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Identification, Characterization, and Application of Three Enoate Reductases from *Pseudomonas putida* in In Vitro Enzyme Cascade Reactions

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Enoate reductases are versatile enzymes for the enantio- and regioselective addition of hydrogen to double bonds. We identified three EREDs (XenA, XenB, NemA) from *Pseudomonas putida* ATCC 17453 through a sequence motif search. In addition to cloning, functional expression, and biochemical characterization of these enzymes, the enoate reductases were also applied in enzyme cascade reactions in combination with a Baeyer–Villiger monooxygenase and an alcohol dehydrogenase to produce lactones.

Introduction

Enoate reductases (EREDs) are NAD(P)H-dependent oxidoreductases that contain flavin mononucleotide (FMN), which catalyze the stereo- and enantioselective reduction of α,β-unsaturated ketones, aldehydes, nitro alkenes, and carboxylic acids.[1] Christian and Warburg discovered the first ERED in bottom-fermented yeast and gave it the well-known name "yellow enzyme".[2] Later they found another yellow enzyme, and the first enoate reductase was redefined as the "old yellow enzyme" (OYE), which is still used as a term for the ERED enzyme family. Since then various other enzymes from bacteria, yeast, plants, and eukaryotes have been described.[3]

EREDs have been used for the production of (*S*)-citronellal, L-menthol, and semisynthetic opiate pharmaceuticals.[4] Another important application is the bioremediation in the degradation of nitrate esters, nitroglycerine, and other explosive nitro aromatic compounds.[5] This detoxification is also the possible physiological function of EREDs.[3b] Furthermore, their importance in oxidative stress response was demonstrated for the enoate reductases from *Bacillus subtilis* and *Corynebacterium glutamicum*.[6] Additionally, they participate in secondary metabolism such as in the prostaglandin-F2α-syntheses (*Trypanosoma cruzi*) and the aerobic anthranilate metabolism (*Azoarcus evansii*).[7] Plant EREDs from *Oryza sativa* L and *Solanum lycopersicum* are important for the biosynthesis of the hormone jasmonic acid and maybe for the detoxification of oxygen-altered lipids from damaged plant tissue.[8]

The EREDs reported herein originate from *Pseudomonas putida* NCIMB 10007 (= ATCC 17453). Pseudomonades are well known for the microbial degradation of camphor that involves different Baeyer–Villiger monooxygenases (BVMOs) in the pathway.[9] *P. putida* is also known to have multiple OYE homologues.[10] Here we present the recombinant expression, characterization, and comparison of three new EREDs (designated as XenA, XenB, and NemA).[11] Single-step transformations of non-natural substrates by enzymes have been established successfully in the last decades as a highly valuable technology for the synthesis of chiral building blocks.[12] The extension of a single-step biotransformation to cascade reactions is a logical and necessary development. For that reason all three EREDs have been coupled with an alcohol dehydrogenase and a BVMO to enable facile access to more interesting building blocks.

Results and Discussion

Identification of the EREDs

For the identification of new EREDs from *P. putida* NCIMB 10007, nucleotide sequence fragments of this strain (provided by Prof. Littlechild, University of Exeter, UK, see the Experimental Section) were browsed for the occurrence of a sequence motif that had been described to be common among enoate reductases. All contigs of the fragmentary genome sequence were translated into six different respective amino acid sequences (three forward and three reverse). Three different putative EREDs were identified from the motif NXRXDXXGG.[13] One contig encoded the entire nucleotide sequence of *xenA*.

Another contig with a length of 532 base pairs (bp) was found to have homology to the ERED *xenB* from *P. fluorescens*. As the respective contig only contained an intermediate part

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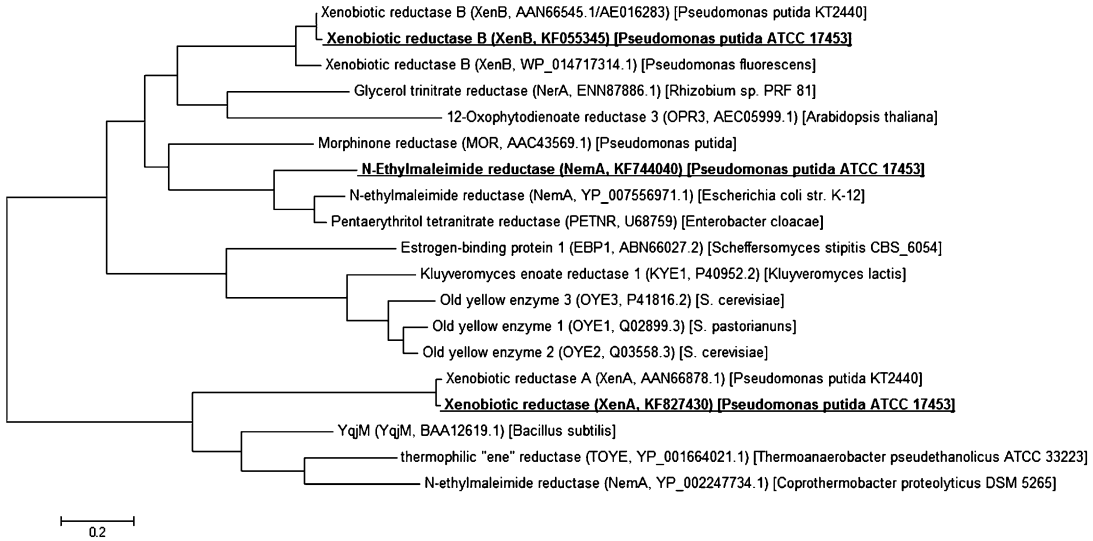


