An (R)-Imine Reductase Biocatalyst for the Asymmetric Reduction of Cyclic Imines

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Although the range of biocatalysts available for the synthesis of enantiomerically pure chiral amines continues to expand, few existing methods provide access to secondary amines. To address this shortcoming, we have over-expressed the gene for an (R)-imine reductase ([R]-IRED) from Streptomyces sp. GF3587 in Escherichia coli to create a recombinant whole-cell biocatalyst for the asymmetric reduction of prochiral imines. The (R)-IRED was screened against a panel of cyclic imines and two iminium ions and was shown to possess high catalytic activity and enantioslectivity. Preparative-scale synthesis of the alkaloid (R)-coniine (90% yield; 99% ee) from the imine precursor was performed on a gram-scale. A homology model of the enzyme active site, based on the structure of a closely related (R)-IRED from Streptomyces kanamyceticus, was constructed and used to identify potential amino acids as targets for mutagenesis.

Chiral amines are key structural motifs widely found in many natural products as well as pharmaceuticals, agrochemicals, and fine chemicals.¹ Methods for their preparation in optically pure form include organo- and transition-metal-catalyzed asymmetric synthesis,² although approaches based on classical resolution through crystallization or kinetic resolution³ often remain the method of choice in industry despite the maximum theoretical yield capped at 50%.⁴ An alternative and attractive strategy for chiral amine synthesis is to employ enzymes, in particular biocatalysts the properties of which have been tailored and enhanced by protein engineering and directed evolution.⁵ Notable developments in this area include the use of monoamine oxidases (MAO-N),⁶ phenylalanine ammonia lyases,⁷ and aminotransferases (transaminases, Scheme 1).⁸

Additionally, an L-amino-acid dehydrogenase has recently been subjected to protein engineering and shown to catalyze the asymmetric reductive amination of ketones (amine dehydrogenases, Scheme 1).⁹ In a number of cases, these biocatalysts have been implemented in large-scale manufacturing processes for the production of active pharmaceutical ingredients as well as their key intermediates.¹⁰

With the exception of MAO-N, which can be used for the chemoenzymatic deracemization of amines with broad structural features, the majority of these biocatalysts generate only primary amines, with no direct access to secondary or tertiary amines. In contrast, the asymmetric reduction of imines provides an alternative method for generating these motifs. Such an approach has been explored by the development of chemo-catalytic processes¹¹ and also the creation of artificial metalloenzymes,¹² which have been successfully used in tandem with biocatalysts in cascade reactions.¹³ However, the enzymatic equivalent of this process remains largely unexplored.¹⁴

Recently, an imine reductase ([S]-IRED) from Streptomyces sp. GF3546 expressed in Escherichia coli¹⁵ was shown to catalyze the enantioselective reduction of a range of different imines, including 2-substituted cyclic imines, dihydroisoquinolines, β-carbolines, and also iminium ions.¹⁶ This ([S]-IRED was found to be exclusively dependent on the cofactor NADPH as a source of hydride. The identification of enantiocomplementary enzymes that provide access to both enantiomers of target products remains a key challenge in biocatalysis, which has been addressed recently by the development of (R)-selective transaminases¹⁷ and an (R)-selective amine oxidase.¹⁸ Herein

Scheme 1. Reactions of enzymes employed in the synthesis of chiral amines, with imine reductase (bottom) representing a recent addition to available biocatalysts.
we report the heterologous expression and characterization of an enantiocomplementary (R)-IRED from *Streptomyces* sp. GF3587, which has been previously described and found to act on 2-methyl-1-pyrroline 1a, giving some indication of substrate preference. In this communication, the (R)-IRED is shown to possess a wide substrate scope and has been applied to the synthesis of the alkaloid conine 4i on a gram scale. Additionally, the imine reductase displayed significantly higher catalytic activity than previously reported IREDS, with a preference for reducing 6-membered ring piperideine substrates, which may provide insight into its natural substrate.

To explore the substrate scope of the (R)-IRED, a panel of cyclic imines was synthesized and screened for activity. With the exception of 1a, all 2-substituted imines were prepared by a previously reported method involving the addition of an organometallic Grignard reagent to an N-Boc-protected lactam (Boc = tert-butoxycarbonyl), followed by deprotection of the amine, which undergoes spontaneous cyclization to give the free-base cyclic imine. This flexible approach allowed access to cyclic imines of varying ring sizes with a range of substituents. The imines were isolated as their hydrochloride salts to enhance their stability as well as water solubility.

