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Authors: Michael Müller, Sebastian Roth, Matthew Kilgore, and Toni Kutchan

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COMMUNICATION

Exploiting Catalytic Diversity of Short-Chain Dehydrogenases/Reductases: Versatile Enzymes from Plants with Extended Imine Substrate Scope

Sebastian Roth,[a] Matthew B. Kilgore,[b] Toni M. Kutchan,[b] and Michael Müller*[a]

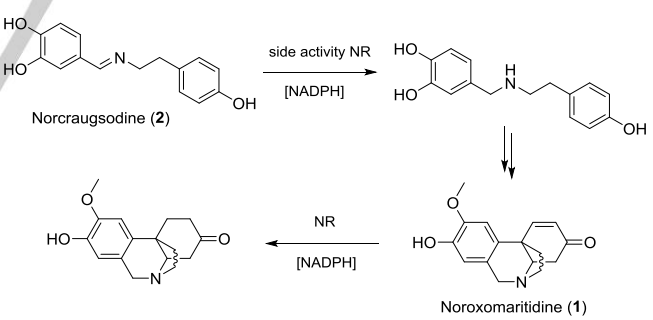
Abstract: Numerous short-chain dehydrogenases/reductases (SDRs) have found biocatalytic applications in C=O and C=C (enone) reduction. For NADPH-dependent C=N reduction, imine reductases (IREDs) have primarily been investigated for extension of the substrate range. Here, we show that SDRs are also suitable for a broad range of imine reductions. The SDR noroxomaritidine reductase (NR) is involved in Amaryllidaceae alkaloid biosynthesis, serving as an enone reductase. We have characterized NR using a set of typical imine substrates and have established that the enzyme is active with all four tested imine compounds (up to 99% conversion, up to 92% *ee*). Remarkably, NR reduced two keto compounds as well, highlighting this enzyme family's versatility. Using NR as a template, we have identified an as yet unexplored SDR from the Amaryllidaceae *Zephyranthes treatiae* with imine-reducing activity (up to 95% *ee*). Our results encourage the future characterization of SDR family members as a means of discovering new imine-reducing enzymes.

The biocatalytic reduction of C=N bonds is of rising importance as an approach to chiral amines.[1] This transformation has almost exclusively been the domain of imine reductases (IREDs), a class of NADPH-dependent oxidoreductases, whose members possess an extended substrate scope.[2] There are only a few reports of other enzymes catalyzing imine reduction; nevertheless, these enzymes usually display strict substrate specificity or their substrate scope has not been investigated in detail.[1] From a mechanistic point of view, one explanation for the particular position of IREDs could be the lower intrinsic reactivity of C=N bonds relative to C=O bonds towards nucleophiles. While IREDs may have evolved a mechanism to overcome this obstacle, details of their catalytic mechanism remain elusive, despite many structural and kinetic studies.[2] Moreover, the ability of IREDs to reduce imine substrates might be a promiscuous activity, as their physiological function is not known.[3] Given the basic mechanism of biocatalytic NAD(P)H-dependent reduction is the transfer of a proton and a hydride regardless of the receiving electrophile,[4] we expect that oxidoreductases other than IREDs would also be applicable for imine reduction.[5] This idea is supported by the recent work of Lenz et al. who optimized β-hydroxy acid dehydrogenases for imine reduction.[6]

For the asymmetric hydrogenation of C=O and C=C bonds, biocatalysis is a beneficial and established tool.[7,8] A pool of oxidoreductases from different enzyme families can be easily screened in search of an appropriate catalyst for a desired transformation. Oxidoreductases that catalyze asymmetric reductions are (sub)classified based on sequence similarity, substrate scope, or cofactor dependency. Members of different enzyme (sub)families can be suited for the same purpose.[9]

Most members of the short-chain dehydrogenase/reductase (SDR) family belong to the EC class 1 (oxidoreductases). Despite low sequence similarity between distinct members, these enzymes display a common scaffold that comprises the Rossmann fold for binding of the cofactor NAD(P)H and facilitates the above-noted proton and hydride transfer.[10] Thus, such enzymes are putatively predestined for (imine) reduction.[11]

In a previous paper, we reported the promiscuous iminium reductase activity of glucose dehydrogenase (GDH).[12] As this enzyme is a prototypical SDR, we searched for further members of this enzyme family that might be suited for imine reduction. Some of us have recently identified noroxomaritidine reductase (NR), an SDR from the plant *Narcissus pseudonarcissus* that is involved in the biosynthesis of Amaryllidaceae alkaloids. NADPH-dependent NR selectively reduces the C=C bond of the enone moiety of noroxomaritidine (**1**). In addition, this SDR exhibits an imine reductase activity with norcraugsodine (**2**), a precursor of **1**, albeit with a 1/400 of its native activity (Scheme 1).[13]



Scheme 1. Involvement of noroxomaritidine reductase (NR) in Amaryllidaceae alkaloid biosynthesis (modified from Kilgore et al.[13]).