Figure 1. Phylogenetic tree that illustrates the integration of NemA, XenA, and XenB in the OYE family. The tree was constructed by using Clustal OMEGA[15] and edited with Mega 5.2.[16]

of the putative *P. putida xenB* gene, the missing C and N termini were identified by gene walking polymerase chain reaction (PCR) with genomic DNA from *P. putida*.[14] The nucleotides of a third contig exhibited a high homology to the C terminus of a putative *N*-ethylmaleimide reductase and the missing nucleotides were identified by gene walking PCR. The complete genes of *xenA* and *xenB* were amplified by using specific primers with restriction sites and were cloned into the expression vector pGaston. The gene of *nemA* was cloned by *FastCloning* into the expression vector pET22b(+). The obtained constructs have a C-terminal hexahistidine-tag (His-tag) fused to the gene to allow easy protein purification of XenA, XenB, and NemA by affinity chromatography. The phylogenetic relationships between the newly identified enzymes and known EREDs are shown in Figure 1. From this it is clear that the three enzymes belong to different subgroups of the ERED family. NemA from *P. putida* is most closely related to the PETN reductase from *Enterobacter cloacae* with 69.3% identity followed by NemA from *E. coli* with 69.1% identity. XenB is located on a common branch shared with XenB from *P. putida* KT2440[10,1b] with 98.3% identity and with XenB from *P. fluorescens* with 87.4% identity. XenA has high identity (97.5%) to the XenA from *P. putida* KT2440.[17] Both are clustered together with the YqjM from *Bacillus subtilis* (38.4% identity).

Expression and purification of the recombinant EREDs

The utilization of *E. coli* BL21 (DE3) as an expression host yielded primarily soluble XenA and XenB protein after cultivation in Luria Bertani (LB) medium. The theoretical protein mass of the His-tagged XenA (41 kDa) and the His-tagged XenB (40 kDa) was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Soluble NemA was expressed at 25 °C in terrific broth (TB) medium with a clear band at ~40 kDa, which matches the theoretical molecular weight of 39.5 kDa for the His-tagged protein. After the successful expression of soluble proteins, they were purified by affinity chromatography with the subsequent removal of imidazole by size-exclusion chromatography or dialysis to result in pure and active enzymes. All purified enzymes were yellow, which indicates that the cofactor FMN is present.

Characterization of XenB and NemA

As the biochemical characterization of related XenAs from other *P. putida sp.* is well documented in the literature,[17] only XenB and NemA were thoroughly characterized.

Initial tests showed that both enzymes favor NADPH over NADH as a cofactor. Next, the pH profile, temperature optimum, and stability of XenB and NemA were determined with substrate **1** (Figure 2). Both NemA and XenB are active over a broad range of pH values with pH 7.5 (4.3 U mg^{-1}) as the optimum for XenB and pH 6.5 (28.8 U mg^{-1}) as the optimum for NemA (Figure 3). Thus, all following experiments were conducted with sodium phosphate buffer (50 mM) at pH 7.5. The

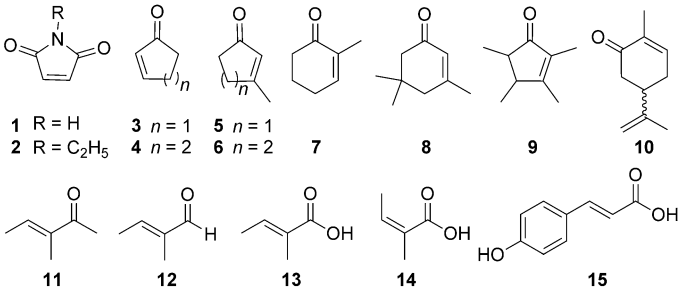


Figure 2. Substrates studied with the EREDs.

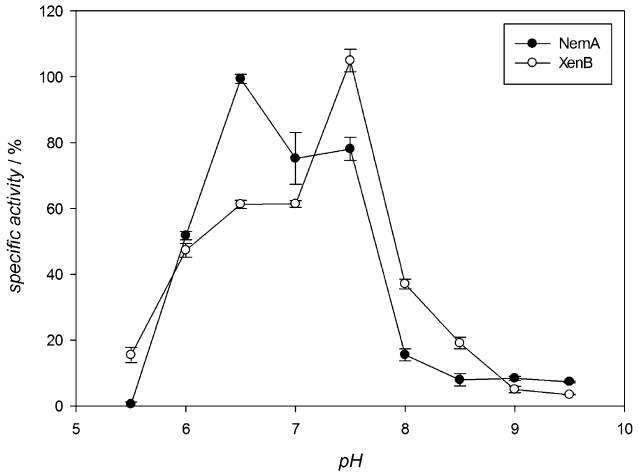


Figure 3. Activity profile of XenB and NemA correlated to pH.

optimum temperature of NemA was 40 °C (Figure S5). However, its stability decreased rapidly >30 °C (Figure S7). XenB shows a temperature activity plateau between 35–60 °C (Figure S6) but it quickly loses activity at temperatures >40 °C (Figure S8). As incubation at 20 or 30 °C for 24 h resulted only in approximately 20% activity loss, all subsequent biocatalysis reactions were performed at 30 °C.

