

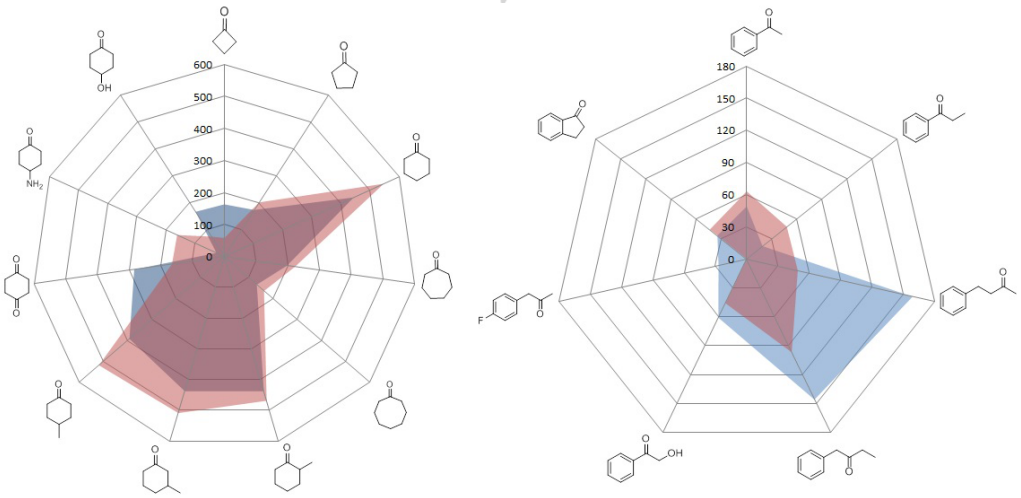


Figure 3. Activity plot for the conversion of cyclic ketones (left) and aromatic ketones (right) catalyzed by imine reductase IR-Sip (blue) and IR-20 (red) with methylamine as co-substrate. Activities are shown in mU/mg and were determined by photometric measurements.

Based on these initial results we extended the photometric assay to a sufficient substrate screening. 46 keto substrates containing aldehydes, aliphatic ketones, cyclic ketones,

aromatic ketones and keto acids were combined with 18 amines, including ammonia, primary and secondary amines. Similar to the reductive methylamination of cyclohexanone, control reactions with most carbonyl substrates were in the range of 10 $mUmg^{-1}$. However, control reactions with seven carbonyl substrates and one amine gave significant absorbance changes. These substrates were thus withdrawn, resulting in 663 substrate combinations. Deamination was only assayed with the substrate amines, as amine standards of most of the product amines were not available.

Table 1 shows activities of exemplary reactions (data of all combinations are shown in the Supporting Information Table S2).

Importantly, the screening results are in accordance with the trends observed in model reactions of previous studies: substrate combinations known to be active from GC-based assays of biocatalysis reaction were also active in our study, and inactive biocatalytic reactions gave no signal in the photometric assay. Furthermore, we confirmed selected activities not described previously by GC-MS analytics (*see* Table 1 and the Supporting Information Figure S2).

From the screened 17 amines, methylamine and ethylamine were by far best accepted by both enzymes. IR-Sip shows a much broader amine nucleophile specificity, also including secondary amines, which are converted to tertiary amines, as shown in Figure 1 (conversions with heptanal as carbonyl substrate). This highlights the more flexible substrate scope of this enzyme compared to IR-20. Besides pyrrolidine, IR-Sip converts the seven-membered cyclic azepane with lower activity, but not 4-methyl piperidine (we could not use piperidine due chemical regulations). The enantioselectivity cannot be accessed in the reductive amination direction of the assay. Therefore we reacted (*R*)-3-methylcyclohexanone with methylamine, dimethylamine and pyrrolidine in analytical scale reactions and obtained the products in 94-96 %de (Table 1). The asymmetric synthesis of secondary amines with IREDs was already established.[7c, 8, 10] Our experiments confirm for the first time that also tertiary amines can be accessed by IREDs by asymmetric reductive amination with high stereo selectivity.

