A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201806893
Angew. Chem. 10.1002/ange.201806893

Link to VoR: http://dx.doi.org/10.1002/anie.201806893
http://dx.doi.org/10.1002/ange.201806893

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10.1002/anie.201806893

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Biocatalytic Synthesis of Chiral *N*-Functionalized Amino Acids

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Abstract: *N*-Functionalized amino acids are important building blocks for the preparation of diverse bioactive molecules including peptides. The development of sustainable manufacturing routes to chiral *N*-alkylated amino acids remains a significant challenge in the pharmaceutical and fine chemical industries. Herein we report the discovery of a structurally diverse panel of biocatalysts which catalyze the asymmetric synthesis of *N*-alkyl amino acids via the reductive coupling of ketones and amines. Reactions have been performed on a gram scale to yield optically pure *N*-alkyl functionalized products in high yields.

Chiral non-natural amino acids represent a class of molecules which have gained increased attention within the pharmaceutical and agrochemical industries in recent years. They are key components of many active pharmaceutical ingredients (APIs) and have been incorporated into various biologically active peptides[1] such as cyclosporins,[2] vancomycin,[3] and actinomycins,[4] to generate analogues that can display enhanced bioavailability and metabolic stability. As a result, the development of sustainable and cost-effective methods for the synthesis of functionalized α-amino acid derivatives is highly desirable. Current chemical methods for the synthesis of *N*-functionalized amino acids rely on either *N*-alkylation processes, which often require toxic reagents and hazardous solvents, or reductive amination which utilizes stoichiometric quantities of hazardous hydrides and requires complex work-up procedures which generate significant waste.[5] Numerous asymmetric reductive amination strategies have also been reported[6] including biomimetic routes,[7] however they are limited by their narrow substrate range, the use of heavy metals, and the requirement to pre-form imine intermediates to avoid undesired ketone reduction.[5a] Biocatalytic approaches to chiral amino acid synthesis provide a 'greener alternative' to traditional chemical methods. Ammonia lyases[8] and amino acid dehydrogenases[9] have been successfully employed in the synthesis of a broad range of primary amino acids, however, they are limited to the use of ammonia as a nitrogen source. In recent years, a family of $NAD(P)^+$ dependent oxidoreductases including imine reductases (IREDs),[10] opine dehydrogenases,[11] and reductive aminases,[12] have emerged as useful tools for the asymmetric synthesis of a broad range of amines. Despite the substrate promiscuity of these classes of enzymes, there are few examples of catalysts with activity towards keto acids for the synthesis of *N*-functionalized amino acids.[12, 13]

N-Methylamino acid dehydrogenases (NMAADHs, EC 1.5.1.1 and EC 1.5.1.21) (also known as dPkAs or Δ^1-piperidine-2-carboxylate (Pyr2CR)/ Δ^1-pyrrolidine-2-carboxylate reductases (Pip2CR)),[14] ketimine reductases (KIREDs, EC 1.5.1.25)[15] and Δ^1-pyrroline-5-carboxylate reductases (P5CR, EC 1.5.1.2)[16] are structurally unrelated enzyme classes which catalyze the reduction of cyclic imino acids in Nature (Figure 1).[17]

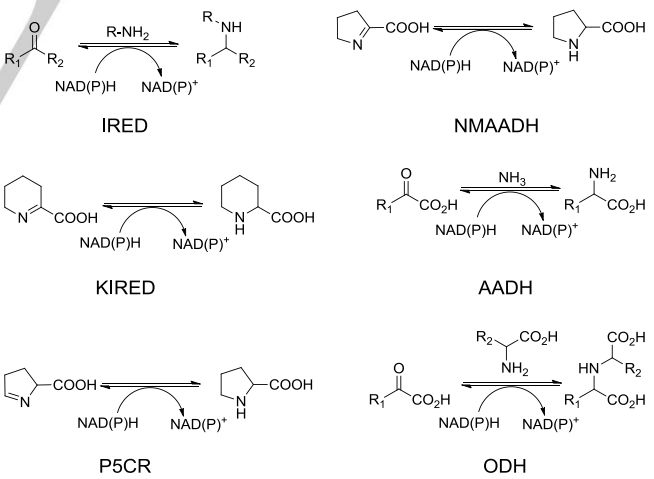

Figure 1. Examples of reductive aminations catalysed by: imine reductases (IREDs), *N*-methylamino acid dehydrogenases (NMAADHs), ketimine reductases (KIREDs), amino acid dehydrogenases (AADHs), fungal Δ^1-pyrroline-5-carboxylate reductases (P5CR), and opine dehydrogenases (ODHs).