Substrate scope of the EREDs

To obtain a reasonable impression of the substrate scope, a series of structurally diverse aliphatic and cyclic alkenes that bear ketone, aldehyde, carboxylic acid, or cyclic imide functionalities as electron-withdrawing groups were investigated (Figure 2).

All enzymes showed highest activity against the unsaturated imide **1** (Table 1). XenA and XenB revealed a similar substrate scope. Substrates **9** and **13**–**15** were not converted by any of the three enzymes. Substrates with a substitution at the Cα position, such as **7** and **10**, were converted preferably over

Table 1. Conversion of various substrates with purified EREDs from *P. putida* as determined by GC or GC–MS after 1 h.[a]

Substrate	Conversion [%]/*ee* [%] XenA	XenB	NemA
1	58/n.a.	47/n.a.	63/n.a.
2	35/n.a.	33/n.a.	39/n.a.
3	85/n.a.	82/n.a.	61/n.a.
4	92/n.a.	90/n.a.	75/n.a.
5[b]	n.d./n.d.	11/>99	32/>99
6[b]	4/>99 (*S*)	8/>99 (*S*)	26/>99 (*S*)
7	52/>99 (*S*)	46/>99 (*R*)	37/>99 (*R*)
8[b]	3/69 (*S*)	6/33 (*R*)	7/54 (*R*)
(−)-**10**	73/>99 (2*R*,5*R*)	75/>99 (2*R*,5*R*)	n.d./n.d.
(+)-**10**	62/>99 (2*R*,5*S*)	60/>99 (2*R*,5*S*)	n.d./n.d.
11	30/80 (*S*)	53/44 (*R*)	33/42 (*R*)
12	26/n.d.	50/n.d.	63/n.d.

[a] All reactions were performed with 3 mM substrate. [b] $t = 22$ h.

substrates with a methyl group at the Cβ position (**5**, **6**, or **8**). The reason for this phenomenon could be the steric hindrance caused by the methyl group at the Cβ position during the hydrogenation by FMN. The results from Yanto et al. confirm this assumption, as they observed 20% conversion for the Cα-substituted **7** but only 0.4% conversion for Cβ-substituted **6**.[17d] The relatively good activity of XenA towards substrate **10** is contradictory to the results of Chaparro-Riggers et al. who reported no activity of XenA for this compound.[17b]

NemA revealed a high specific activity towards **1** (14.3 U mg^{-1}) and **2** (11.2 U mg^{-1}). Interestingly, NemA from *P. putida* differs in many aspects from the corresponding NemA from *E. coli*. The most important disparity is the different cofactor usage: the enzyme from *E. coli* prefers NADH[18] but also uses NADPH, whereas the *P. putida* NemA takes only NADPH and shows no conversion with NADH. Müller et al. reported also conversions of **5** and **10** by the *E. coli* NemA,[18] whereas the enzyme from *P. putida* shows no activity. These two examples suggest strongly that the automatic annotation (and here also naming) of an enzyme can be misleading as clearly the NemAs substantially differ from each other. For the *E. coli* NemA, Williams and Bruce detected a twofold higher activity[3b] (4.4 U mg^{-1}) against **4** than we could find for the *P. putida* enzyme (2.4 U mg^{-1}). This higher basal activity of the *E. coli* enzyme could be the reason why this enzyme can even convert the Cβ-methylated cyclopentenone **5**.

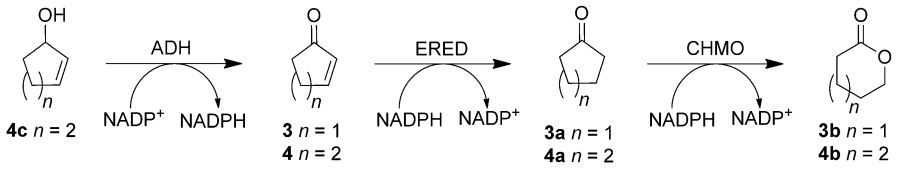
The degrees of conversion for **3** and **4** had substantial similarity to the observations reported by Padhi et al.[19] They reported that the catalytic efficiency for OYE1 generally increases with the substrate size. However, for substrates **5** and **6**, which bear a methyl group at the Cβ position, we could detect a complementary preference for XenA and XenB. In contrast, the conversion rates also increased with NemA for Cβ-methyl-substituted substrates with their size. The biocatalysis of XenA with substrates **4** and **6** resulted in conversions similar to the data reported by Yanto et al.[17d]