Since the only previously reported substrate for the (R)-IRED was 1a, which gave excellent conversion (> 98%) and ee (> 98%), our initial focus was directed towards screening a range of 2-substituted pyrrolines. Biotransformations using resting *E. coli* cells expressing the (R)-IRED were conducted at substrate concentrations of 5 mM with added glucose to aid cofactor recycling (see Supporting Information for the conditions used for cultivation of the recombinant strain and expression of the (R)-IRED). Negative controls using cells harboring the empty plasmid vector were run in parallel to ensure that any observed activity occurred as a result of the IRED biocatalysts.

The (R)-IRED was shown to catalyze the reduction of pyrrolines containing both aliphatic and aromatic substituents at C-2 (Table 1). In addition to the reduction of 1a to (R)-2a (from which the designation as (R)-IRED was derived) the phenyl- and cyclohexyl-substituted pyrrolines 1b and 1e, respectively, were fully converted after 24 h; however, a significant reduction in enantioselectivity was observed with these bulkier substituents. When a para-methoxy substituent was introduced on the benzene ring, conversion fell to 20% but the reaction proceeded with excellent selectivity to give (S)-2c in >98% ee. Under the same conditions, the (R)-IRED gave significantly higher conversions than the previously reported (S)-IRED for pyrroline substrates 1a–d.

To further investigate the substrate scope of the (R)-IRED, whole-cell biotransformations were performed with larger 6- and 7-membered-ring cyclic imine scaffolds. Tetrahydrozepines 3a and 3c were both converted with excellent selectivity. The (R)-IRED was found to display particularly high activity towards 6-membered rings, yielding 2-substituted piperidines, which are important scaffolds in many biologically significant compounds. The high activity towards this class of compound is apparent if comparing the conversions of para-methoxyphenyl-substituted homologues 1c, 3c, and 5c (conversions = 20, 50, and > 98%, respectively). The biocatalytic reduction of a large panel of piperideines 5a–m was performed to fully gauge the effects of varying substituents (Table 2).

Excellent conversion was observed after 24 h with piperideines 5a–l. Reduced conversion of 54% was observed with

| Table 1. Reduction of 5- and 7-membered-ring imines by (R)-IRED. |
|----------------------------------------|
| Substrate | R     | Conversion [%] | ee [%] | Absolute configuration |
| 1a | Me   | > 98   | > 98 | (R) |
| 1b | Ph   | > 98   | 8    | (S) |
| 1c | p-MeOPh | 20    | > 98 | (S) |
| 1d | p-FPh | 86    | 26   | (S) |
| 1e | cyclohexyl | > 98 | 66  | (S) |
| 3a | Me   | > 98   | > 98 | (R) |
| 3c | p-MeOPh | 50    | > 98 | (S) |

[a] Conversion of starting imine measured after 24 h. [b] Absolute configuration determined by comparison with previously reported biotransformations with MAO-N. [c] Configuration assigned by analogy of compound to selectivity observed with 1c and 3c.

| Table 2. Reduction of 6-membered-ring imines by the (R)-IRED. |
|----------------------------------------|
| Substrate | R     | Conversion [%] | ee [%] | Absolute configuration |
| 5a | Me   | > 98   | > 98 | (R) |
| 5b | Ph   | > 98   | 37   | (S) |
| 5c | p-MeOPh | > 98 | > 98 | (S) |
| 5d | p-FPh | > 98   | 91   | (S) |
| 5e | cyclohexyl | > 98 | 66  | (S) |
| 5f | m-MeOPh | > 98 | 89   | (S) |
| 5g | o-MeOPh | > 98 | 77   | (S) |
| 5h | p-tolyl | > 98 | > 98 | (S) |
| 5i | n-propyl | > 98 | > 98 | (R) |
| 5j | isopropenyl | > 98 | 96   | (S) |
| 5k | isopropyl | > 98 | > 98 | (S) |
| 5l | 2-thienyl | > 98 | 78   | (S) |
| 5m | benzyl | 54    | 78   | (S) |
| 5n | 1-naphthyl | 4   | 27   | (S) |
| 7 | H     | > 98   | n/a  | n/a |
| 9a | Me   | > 98   | 71   | (R) |
| 9a | Me   | 24    | 74   | (R) |