Examples of enzymatic reduction promiscuity have been reported for highly reactive iminium[12] and activated keto substrates.[14] NR, however, displays catalytic versatility with non-activated substrates, occurring in vivo.[13] This makes the enzyme an interesting candidate to investigate its intrinsic versatility for the biocatalytic reduction of imines. Here, we outline plant SDRs that can reduce a broad range of imines, thereby expanding the spectrum of imine-reducing enzymes. In addition, these enzymes accept keto substrates and can serve as enone reductases. This demonstrates that one and the same enzyme can possess the ability to catalyze C=N, C=O, and C=C reduction.

[a] S. Roth, Prof. Dr. M. Müller
Institut für Pharmazeutische Wissenschaften
Albert-Ludwigs-Universität Freiburg
Albertstrasse 25, 79104 Freiburg (Germany)
E-mail: michael.mueller@pharmazie.uni-freiburg.de

[b] Dr. M. B. Kilgore, Prof. Dr. T. M. Kutchan
Donald Danforth Plant Science Center
975 N. Warson Rd., St. Louis, MO 63132 (USA)

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The gene encoding NR was cloned in pET28a with an N-terminal His tag. After overexpression in *Escherichia coli* BL21-CodonPlus (DE3)-RP x pL1SL2, the enzyme was purified by Ni-NTA affinity chromatography.[15] To obtain an overview of the substrate range and stereoselectivity of NR-catalyzed reductions, we chose previously described, representative imine compounds: the well-known monocyclic 2-methyl-1-pyrroline (**3**), an isoquinolinium compound (**4**), an exocyclic imine (**5**), and a β-carboline (**6**).[16–19] The two keto substrates **7** and **8** complemented the substrate panel (Figure 1).

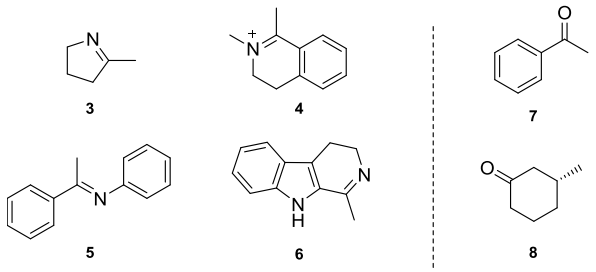

Figure 1. Structures of imines (**3**–**6**) and ketones (**7**, **8**) tested as substrates in this study.

With 5 mol% $NADP^+$, a D-glucose/GDH system was applied for cofactor regeneration. As GDH is active with isoquinolinium compounds and also proved to be active with substrate **8**, reactions containing **4** and **8** were performed with NADPH in an equimolar amount or with malate dehydrogenase/L-malate cofactor regeneration. GC-MS analysis or ^{1}H NMR spectroscopy was used to analyze product formation, which indicated NR was active with the tested imine and iminium compounds **3**–**6**. The conversion, determined by HPLC analysis or ^{1}H NMR spectroscopy, however, differed significantly (Table 1). While the monocyclic (**3**) and exocyclic (**5**) compounds were poorly converted, our data suggest a preference for polycyclic compounds **4** and **6**, which are structurally more similar to the physiological substrate **1**; conversion of **4** (alongside malate dehydrogenase/L-malate) and **6** surpassed 90%.

Table 1. Conversion of substrates **3**–**8** with SDRs and stereochemistry of the resulting reduced products **3a**–**8a** (in parentheses).[a]

	Conversion [%], (enantiomeric or diastereomeric excess [%] and absolute or relative configuration, respectively)					
Enzyme	**3**[b]	**4**[b,d]	**5**[c,e]	**6**[b,e]	**7**[c,e]	**8**[b]
NR	10, –	>99, (92, *R*)	11, –	90, (60, *S*)	17, (81, *S*)	>99, (*cis*/*trans*)
Pb_SDR	n.d.	n.d.	n.d.	n.d.	>99, (>99, *R*)	>99, (>99, *trans*)
Pb_SDR_ R107N	n.d.	n.d.	traces[f]	n.d.	>99, (>99, *R*)	>99, (~45, *trans*)
Ao_SDR	n.d.	n.d.	n.d.	traces[f]	>99, (81, *S*)	>95, (~60, *cis*)
Zt_SDR	14, –	>99, (60, *R*)	18, (95, *S*)	87, (50, *S*)	3, –	46, (*cis*/*trans*)