NMAADHs have also been reported to promote the intermolecular coupling of α-keto acids with methylamine,[18] however, their widespread use as biocatalysts for the synthesis of enantiopure *N*-methyl amino acids has been limited by their requirement for large excesses of the amine coupling partner

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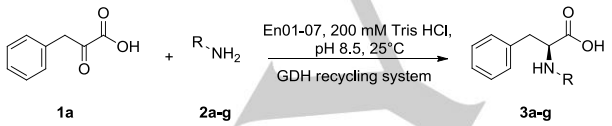
and their narrow substrate specificity. Herein, we have evaluated a panel of diverse NMAADHs KIREDs and P5CRs for the reductive amination of α-keto acids. We have shown that together these enzymes can accept a broad range of both aliphatic and aromatic amines and we demonstrate their synthetic utility in the synthesis of *N*-functionalized amino acids on a preparative scale. Although NMAADHs, KIREDs and P5CRs catalyze similar reactions in Nature, there is low sequence homology between clusters (Figure 2 and Figure S8). While all exist as dimers and contain an NADPH binding domain, they have diverse folding architectures.

We selected seven enzymes (En01-En07) from three distinct clusters for evaluation. En01-En03 (from *Pseudomonas putida,*[14c] *P. syringae*[19] and *P. fluorescens,*[20] respectively) belong to the NMAADH cluster, En04-En06 (from *Rattus norvegicus,*[21] *Homo sapiens,*[15c] and *Bos taurus*[16a] respectively) are KIREDs, and the fungal enzyme En07 (from *Neurospora crassa*[16a]) is from the P5CR superfamily. The selected enzymes are not only structurally diverse but also originate from different species (mammalian, fungal, and bacterial) (See Table S1). En01-07 were overexpressed in *E. coli*, purified and the activity towards the reductive coupling of phenyl pyruvate **1a** and amines **2a-g** was evaluated using HPLC analysis. Interestingly, the different classes of enzymes displayed complementary reactivities and together were able to tolerate a range of primary amine substrates (Table 1). Under optimized reaction conditions (200 mM Tris HCl buffer pH 8.5) using 10 equivalents of methylamine (**2a**), the NMAADHs (En01-En03) proceed with almost complete conversion to the product **3a**. En01-En03 also accept amines **2b-2c** and have good activity towards propargylamine (**2d**) and cyclopropylamine (**2e**). The KIREDs (En04-En06) have the broadest substrate tolerance and are active towards larger amines containing aromatic functionality such as **2f** despite of reduced activity for **2g**. Unlike the NMAADHs and KIREDs, P5CR En07 has a preference for propargylamine (**2d**) over methylamine (**2a**) and the product **3d** is formed with 82% conversion. *n*-Propylamine, *i*-propylamine, dimethylamine, and aniline derivatives were not tolerated by any of the enzymes evaluated. All biotransformations were (*S*)-selective and yielded secondary amine products with excellent *ee* as confirmed by chiral HPLC and comparison to optically

Table 1. Comparison of enzymatic reductive amination activity between phenyl pyruvate (**1a**) with different amines (**2**).[a]



1a 2a-g 3a-g

Entry	Amine	Amine Equiv.	Product	En01		En02		En03		En04		En05		En06		En07	
				conv.[b]	ee[c]	conv.[b]	ee[c]	conv.[b]	ee[c]	conv.[b]	ee[c]	conv.[b]	ee[c]	conv.[b]	ee[c]	conv.[b]	ee[c]
1	**2a**, R=Me	10	**3a**	97%	>99%	98%	>99%	98%	>99%	51%	>99%	81%	>99%	32%	>99%	33%	>99%
2	**2a**, R=Me	1	**3a**	55%	>99%	47%	>99%	50%	>99%	14%	>99%	23%	>99%	10%	>99%	8%	>99%
3	**2b**, R=Et	10	**3b**	16%	>99%	6%	>99%	5%	>99%	5%	>99%	9%	>99%	2%	nd[d]	2%	nd[d]
4	**2b**, R=Et	1	**3b**	2%	nd[d]	1%	nd[d]	1%	nd[d]	-[e]		-[e]		-[e]		--[e]	
5	**2c**, R=Allyl	10	**3c**	12%	>99%	6%	97%	5%	>99%	16%	>99%	30%	>99%	10%	>99%	23 %	>99%
6	**2c**, R=Allyl	1	**3c**	2%	nd[d]	1%	nd[d]	1%	nd[d]	3%	nd[d]	5%	>99%	2%	nd[d]	3%	nd[d]
7	**2d**, R=Propargyl	10	**3d**	92%	>99%	52%	>99%	58%	>99%	19%	>99%	24%	>99%	26%	>99%	82%	>99%
8	**2d**, R=Propargyl	1	**3d**	20%	>99%	9%	>99%	10%	>99%	2%	nd[d]	3%	nd[d]	2%	nd[d]	21%	>99%
9	**2e**, R=Cyclopropyl	10	**3e**	59%	>99%	27%	>99%	29%	>99%	43%	>99%	79%	>99%	29%	>99%	2%	nd[d]
10	**2e,** R=Cyclopropyl	1	**3e**	32%	>99%	11%	>99%	11%	>99%	11%	>99%	23%	>99%	1%	nd[d]	-[e]	
11	**2f**, R=Benzyl	10	**3f**	-[e]		-[e]		-[e]		18%	>99%	17%	>99%	7%	>99%	-[e]	
12	**2f**, R=Benzyl	1	**3f**	-[e]		-[e]		-[e]		7%	>99%	18%	>99%	4%	>99%	-[e]	
13	**2g**, R=CH_2CH_2Ph	10	**3g**	-[e]		-[e]		-[e]		3%	nd[d]	5%	>99%	1%	nd[d]	2%	nd[d]
14	**2g**, R=CH_2CH_2Ph	1	**3g**	-[e]		-[e]		-[e]		3%	nd[d]	3%	nd[d]	2%	nd[d]	1%	nd[d]