Interestingly, the amino donor specificity is affected by the carbonyl substrate employed (Figure 1), as can be seen by comparing activities with cyclohexanone and heptanal. In the reaction with methylamine, heptanal has a lower (65%) relative activity compared to cyclohexanone (100%). Whereas some nucleophiles are converted less efficient with heptanal, such as ammonia and propylamine, several amines including diethylamine and azepan can only be detected with heptanal as carbonyl substrate, and also other amines, especially pyrrolidine, are converted with a 4-6 fold higher relative rate compared with cyclohexanone. The different activities with aldehydes and ketones likely reflects different reactivities for imine formation.[10]

We used five groups of carbonyl substrates to understand the substrate scope of these enzymes. For the investigated keto acids pyruvate, γ-aminobutyric acid and phenylpyruvic acid, we did not detect conversion. Aliphatic aldehydes and ketones are converted with a similar pattern (Figure 2). We screened shorter aldehydes down to acetaldehyde as well as longer aldehydes up to octanal, and the methyl ketones acetone up to 2- nonanone plus the 'internal' ketones 3-hexanone and 4-heptanone. Both enzymes showed a small divergent pattern, IR-Sip with an activity maximum for the conversion of octanal and IR-20 with hexanal as preferred aldehyde (with methylamine). Similarly, activities were highest for ketone substrates with chain lengths of 6-8 carbon atoms. Aldehydes were more reactive than linear ketones, which might reflect their higher electrophilicity and thus a better imine formation. Also cyclic ketones appeared to be more reactive: ≈550 mU/mg was measured with cyclohexanone, which is the best accepted substrate. Both enzymes showed a very similar pattern: There is no significant influence if a methyl group is attached somewhere to the cyclohexanone ring, but activity drops if a polar substituent (keto, amine or hydroxyl group) is present in 3-position. Activities also decrease if the ring size shrinks to cyclobutanone or increases to cyclooctanone.

Finally, we investigated the conversion of cyclic and aryl(aliphatic) ketones considering the relevance for these compounds as building blocks in fine chemistry. The acceptance of these ketones differ more between the two tested enzymes (Figure 3). IR-Sip showed reasonable activities for 4-fluoro phenyl acetone and 4-phenyl-2-butanone. In contrast to IR-20, just 4-fluoro phenyl acetone was well converted (97 mU/mg). Except of two more bulky substrates, acetophenone derivatives were converted, but with relatively low activity. Based on these findings, we can say that cyclic substrates, secondary cyclic amines and as well cyclic ketones, are favored substrates for reductive amination using IR-20 and IR-Sip. Considering to the probable natural function in synthetic pathways of antibiotics, the preference of cyclic substrates is plausible for the enzymes.

We also employed crude extracts as enzyme source to measure activity of selected enzymes. As anticipated, this results in higher levels of background reactions, and thus, the measurements are not as sensitive. Although the higher measurement error will increase the possibility to detect false positive or negative activities, libraries of IRED variants can be screened if well or moderately accepted substrate pairs are employed, and the enzymes are sufficiently over-expressed (see Fig. S3 in the Supporting Information for a SDS-PAGE gel).

In conclusion, this study gives a comprehensive overview of the substrate specificity in the reductive amination catalyzed by the two IREDs IR-20 and IR-Sip, although enantioselectivity cannot be measured in the reductive amination mode. Besides selected substrate combinations that were investigated previously, we detected activity with a rather broad range of further substrates, ranging from secondary amines to aldehydes and bulky ketones, which can be used as precursor for pharmaceutical relevant amines. We further demonstrated that the photometric assay works when crude extract is employed as enzyme source and moderately or well accepted substrate pairs are screened. This opens the possibility for high-throughput screening in protein engineering studies.