[a] Reaction conditions: 1 mg mL^{-1} SDR, 10 mM substrate, 20 mM D-glucose, 0.5 mM $NADP^+$, 5 mM $MgCl_2$, 0.25 mg mL^{-1} GDH, 30 °C, 20 h. Reactions with **4** and **8** were performed with NADPH (1 equiv) or contained malate dehydrogenase/L-malate as cofactor regeneration system. [b] Conversions and *de* values of **8a** were determined by ^{1}H NMR spectroscopy. [c] Conversions were determined by HPLC analysis. [d] *ee* values were determined by chiral-phase GC analysis. [e] *ee* values were determined by chiral-phase HPLC analysis. [f] GC-MS analysis. – = not determined due to low conversion; n.d. = no product detected; *cis*/*trans* = no significant excess of one diastereomer.

In the cases of more than 15% conversion, the enantiomeric excess (*ee*) or diastereomeric excess (*de*) of the enzymatic products **3a**–**8a** was determined by chiral-phase GC or HPLC analysis or ^{1}H NMR spectroscopy (Table 1). NR reduced imine **4** with good and **6** with moderate *ee* values, and displayed different stereoselectivity depending on the substrate. Moreover, NR exhibited substantial conversion of ketones **7** and **8**, although with a low to moderate stereocontrol (Table 1).

Encouraged by these results, we explored the catalytic scope of NR homologues. Using the Basic Local Alignment Search Tool (BLAST) with NR as a template, proteins with overall sequence identity of 60–70% were found. The majority of the proteins identified are annotated as tropinone-reductase-like SDRs. Such enzymes typically accept keto compounds as substrates.[20] Although a crystal structure of NR is available,[13] the prediction of crucial amino acid residues for imine reduction remains speculative. Therefore, we decided to take a biosynthesis-guided approach and initially restricted the search to alkaloid-producing organisms. It is known that members of the genera *Pinus* and *Picea* contain alkaloids that might result from imine reduction.[21,22] As we have shown that NR is capable of catalyzing such reactions, we hypothesized that an SDR might be responsible for the assumed, as yet unknown, biosynthetic step(s). In a transcriptome of *Pinus banksiana*,[23] we located a candidate (Pb_SDR, see Supporting Information) with 59.4% sequence identity to NR. A BLAST search using this sequence as a template revealed highly similar sequences from other *Pinus*/*Picea* species for which alkaloids have been reported, followed by the above-noted hypothetical tropinone-reductase-like SDRs. Hence, we chose Pb_SDR as a candidate to be tested for imine reduction. In addition, acetophenone (**7**) and (*R*)-3-methylcyclohexanone (**8**) were tested. Ketone **8** has been identified as a substrate of tropinone-reductase-like SDRs.[20] Based on the protein sequence of Pb_SDR, a synthetic gene fragment was ordered, which was codon-optimized for *E. coli*, and cloned into pET28a via In-Fusion® cloning. After overexpression in *E. coli* BL21-Gold(DE3), the N-terminally His-tagged enzyme was purified by Ni-NTA affinity chromatography. Pb_SDR was active with ketones **7** and **8** (Table 1), which were quantitatively converted. The products were obtained with excellent *ee* [>99% (*R*)-**7a**] and *de* (>99% *trans*-**8a**). Subsequently, we applied Pb_SDR for imine reduction; however, no amine product was detected for substrates **3**–**6**. As an inherent inactivity of Pb_SDR was ruled out, we examined the Pb_SDR sequence. For this purpose, we combined information derived from the NR structure and from literature concerning tropinone reductases[24] and applied it to Pb_SDR, assuming a highly similar SDR fold. In tropinone reductase II from *Datura stramonium*, a histidine residue contributes to charge distribution in the active site. Featured at the equivalent position in Pb_SDR is an arginine residue (R107) whose permanent positive charge might hinder the entrance/orientation of

(protonated) imine compounds in the enzyme's active site. As hypothesized, the rationally designed variant Pb_SDR_R107N showed minimal, but measurable (GC-MS, Figure S6), conversion of **5**, while the ketoreductase capability remained unchanged. This reveals that SDRs bear, in principle, the prerequisites for ketone and imine reduction.