[a] Conversion of starting imine measured after 24 h. [b] Configuration assigned by analogy of compound to selectivity observed with 5c, 5f, and 5h. [c] Absolute configuration determined by comparison with previously reported biotransformation with (S)-IRED. [d] Absolute configuration undetermined.
benzyl-substituted derivative 5m and detectable conversion was also observed with the bulky 1-naphthyl-substituted 5n, suggestive of an enzyme possessing a large active site. For substrates bearing phenyl derivatives, the introduction of a substituent on the ring led to an increase in enantioselectivity. Both electron-donating and electron-withdrawing groups were readily accepted, with high conversion and ee observed. Interestingly, a moderate decrease in selectivity was observed if moving the position of the methoxy-substituent from para to meta and ortho, signaling an increasing contribution of a competing binding mode in which the opposite face of the imine is exposed for reduction by NADPH. The (R)-IRED readily accepted other heterocyclic motifs including a thiophene ring (5l).

With simple alkyl and alkenyl substituents, the (R)-IRED showed excellent enantioselectivity. In addition, reduction of α,β-unsaturated imine 5j proceeded exclusively at the carbon–nitrogen double bond with no reduction of the alkenyl observed. Reduction of n-propyl-substituted piperideine 5i resulted in the formation of the more active (R)-enantiomer of the natural product conine in >98% ee. To demonstrate the application of the (R)-IRED biocatalyst for preparative-scale synthesis of chiral amines, the reduction of 5j was performed on a 1.0 g (25 mm) scale, yielding (R)-conine 6i (90% yield, >98% ee), which was isolated as its hydrochloride salt. In addition to simple 2-substituted piperideines, 3,4-dihydroisoquinolines 7 and 7a were also confirmed to be substrates for the enzyme. Significantly, the corresponding N-methyl iminium derivatives 9 and 9a were also reduced, with chiral amine 10a produced with comparable selectivity (74% ee), albeit with reduced conversion, demonstrating the potential application of this biocatalyst for the synthesis of tertiary chiral amines.

Biotransformations of several substrates conducted with the isolated enzyme, coupled with an NADPH cofactor recycling system using glucose dehydrogenase 2 from Bacillus megaterium,[22] showed no significant change in enantioselectivity if compared to their whole-cell equivalents, with the exception of dihydroisoquinoline 7a for which unusually the selectivity appeared to diminish to give (R)-8a in 47% ee (see Supporting Information). As the whole-cell biocatalyst requires only the addition of glucose for cofactor recycling and negates the necessity of using large amounts of NADPH or an additional cofactor recycling system, this approach was used for any preparative biotransformations.

Kinetic parameters were determined for representative substrates (Table 3). Kinetic constants with the previously reported (S)-IRED from Streptomyces sp. GF3546 have been described with substrates 1a, 3a, 5a, 7a, and 9 (i.e., kcat = 0.024 s⁻¹, 0.039 s⁻¹, 0.137 s⁻¹, 0.445 s⁻¹, 0.040 s⁻¹, and 0.483 s⁻¹, respectively).[16] Excluding 7, significantly higher kcat values and lower Michaelis constants (Km) were observed for the (R)-IRED described herein (Table 3). The previously reported substrate 2-methyl-1-pyrrole 1a had a notably lower kcat/Km value (catalytic efficiency) than several piperideine substrates tested. In particular, we observed a greater than sixty-fold increase in kcat/Km for 2-methyl-1-piperideine 5a over pyrrole 1a. This preference for 6- and 7-membered ring systems is shared with the (S)-IRED and may allude to the natural function of this class of enzyme. The (R)-IRED also displayed greater activity towards simple alkyl- and alkenyl-substituted imines compared with those bearing aromatic moieties.

A selection of ketones and oximes were screened for activity with the (R)-IRED, with no conversion detected (see Supporting Information). The absence of activity towards ketones suggests future potential applications of this enzyme for intermolecular reductive aminations, which remains a significant challenge in organic synthesis.

We have recently determined the structure of oxidoreductase Q1EQE0 from Streptomyces kanamyricus,[23] which shares 50% sequence identity with the (R)-IRED, and which also catalyzes the (R)-selective reduction of imine substrates such as 2-methyl-1-pyrrole 1a. The structure of Q1EQE0 revealed a dimeric association in which two monomers associate very closely through domain swapping. The active site, containing NADPH, is a channel that traverses the width of the dimer. The active site also revealed residues which may be responsible for catalysis, including Asp187, which was proposed to be the catalytic residue for protonation of the imine in IRED-mediated catalysis.[22] The similarity between the two sequences gave us confidence to build a model of the (R)-IRED (Figure 1a) using the Phyre2 server.[24] In the model, Asp187 in Q1EQE0 is conserved in the (R)-IRED as Asp172 and many of the other residues within the region of the active site also appear to be conserved between the two enzymes (Figure 1b).