Biotransformation with **10** revealed that XenA and XenB only formed the *R* enantiomer from (+)-**10** or (−)-**10** to result in either (+)-(2*R*,5*R*)-dihydrocarvone from (−)-**10** with >99% *de* or (−)-(2*R*,5*S*)-dihydrocarvone from (+)-**10** with >99% *de*. With both enzymes, higher enantioselectivities against (+)-carvone were achieved compared to data reported (86% *de*) by Chen et al.[20] who used LacER from *Lactobacillus casei*. The enantioselectivities for aliphatic ketones such as **11** are low for all three enzymes. XenB produces the *R* enantiomer with 44% *ee* and NemA with 42% *ee*. The complementary *S* enantiomer was produced in 80% *ee* by using XenA. For the methyl-substituted cyclohexenones and cyclopentenones (**5**, **6**, and **7**), all three enzymes show excellent enantioselectivities over 99% *ee*. The presence of a further methyl group as in substrate **8** increased steric hindrance and resulted in lower optical purities (33 and 69% *ee* with XenB and XenA, respectively).

Finally, to demonstrate the applicability of the presented enzymes for the synthesis of high-value compounds, a preparative-scale experiment was performed. *N*-Ethylmaleimide (**2**; 75 mg) was converted completely with freshly purified NemA to the desired *N*-ethylsuccinimide in 73% isolated yield.

Enzyme cascade reactions

Within the community of synthetic chemists, in recent years attention has been focused on the concept of multistep one-pot reactions.[21] Enzymatic cascade reactions that consist of two or more transformations that are performed simultaneously in the same vessel can substantially decrease the amount of chemicals used for each reaction and facilitate downstream processing. Such cascades also offer advantages if unstable or toxic intermediates are involved as these do not accumulate and they improve processes by saving time and reducing waste.[21c] Recently, several examples of enzymes cascades have been published, which include our recent work.[22] Here, we describe a cascade reaction that was developed to convert substrates **3** and **4** to **3b** and **4b** by using the three purified EREDs in combination with the well characterized BVMO cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* (Scheme 1). Purified enzymes were used to obviate any undesired effects caused by *E. coli*'s own enzymes, such as the *E. coli* NemA. Accumulation of the expected lactones was observed with all three EREDs. The cascade with XenA gave 84% conversion to **4b** in 1 h, and the other enzymes gave lower conversions (Table 2).



Scheme 1. Cascade composed of an alcohol dehydrogenase, an ERED, and a CHMO.

Table 2. Conversions achieved in the cascade reactions. Data were determined by GC analysis after 1 h starting from 3 mM substrate concentration.

Substrate	Products	XenA [%]	XenB [%]	NemA [%]
3[a]	**3b**	63	46	19
4[a]	**4b**	84	56	40
4c[b]	**4b**	79	67	99

[a] ERED and CHMO, [b] LK-ADH, ERED, and CHMO.

Next, the cascade was extended to include the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) to convert cyclohexenol (**4c**; Scheme 3) into the lactone **4b**. A conversion of 99% could be achieved, which we also attributed to the immediate conversion of $NADP^+$ from the equilibrium to avoid product inhibition as reported by Bühler and Simon[23] for the EREDs from *Clostridium sp.* La 1 and *Clostridium kluyveri*. In light of this behavior, it was no surprise that the three-enzyme cascade gave very good conversions between 67 and 99% after only 1 h. Thereby the LK-ADH consumed the produced $NADP^+$ so that the inhibitory effect on the EREDs was reduced and better overall conversions and reaction rates were achieved.

Conclusions

Three new enoate reductases from *P. putida* were identified, cloned, and expressed recombinantly. Two of these enzymes were characterized biochemically, and all three were applied in biocatalysis. The enzymes accepted aliphatic and cyclic ketones, aldehydes as well as cyclic imides and carvones. Especially with XenA and XenB, excellent stereoselectivities were obtained in the conversion of chiral carvones. The deviating substrate scope of the newly described enoate reductases compared to candidates established previously broadens the range of possible applications of these enzymes as versatile catalysts in selective hydrogenation chemistry considerably. Moreover, the enzymes were integrated successfully into cascade reactions that involve two or three enzymatic steps to result in moderate to full conversion of unsaturated cyclic ketones or alcohols within only 1 h.

Experimental Section

Chemicals and materials

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany), Merck (Darmstadt, Germany), VWR (Hannover, Germany), or Carl Roth (Karlsruhe, Germany) and were used without further purification unless otherwise specified. Restriction enzymes and polymerases were obtained from New England Biolabs GmbH (NEB, Frankfurt am Main, Germany), T4-Ligase was from Fermentas (St. Leon-Rot, Germany), and primers were ordered from Invitrogen (life technologies GmbH, Darmstadt, Germany). The HisTrap FF and the HiTrap Desalting columns were ordered from GE Healthcare (Uppsala, Sweden).