[a]Reaction parameters: keto acid **1a** (10 mM), amines **2a-g** (10 mM or 100 mM), NADPH (1 mM), En01-07 (0.1 μM), GDH (1 mg/mL), D-glucose (30 mM), Tris HCl buffer (200 mM, pH 8.5), 25 °C, 24 h. [b] Conversion to the (*S*)-product determined by HPLC. [c]Determined by chiral HPLC and absolute configuration assigned in comparison to racemic and authentic (*S*)-product standards . [d]Not determined due to low conversion. [e]No conversion to the product observed.

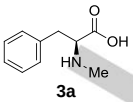
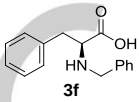
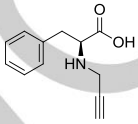
10.1002/anie.201806893

pure standards. Biotransformations performed using equimolar ratios of ketone and amine gave reduced conversions to the amino acid products, due to a combination of increased recovery of keto acid starting materials and low levels of competing enzyme-catalyzed ketone reduction (< 15%). Nevertheless, in favourable cases substantial conversions could be acheived (up to 55%) using stoichiometric substrate ratios, with considerable potential to further optimize these processes through combinations of reaction engineeing and enzyme evolution.

The substrate promiscuity of En01-En07 was further evaluated using α-keto acids 2-oxo-4-phenylbutanoic acid (**1b**) and 3-(4-hydroxy-3-methoxyphenyl)-2-oxopropanoic acid (**1c**), with methylamine (**2a**) and propargylamine (**2d**) as co-substrates (Supporting Information). All biocatalysts tolerated substrates with an increased linker length (**1b**), with product yields comparable to reactions with substrate **1a**. Keto acid **1c** containing substituted aromatic groups was also tolerated by all enzymes screened.

To demonstrate the practical utility of these biocatalysts for the synthesis of *N*-functionalized amino acids, selected reactions were performed on a preparative scale using clarified lysates containing an enzyme from each of the families evaluated. Reactions catalyzed by En01 and En04 resulted in excellent yields of *N*-methylphenylalanine (**3a**) and *N*-benzylphenylalanine (**3f**) respectively. The conversion to **3f** is higher than previously reported using purified En04 (Table 1) and this can be attributed to higher catalyst loading. The En07-catalyzed reaction between **1a** and propargylamine (**2d**) resulted in a modest yield, due to competing ketone reduction catalyzed by endogenous *E. coli* enzymes present in the cell lysate.

Table 2. Preparative scale synthesis of *N*-alkyl amino acids **3a**, **d**, and **f**.[a]

Enzyme	Amount of **1a** (g)/ Amine	Product	Product conv (%)[b] / isolated yield (%)	3-Phenyl lactic acid conv (%)[c]	ee (%) (*S*)
En01	3.0/**2a**	 **3a**	82/80	5	>99
En04	0.2/**2f**	 **3f**	98/9[d]	2	>99
En07	0.2/**2d**	 **3d**	32/21	67	>99

[a]Reaction parameters: Phenyl pyruvate **1a** (10 mM), amine **2a** (700 mM) or **2d**, **2f** (100 mM), En01 (60 mL cell free extract from 7.5 g wet cell pellet), En04, En07 (40 mL cell free extract from 5.0 g wet cell pellet), NADPH (1 mM), GDH (1 mg/mL), D-glucose (20 mM), 25 °C, 24 h. Tris HCl buffer (200 mM, pH 8.5), 27 h for entry 1 and 24 h for entries 2-3. [b]% HPLC conversion to desired product **3**. [c]% HPLC conversion to 3-phenyl lactic acid. [d]Product isolation was performed using preparative LC-MS (mass-directed autopurification system, emphasizing purity over yield).