The role of Asp172 in the catalytic mechanism was further probed by generation of the Asp172Ala and Asp172Lys point mutants by site-directed mutagenesis. Interestingly, both mutants retained catalytic activity although conversions were generally lower, for example, for 5c the conversion was reduced from 92% for the wild-type isolated protein to 40% and 72% for the Asp172Ala and Asp172Lys mutants, respectively. Remarkably, reduction of 5a by the mutants showed no change in enantioselectivity whereas for 7a the ee value was increased to 81% for the Asp172Ala variant (Table 4). These initial studies highlight the importance of this residue as a hotspot for mutagenesis to improve conversions and/or enantioselectivity.

In summary, the gene for Streptomyces sp. GF3587 (R)-IRED was overexpressed in E. coli to produce a recombinant whole-cell biocatalyst possessing broad substrate scope, suitable for

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**Table 3. Kinetics data for cyclic imine substrates 1–7.**

| Substrate | R     | Vmax  | kcat  | Km    | kcat/Km |
|----------|-------|-------|-------|-------|---------|
| 1a       | Me    | 0.633 | 0.351 | 1.88  | 0.187   |
| 3a       | Me    | 6.57  | 3.64  | 5.22  | 0.698   |
| 5a       | Me    | 7.60  | 4.21  | 0.371 | 11.4    |
| 5c       | p-MeOPh | 0.474 | 0.263 | 1.05  | 0.250   |
| 5e       | cyclohexyl | 0.0388 | 0.0215 | 1.55 | 0.0139  |
| 5i       | n-propyl | 8.35  | 4.63  | 0.804 | 5.76    |
| 5j       | isopropenyl | 4.24  | 2.35  | 1.77  | 1.33    |
| 5l       | 2-thienyl | 1.16  | 0.643 | 0.244 | 2.64    |
| 7        | H     | 0.255 | 0.141 | 0.317 | 0.447   |
| 7a       | Me    | 0.340 | 0.189 | 0.155 | 2.22    |
| 9        | H     | 1.905 | 1.057 | 0.481 | 2.195   |
Although wild-type IREDs appear to possess relatively broad substrate specificity, further engineering through directed evolution and rational design, using the protein model presented herein, will undoubtedly lead to more active biocatalysts. Moreover, the opportunity to combine IREDs with existing biocatalysts in cascade reactions should result in greater application of these enzymes in asymmetric synthesis.

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Figure 1. a) Homology model of the (R)-IRED, shown in ribbon format, based on the known structure of Q1EQE0,[13] with which it shares 50% sequence identity and 70% similarity. Subunit A is shown in green; subunit B in coral. NADPH is shown bound at the dimer interface in cylinder format, with carbon atoms in grey. b) Detail of active site, illustrating conservation of active residues between Q1EQE0 and (R)-IRED using (R)-IRED numbering. All labeled residues are conserved, except Trp206 (replaced by Phe in Q1EQE0) and Phe217 (Trp232), both shown in red, and Met122 (Leu137) and Ala245 (Thr258), shown in the active-site region close to the NADPH nicotinamide ring. Asp172, which, as Asp187 in Q1EQE0 was suggested to be a catalytic residue,[22] was selected for mutational studies.

| Table 4. Reduction of representative imines by purified (R)-IRED and D172A and D172L mutants. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Substrate       | Wild-type (R)-IRED | D172A (R)-IRED | D172L (R)-IRED |
|                 | Conv.[a] (%) | ee (%) | Conv.[a] (%) | ee (%) | Conv.[a] (%) | ee (%) |
| 1a               | > 98          | 98    | > 98          | 97    | > 98          | 93    |
| 5a               | > 98          | > 98  | > 98          | > 98  | > 98          | > 98  |
| 5c               | 92            | > 98  | 40            | 2     | 72            | 80    |
| 5i               | > 98          | 98    | > 98          | 93    | > 98          | 93    |
| 7a               | 97            | 47    | 41            | 81    | 41            | 68    |
| 9a               | 96            | 76    | 97            | 35    | 97            | 54    |

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