Experimental Section

Enzyme preparation was done as described previously.[7c, 12] Activities were determined by a continuous photometric NADPH assay: in contrast to $NADP^+$, NADPH absorbs light at a wavelength of 340 nm (in the microplate with a path length of 5 mm, a value of ε_{340} = 3.2 mM^{-1} was determined in a calibration experiment. For calculation of enzyme activity, the slope of a control reaction without enzyme was substracted. One U corresponds to a NADPH consumption of 1 µmol/min. The screening was performed under the following reaction conditions: 200 mM amine buffer (the pH of the aqueous amine solution was adjusted with HCl to pH 9.5), 10 mM ketone, 0.5 mM NADH and 0.1-0.15 mg/ml enzyme concentration. For an efficient screening, a master deep-well plate contained every keto substrate as duplicate in DMSO at a concentration of 200 mM. The reaction mixture was prepared on a microtiter plate, 10 µl of each substrate was added and the reaction was followed for 10 min at 30°C after addition of the enzyme. For establishing the assay using purified enzymes and crude extract, 7 replicates were measured and a detection limit of 15 mU/mg was found. For the subsequent in-depth characterization, measurements were performed in duplicates. The Deamination of the high-concentrated substrate amines were assayed separately by adding enzyme solution and $NADP^+$ to the amine buffers and obtained the absorbance at 340 nm. An increase of absorbance, which would indicate an undesirable deamination, was not observed.

For confirming selected activities with GC, 1 ml scale biotransformations were performed with purified enzymes for a reaction time of 20 h under the following conditions: 200 mM amine buffer, pH 9.5, 10 mM carbonyl substrate, 0.5 mg/mL purified IRED, and for recycling of NADPH, the reaction contained 0.1 mg/mL of glucose dehydrogenase (Codexis GDH-105), 60 mM D-glucose, and 0.5 mM NADPH. The reaction was stopped with concentrated sodium hydroxide (end concentration 1.7 M). 240 µl of the solution was extracted twice with 120 µL ethyl acetate before analysis in GC-MS. The GC analytics was performed on a BPX-5 column with following program: 60°C hold for 10 min, 220°C (10°C/min) hold for 5.5 min, 260°C (5°C/min). The inlet pressure and temperature was set to 78.9 kPa and 300°C. Chromatograms are shown in the Supporting Information. For analysing stereoselectivity, we synthesized the racemic product standards according to an established protocol.[13] The ^1H and ^{13}C-NMR spectra and GC-MS analysis confirmed the product identity. Separation of the diastereomers was achieved by GC (please see Fig. S4 in the Supporting Information).

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Keywords: enzyme catalysis, amines, imine reductase reductive amination, photometric assay

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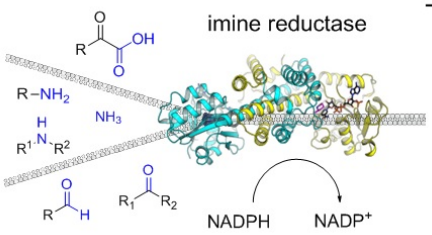

ketones \ amines							
	0	103	77	0	0	21	0
	10	60	*12*	0	0	*7*	*5*
	6	121	172	*6*	0	37	*5*
	13	108	31	0	0	0	0
	0	212	224	0	0	90	0
	8	165	43	0	0	0	0
	17	191	239	17	0	96	16
	42	245	93	*9*	0	*7*	0
	18	223	273	18	*7*	120	19
	40	322	105	24	0	0	*9*
	0	291	275	0	0	139	36
	24	196	75	15	0	0	0

P. Matzel, L. Krautschick, M. Höhne

Page No. – Page No.

Photometric characterization of the reductive amination scope of the imine reductases from Streptomyces tsukubaensis and Streptomyces ipomoeae

Two enzymes, 663 combinations: The substrate scope in the reductive amination catalyzed by two promising IREDs was established in a rapid photometric NADPH assay. Substrates ranging from aldehydes to ketones, and from primary to secondary amines are accepted giving access to various secondary and tertiary amine products.