Bacterial strains, culture conditions, and plasmids

P. putida NCIMB 10007 (equivalent to ATCC 17453) was purchased from the German National Resource Center for Biological Material (DSMZ, Braunschweig, Germany). For cultivation of *P. putida*, LB medium without antibiotics at 26 °C was used. *E. coli* TOP10 [F'laclq, Tn10(TetR) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80 LacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu)7697 galU galK rpsL (StrR) endA1 nupG] was obtained from Invitrogen (Carlsbad, CA, USA). *E. coli* BL21 (DE3) [fhuA2 [lon] ompT gal (λ DE3) [dcm] ⊿hsdS] was purchased from New England Biolabs (Beverly, MA, USA). *E. coli* strains were cultured routinely in LB or TB medium, and if necessary were supplemented with ampicillin (100 μg mL^{-1}) or kanamycin (50 μg mL^{-1}). Bacterial cultures were incubated in baffled Erlenmeyer flasks in orbital shakers (InforsHT Multitron 2 Standard) at 200 rpm at 37 °C. Bacteria on agar plates were incubated in a Heraeus Instruments FunctionLine incubator under air. All materials and biotransformation media were sterilized by autoclaving at 121 °C for 20 min. Aqueous stock solutions were sterilized by filtration through 0.20 μm syringe filters. Agar plates were prepared with LB medium supplemented by 1.5% (w/v) agar. The plasmid pET22 LK-ADH (GenBank: AY267012.1) was provided by Prof.

Hummel (University of Düsseldorf, Germany). The CHMO gene (GenBank: BAA86293.1) was cloned readily into pET28b(+). Transformations of *E. coli* strains with the plasmids were performed with the heat shock method as described by Chung et al.[24]

Genetic methods and sequence analysis

Total genomic DNA from *P. putida* NCIMB 10007 was extracted by using a DNeasy Blood and Tissue Kit from Qiagen (Hilden, Germany). Plasmid isolation (Analytik Jena, Jena, Germany or Fermentas, St. Leon-Roth, Germany), PCR purification, and gel extraction (Qiagen, Hilden, Germany or Roche, Mannheim, Germany) as well as QuikChange (Agilent Technologies, Santa Clara, CA, USA) mutagenesis were performed according to the manufacturers' instructions. The StrataClone PCR Cloning Kit from Agilent Technologies (Santa Clara, CA, USA) was used to subclone DNA fragments. DNA sequencing was conducted by GATC (Konstanz, Germany) or Eurofins MWG Operon (Ebersberg, Germany), and analyses were performed by using the software Geneious.[25]

Gene walking

Starting from sequence fragments obtained from *P. putida* (provided by Prof. Littlechild), a BLAST[26] search was performed to identify full gene lengths. The 3′-sequence of the *xenB* gene was identified by using an oligonucleotide forward primer:

5′-CCA TTG AAG TGT GGG GCG CGA AC-3′

and the 5′-sequence of the *xenB* gene was identified by using two reverse primers:

5′-GTG AAC TGG GCA AGC GTG GCA TTG-3′,

5′-CTG CAA CTG CCC ACC GCA TCA TC-3′.

After initial denaturation for 4 min at 95 °C, the cycling program was followed for 30 cycles: 30 s, 95 °C denaturation; 30 s, 55 °C primer annealing; 3 min, 72 °C elongation; unspecific annealing of the primer: 30 s, 95 °C denaturation; 30 s, 40 °C primer annealing; 3 min, 72 °C elongation; and complementary strand synthesis for 30 cycles: 30 s, 95 °C denaturation; 30 s, 60 °C primer annealing; 3 min, 72 °C elongation. The final elongation step was performed over 15 min at 72 °C. The DNA fragments obtained were subcloned by using the StrataClone PCR Cloning Kit, transformed into *E. coli* TOP10 cells, and colonies were examined for the presence of inserts in a colony PCR with M13 primers:

forward: 5′-TGT AAA ACG ACG GCC AGT-3′,

reverse: 5′-CAG GAA ACA GCT ATG ACC-3′.

After initial denaturation for 10 min at 95 °C, the cycling program was followed for 25 cycles: 30 s, 95 °C denaturation; 30 s, 56 °C primer annealing; 3 min, 72 °C elongation. The final elongation step was performed over 10 min at 72 °C. Plasmids of colonies, which contained DNA fragments, were subsequently isolated and sequenced. The *xenB* gene is 1050 bp long (GenBank code: KF055345). The nucleotide sequence of a third contig showed high homology to the C terminus of a putative *N*-ethylmaleimide reductase. To elucidate the missing N terminus, the forward primers:

5′-CAA TAC GTG CGC GGT TTT CGA CG-3′,

5′-CCT GGT CTT CGC CAT TGT CCA CG-3′

were used following the procedure given above. The GenBank code is KF744040.

Cloning

Cloning of the *nemA* gene into pET22b(+) was performed applying the *FastCloning* method[27] so that the C-terminal His-tag was in frame. Primers for amplification of the gene from genomic DNA:

5′-CGG CCG CAA GCT TAG CCT GCT TCA GG-3′,

5′-CGG AAT TAA TTC GGA TCC ATG AAA CTC TTG CAA CCG C-3′.