For the choice of the next candidate, we applied the following criteria to scan the BLAST hits: sequences without a glycine residue at the position equivalent to G192 in NR were excluded, as this glycine is conserved in active SDR/tropinone reductases.[20,25] Sequences with a basic residue at the equivalent position to R107 of Pb_SDR were also excluded, as the positive charge seems to hinder imine reductase activity. Moreover, the candidate should feature at least one aromatic residue at the equivalent positions to residues Y114 and F216 in the active site of NR. These residues are presumably involved in substrate binding and orientation, which might be reflected in the preference of NR for the polycyclic substrates **4** and **6**.[13] We identified a sequence from *Asparagus officinalis* (Ao_SDR, see Supporting Information) annotated as a tropinone-reductase-like SDR with a sequence identity of 66% to NR and of 61% to Pb_SDR. Ao_SDR was obtained as a His-tagged protein, analogously to Pb_SDR. Ao_SDR was active with ketones **7** and **8** and, in contrast to wild-type Pb_SDR, showed minimal conversion of compound **6** (Table 1, Figure S9). Hence, despite a relatively elevated sequence similarity of Pb_SDR and Ao_SDR to NR, the activity profiles of the wild-type enzymes differ. Whereas NR accepts both keto and imine compounds, Pb_SDR and Ao_SDR seem to be almost exclusively ketoreductases.

Recently, the transcriptomic data from the 1000 Plants project (1KP) were made publically available,[26–29] thus expanding the data pool for finding new sequences to be tested. To identify putative homologues of NR, the data of the included Amaryllidaceae species were used for a BLAST search that gave access to hits with a sequence identity of up to 88% to NR. Applying the above-noted criteria, a hypothetical SDR from *Zephyranthes treatiae* (Zt_SDR, see Supporting Information) was chosen which displayed 87% overall sequence identity to NR. Relative to NR, Zt_SDR showed slightly higher conversions of the imine substrates **3** and **5** but was less active with the keto compounds **7** and **8** (Table 1). This demonstrates that homologues of NR can possess a comparable activity profile. Prerequisite for this finding was the custom BLAST search on transcriptomic data. Our results indicate that this approach is appropriate for the identification of novel imine-reducing enzymes. In addition, our finding is in line with the recent discovery of an iminium-reducing SDR by plant genome mining.[30]

In summary, we have shown that members of the SDR enzyme family are suitable to be applied as biocatalysts for imine reduction, in addition to their well-established ability to reduce C=O and C=C (enone) bonds. The SDR NR from *N. pseudo-narcissus* is capable of reducing C=N bonds of imine/iminium compounds with different scaffolds. With NR as a template and using transcriptomic data for the BLAST search, we identified the imine-reducing SDR Zt_SDR from the Amaryllidaceae *Z. treatiae*. Thus, we have broadened the spectrum of imine-reducing enzymes, underpinning our serendipitous findings for GDH.[12]

With the SDR family being one of the largest enzyme families, these results suggest that it harbors additional enzymes with an extended imine substrate scope. Moreover, NR and Zt_SDR also accept "common" ketones as substrates. This is remarkable as, to date, promiscuous reductase activity of SDRs with C=N bonds, and vice versa of IREDs with C=O bonds, has only been observed with highly reactive compounds.[12,14] Along with the physiological enone reductase activity of NR, this highlights the pluripotency featured by some SDRs.[31] With regard to the tested substrates, the reductase capability of Pb_SDR and Ao_SDR shifts towards the keto compounds. Nevertheless, the four enzymes share the SDR-typical catalytic triad and should have highly similar overall structures.[10] Hence, the challenging question arises as to which factors lead to the discrimination or the acceptance of keto and imine compounds.

Experimental Section

The biotransformations were performed on an analytical scale (0.5 mL, unless indicated otherwise) and the SDRs were applied as purified enzymes. The substrates **3**–**8** were added from a stock solution (1 M in MeOH), resulting in a 10 mM substrate concentration. Each reaction was started by addition of a mixture (125 μL) containing the other reaction components dissolved in HEPES buffer (pH 7.5, 100 mM). The reaction mixture consisted of purified SDR (1 mg mL^{-1}), substrate (10 mM), $NADP^+$ (0.5 mM), and the cofactor regeneration system: a) GDH (0.25 mg mL^{-1}), D-glucose (20 mM), and $MgCl_2$ (5 mM) or b) malate dehydrogenase (evocatal, 7.5 U), L-malate (50 mM), and $MnCl_2$ (10 μM). After incubation for 20 h at 30 °C and 400 rpm, the reaction was stopped by removing the enzyme (see the Supporting Information). For the analyses of product formation and the determinations of enantiomeric/diastereomeric excesses, see the Supporting Information.

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Keywords: alkaloids • asymmetric reduction • biocatalysis • biosynthesis • imine reduction

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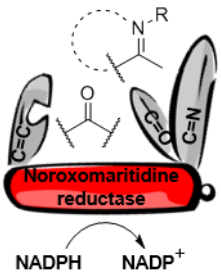
One enzyme, three activities: The plant SDR noroxomaritidine reductase (NR) serves as an enone reductase in alkaloid biosynthesis. Here, we show that NR accepts different imine compounds and features a keto reductase activity. With NR as a template, we have identified a novel imine-reducing SDR.



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