To gain insights into the origins of reaction selectivities, we performed docking studies with *N*-methyl-L-phenylalanine (**3a**) using available crystal structures of En01, En05 and En07, representing each of the three oxidoreductase families investigated. The models reveal that hydrogen bonding interactions between the substrate/product carboxylate and a conserved active site arginine, position the *Re*-face of the imine intermediate adjacent to the nicotinamide ring of NADPH in a productive conformation for hydride transfer. However in P5CR En07, the carboxylate appears to interact with a threonine (Thr209) and the backbone NH of Thr154. All proteins also contain a conserved serine residue, which is suitably positioned to serve as a proton donor (Figure 2).

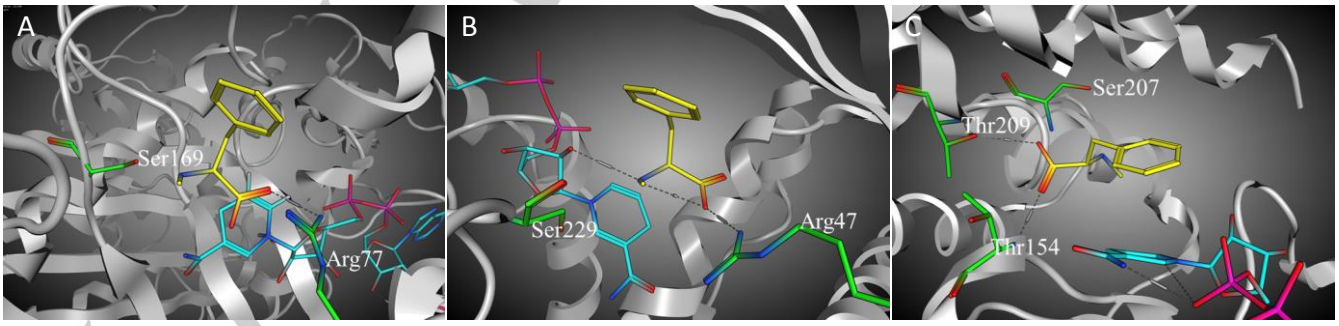


Figure 2. Models of En01 (A), En05 (B) and En07 (C) with the product (*S*)-*N*-methylphenyalalanine, **3a** (yellow) docked into the active site. Models of the enzymes were generated using MOE modeling software and x-ray crystal structures available in the PDB, 2CWH (En01)[22], 4BVA (En05)[23] and 5BSF (En07)[24]. $NADP^+$ is shown in cyan.

In summary, we have identified and evaluated a panel of biocatalysts from three distantly related enzyme classes for the synthesis of *N*-functionalized amino acids. Our study demonstrates the first examples of KIRED and P5CR catalyzed reductive amination reactions, and shows that these enzymes are useful biocatalysts with complimentary substrate specificities to NMAADHs. The synthetic utility of this enzyme panel has been demonstrated through gram-scale biotransformations. Although a ten-fold excess of amine was typically required to achieve high conversions, we have also demonstrated that biotransformations can be performed using 1 equivalent of amine, with significant levels of conversion achieved in favourable cases. It is anticipated that increased conversions and productivities can be achieved through a combination of reaction engineering and laboratory evolution,[25] leading to the development of industrially viable processes. The synthetic utility of this enzyme panel has also been demonstrated on a gram scale. Future work will focus on the acquisition and characterization of further family members as well as further improving the substrate scope and stability of these enzymes via directed evolution.

Acknowledgements

We thank Tomasz Kubowicz for help with LC-MS and chiral UPLC analysis.

Keywords: *N*-methyl amino acid dehydrogenases • ketimine reductases • α-keto amino acids • biocatalysis • reductive amination

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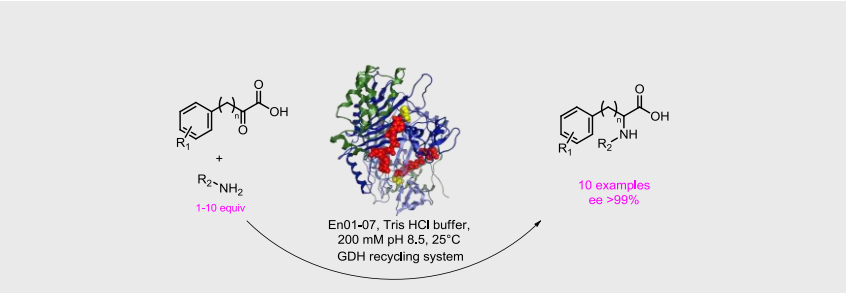
COMMUNICATION



Exploring the reductive amination space: The characterization of a new family of biocatalysts for the asymmetric synthesis of *N*-functionalized amino acids.

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Biocatalytic Synthesis of Chiral *N*-Functionalized Amino Acids