Primers for amplification of the plasmid pET22b(+):

5′-GCG GTT GCA AGA GTT TCA TGG ATC CGA ATT AAT TCC G-3′,

5′-CCT GAA GCA GGC TAA GCT TGC GGC CG-3′.

The genes *xenA* and *xenB* were cloned into pGaston by using the restriction enzymes *Nde*I and *Bam*HI by addition of their recognition sites to gene-specific primers for amplification of the genes from isolated genomic DNA:

XenA: 5′-GGA ATT CCA TAT GTC CGC ACT GTT CGA ACC CTA CAC-3′,

5′-CCA ATT GGA TCC GCG ATA GCG CTC AAG CCA GTG C-3′;

XenB: 5′-GGG AAT TCC ATA TGA CCA CGC TTT TCG ATC CG-3′,

5′-CGC GGA TCC CAA CCG CGG GTA ATC GAT GTA-3′.

The gene fragment and the empty vector were digested, ligated, and transformed into *E. coli* TOP10. In these constructs, XenA and XenB contained a C-terminal His-tag to facilitate purification.

Expression

Expression of XenA and XenB in *E. coli* was performed by inoculation of LB media (400 mL) supplemented with ampicillin (100 μg mL^{-1}) with an overnight culture (4 mL; 1:100). This was incubated at 37 °C at 180 rpm until OD_{600} = 0.6–0.8 was reached. Then expression was induced with 0.2% (w/v) L-rhamnose, and incubation was continued at 25 °C (XenB) or 30 °C (XenA) for 8 h. The cultivation of NemA was performed in the same way except for the used medium (TB), the inductor (1 mM IPTG), and the expression conditions (at 25 °C for 4 h). CHMO and LK-ADH were also cultivated in TB supplied with 50 μg mL^{-1} kanamycin (CHMO) or ampicillin (LK-ADH) as described previously.[22] Expression was induced with 0.1 mM IPTG followed by cultivation for 16 h at 25 °C. Cells were harvested by centrifugation at 4500 × g for 20 min at 4 °C.

Enzyme purification

For cell disruption, the cell pellet was resuspended in 25 mL buffer (sodium phosphate buffer, 100 mM, pH 7.5 supplemented with 300 mM NaCl) that included 30 mM imidazole. Cell disruption was performed by a single passage through a French pressure cell at 2000 psi. By centrifugation at 9000 × g for 20 min, cell debris was separated from the crude cell extract. Purification was performed by affinity chromatography by the C-terminal His-Tag with an automated Äkta purifier system. A 5 mL HisTrap FF column was equilibrated with buffer. After the crude cell extract was applied on the column, unbound protein was eluted with five column volumes of buffer supplied with 39 mM of imidazole. The elution of the desired ERED was accomplished by three column volumes of buffer that contained 300 mM imidazole. Washing, flow through, and elution fractions were analyzed by SDS-PAGE. Desired fractions were collected for desalting by dialysis (XenA and XenB) in dialysis tubes

in sodium phosphate buffer (50 mM, pH 7.5) or using the Äkta purifier (NemA and CHMO) to remove the imidazole and to change the buffer. This was performed by using three coupled 5 mL HiTrap desalting columns. After the column was equilibrated with sodium phosphate buffer (50 mM, pH 7.5), the collected enzyme solution was loaded. Protein fractions were recognized by using the Äkta purifier system by online absorption measurement at 280 nm. The protein content of the crude cell extract as well as of the purified and desalted fractions was determined by using the BCA kit. Standard curves were recorded with bovine serum albumin (BSA) at 0.02–2 mg mL^{-1}. Samples were measured in triplicate at suitable dilutions.

Activity measurements

Activity measurements were performed spectrophotometrically by observing NADPH consumption for 120 s at 340 nm in 1 mL cuvettes. The activities of XenA, XenB, and NemA to generate pH, temperature, and stability profiles as well as substrate scope were measured in sodium phosphate buffer (50 mM, pH 7.5) at RT with 1 mM **1** and 0.3 mM NADPH. Pure and desalted enzyme solution (1–20 μL) was used, and the reaction mixture was adjusted to 1 mL with buffer. The pH profile was generated in Davies Buffer.[28] The activities of CHMO and LK-ADH were also defined by the NADPH assay. For the CHMO, 1 mM **4a** and 100 μL enzyme solution were used. In the case of the oxidation of **4c**, 0.3 μM $NADP^+$ was used to determine the activity of LK-ADH (100 μL crude cell extract).

Biocatalysis and GC analysis

For the two- and three-enzyme cascades and biocatalysis, desalted enzyme solutions of XenA, XenB, NemA, CHMO, and crude cell extract of LK-ADH were used. Different values of units were utilized: 1 U of XenA, 3 U of XenB, 10 U of NemA, 3 U of CHMO, and 0.06 U of LK-ADH. The appropriate volume of enzyme solution and 3 mM substrate (in DMF) plus 3 mM NADPH for normal biocatalysis, 6.4 mM NADPH (for the two-enzyme biocatalysis), or 4.8 mM NADPH (for the three-enzyme biocatalysis) were combined in sodium phosphate buffer (50 mM, pH 7.5). Reaction approaches of 1.5 mL in closed GC vials were incubated at 30 °C and 550 rpm. For the extraction protocol and GC analysis of substrates **4**, **6**, **7**, and **10**, see Ref. [22]. GC method 1 was used for substrate/product **1** and **2**. For **3**, method 2, for **11** and **12**, method 3, for **5**, method 4, and for **8**, method 5 was used. Method 1: Macherey–Nagel BPX70 (30 m, 0.25 mm, 0.25 μm); GC program parameters: injector 250 °C, flame ionization detector (FID) 230 °C, pressure 57.5 kPa, 60 °C/hold 5 min; 230 °C/rate 10 °C per min/hold 10 min. Method 2: Macherey–Nagel Hydrodex-β-3p (25 m, 0.25 mm, 0.25 μm); GC program parameters: injector 200 °C, FID 220 °C, pressure 54.7 kPa, 60 °C/hold 5 min; 160 °C/rate 10 °C per min/hold 10 min. Method 3 used the same column as that used in method 2; GC program parameters: injector 220 °C, FID 220 °C, pressure 36.4 kPa, 40 °C/hold 5 min; 120 °C/rate 5 min per °C/hold 1 min; 180 °C/rate 10 °C per min/hold 2 min. Method 4: Macherey–Nagel Hydrodex-β-TBDAc (25 m, 0.25 mm, 0,25 μm); GC–MS program parameters: injector 220 °C, ion source 200 °C, interface temperature 220 °C, pressure 109.2 kPa, 80 °C/hold 20 min; 90 °C/rate 2 °C per min/hold 5.5 min; 180 °C/rate 20 °C per min/hold 5 min. Method 5 used the same column as that used in method 4; GC–MS program parameters: injector 200 °C, ion source 215 °C, interface temperature 215 °C, pressure 82.9 kPa, 95 °C/hold 10 min; 180 °C/rate 20 °C per min/hold 3.75 min.

Preparative biocatalysis

The NemA was purified for the preparative experiment. Compound **2** (75 mg) was added to NemA (≈100 U) in sodium phosphate buffer (30 mL, pH 7.5, 50 mM) supplemented with glucose (4.5 g L^{-1}). Glucose dehydrogenase (GDH; 15 U) and NADPH (1.2 mM) were used for cofactor recycling. The reaction was performed in a 40 mL glass vial at 30 °C and 180 rpm. For GC analysis, a sample (50 μL) was extracted with ethyl acetate (250 μL) supplemented with 1 mM methyl benzoate to serve as an internal standard. After 100% conversion, the solution was extracted five times with dichloromethane. The combined organic layers were dried over Na_2SO_4, and the solvent was removed under vacuum to yield 56 mg (73%) *N*-ethylsuccinimide.

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Keywords: biocatalysis · enantioselectivity · enoate reductase · enzymes · protein expression

[1] a) K. Durchschein, W. M. F. Fabian, P. Macheroux, K. Zangger, G. Trimmel, K. Faber, *Org. Biomol. Chem.* **2011**, *9*, 3364–3369; b) P. van Dillewijn, R.-M. Wittich, A. Caballero, J.-L. Ramos, *Appl. Environ. Microbiol.* **2008**, *74*, 6820–6823.
[2] O. Warburg, W. Christian, *Naturwissenschaften* **1932**, *20*, 688–688.
[3] a) E. Haas, *Biochem. Z.* **1938**, *298*, 369–390; b) R. E. Williams, N. C. Bruce, *Microbiology* **2002**, *148*, 1607–1614.
[4] a) A. Müller, B. Hauer, B. Rosche, *Biotechnol. Bioeng.* **2007**, *98*, 22–29; b) D. H. Craig, P. C. E. Moody, N. C. Bruce, N. S. Scrutton, *Biochemistry* **1998**, *37*, 7598–7607.
[5] a) N. Marmiroli, B. Samotokin, M. Marmiroli, *Advanced Science and Technology for Biological Decontamination of Sites Affected by Chemical and Radiological Nuclear Agents, Vol. 75*, Springer, **2007**; b) K. Durchschein, M. Hall, K. Faber, *Green Chem.* **2013**, *15*, 1764–1772.
[6] a) T. B. Fitzpatrick, N. Amrhein, P. Macheroux, *J. Biol. Chem.* **2003**, *278*, 19891–19897; b) S. Ehira, H. Teramoto, M. Inui, H. Yukawa, *Microbiology* **2010**, *156*, 1335–1341.
[7] a) B. K. Kubata, Z. Kabututu, T. Nozaki, C. J. Munday, S. Fukuzumi, K. Ohkubo, M. Lazarus, T. Maruyama, S. K. Martin, M. Duszenko, Y. Urade, *J. Exp. Med.* **2002**, *196*, 1241–1252; b) K. Schühle, M. Jahn, S. Ghisla, G. Fuchs, *J. Bacteriol.* **2001**, *183*, 5268–5278.
[8] a) A. Schaller, A. Stintzi, *Phytochemistry* **2009**, *70*, 1532–1538; b) H. Sobajima, M. Takeda, M. Sugimori, N. Kobashi, K. Kiribuchi, E. M. Cho, C. Akimoto, T. Yamaguchi, E. Minami, N. Shibuya, F. Schaller, E. W. Weiler, T. Yoshibara, H. Nishida, H. Nojiri, T. Omori, M. Nishiyama, H. Yamane, *Planta* **2003**, *216*, 692–698.
[9] M. Kadow, K. Loschinski, S. Saß, M. Schmidt, U. Bornscheuer, *Appl. Microbiol. Biotechnol.* **2012**, *96*, 419–429.
[10] P. van Dillewijn, R.-M. Wittich, A. Caballero, J.-L. Ramos, *Appl. Environ. Microbiol.* **2008**, *74*, 6703–6708.
[11] The naming of the EREDs as XenA, XenB, and NemA is based solely on the automatic annotation of protein sequences in the database. This does not imply that the enzymes are identical to the previously described XenA, XenB, or NemA from other organisms.
[12] U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185–194.
[13] M. Gall, M. Thomsen, C. Peters, I. V. Pavlidis, P. Jonczyk, P. P. Grünert, S. Beutel, T. Scheper, E. Gross, M. Backes, T. Geißler, J. P. Ley, J. M. Hilmer, G. Krammer, G. J. Palm, W. Hinrichs, U. T. Bornscheuer, *Angew. Chem. Int. Ed.* **2014**, *52*, 1439–1442; *Angew. Chem.* **2014**, *126*, 1463–1466.

[14] M. Pilhofer, A. P. Bauer, M. Schrallhammer, L. Richter, W. Ludwig, K. H. Schleifer, G. Petroni, *Nucleic Acids Res.* **2007**, *35*, e135.
[15] F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson, D. G. Higgins, *Mol. Syst. Biol.* **2011**, *7*, DOI: 10.1038/msb.2011.75.
[16] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, *Mol. Biol. Evol.* **2011**, *28*, 2731 – 2739.
[17] a) D. S. Blehert, B. G. Fox, G. H. Chambliss, *J. Bacteriol.* **1999**, *181*, 6254 – 6263; b) J. F. Chaparro-Riggers, T. A. Rogers, E. Vazquez-Figueroa, K. M. Polizzi, A. S. Bommarius, *Adv. Synth. Catal.* **2007**, *349*, 1521 – 1531; c) J. J. Griese, R. P. Jakob, S. Schwarzinger, H. Dobbek, *J. Mol. Biol.* **2006**, *361*, 140 – 152; d) Y. Yanto, H.-H. Yu, M. Hall, A. S. Bommarius, *Chem. Commun.* **2010**, *46*, 8809 – 8811; e) O. Spiegelhauer, T. Werther, S. Mende, S. H. Knauer, H. Dobbek, *J. Mol. Biol.* **2010**, *403*, 286 – 298.
[18] N. J. Mueller, C. Stueckler, B. Hauer, N. Baudendistel, H. Housden, N. C. Bruce, K. Faber, *Adv. Synth. Catal.* **2010**, *352*, 387 – 394.
[19] S. K. Padhi, D. J. Bougioukou, J. D. Stewart, *J. Am. Chem. Soc.* **2009**, *131*, 3271 – 3280.
[20] X. Chen, X. Gao, Q. Wu, D. Zhu, *Tetrahedron: Asymmetry* **2012**, *23*, 734 – 738.
[21] a) I. Oroz-Guinea, E. García-Junceda, *Curr. Opin. Chem. Biol.* **2013**, *17*, 236 – 249; b) *Multi-Step Enzyme Catalysis* (Ed.: E. Garcia-Junceda), Wiley-VCH, Weinheim, **2008**; c) E. Ricca, B. Brucher, J. H. Schrittwieser, *Adv. Synth. Catal.* **2011**, *353*, 2239 – 2262; d) C. M. Pirie, M. De Mey, K. L. J. Prather, P. K. Ajikumar, *ACS Chem. Biol.* **2013**, *8*, 662 – 672; e) J.-K. Guterl, V. Sieber, *Eng. Life Sci.* **2013**, *13*, 4 – 18.
[22] N. Oberleitner, C. Peters, J. Muschiol, M. Kadow, S. Saß, T. Bayer, P. Schaaf, N. Iqbal, F. Rudroff, M. D. Mihovilovic, U. T. Bornscheuer, *ChemCatChem* **2013**, *5*, 3524 – 3528.
[23] M. Bühler, H. Simon, *Hoppe-Seyler's Z. Physiol. Chem.* **1982**, *363*, 609 – 625.
[24] C. T. Chung, S. L. Niemela, R. H. Miller, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 2172 – 2175.
[25] Geneious version (6.1) created by Biomatters. Available from http://www.geneious.com/.
[26] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, T. Madden, *BMC Bioinf.* **2009**, *10*, 421.
[27] C. Li, A. Wen, B. Shen, J. Lu, Y. Huang, Y. Chang, *BMC Biotechnol.* **2011**, *11*, 92.
[28] M. T. Davies, *Analyst* **1959**, *84*, 248 – 251.

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