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C.V.-V. and A.Z designed and supervised this study. O.M. and S.D. conducted the biocatalysis work. E.D. performed the MS analyses. C.V.-V., A.Z., O.M., S.D. and V.D.B. wrote the manuscript. M.S. contributed to the design of this study. J.-L.P., V.P., A.D. and A.M. performed the steps required for protein production and purification. C.V.-V. and V.D.B. supervised experiments.

Electronic Supplementary Information (ESI) available: protein gels, HPLC chromatogram, NMR analyses See DOI: 10.1039/x0xx00000x

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Asymmetric reductive amination by a wild-type amine dehydrogenase from the thermophilic bacteria *Petrotoga mobilis*

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The biocatalytic reductive amination of ketone to chiral amine is one of the most challenging reaction. Using a genome-mining approach, we found proteins catalyzing the reductive amination of ketones without carboxylic function in α or β position. The synthesis of (*4S*)-4-aminopentanoic acid (*ee* ≥ 99.5%) was achieved with the thermoactive AmDH4 from *Petrotoga mobilis* in 88 % yield. The high stability and substrate tolerance make this AmDH4 a very good starting point for further discovery of reductive amination biocatalysts with enlarged substrate range. This is the first report of wild-type enzymes with related genes having proper NAD(P)H-amine dehydrogenase (AmDH) activity.

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Introduction

Chiral amines are present in many active compounds and are among the most frequently used chemical intermediates for the production of agrochemicals, pharmaceuticals ingredients,[1] polymers and many high value materials. These secondary or tertiary amines come mostly from the simple alkylation and/or amidation of chiral primary amines, making this latter as convenient building blocks adopted by chemists. Various production procedures have been developed, the major route being the kinetic resolution by diastereoisomeric crystallization of racemic amines. Besides, the most two established chemicals methods are the asymmetric addition of carbanions to aldimines generated from aldehyde and the asymmetric hydrogenation of acetamides and imines obtained from ketones, followed by cleavage of the tertiary amines obtained.[2] None of these methods enable the direct access to chiral primary amine from ketone without stepping through a secondary imine or protecting the amine intermediate. Moreover, most processes require expensive and unsustainable transition metal complexes and generate copious amount of waste via protection and deprotection steps. Thanks to significant advances in genomics, molecular biology and bioinformatics, high number of natural or redesign enzymes are now available to the synthetic organic chemist,[3] making biocatalysis an increasingly attractive manufacturing option.[4] At industrial scale, the ω-transaminases,[5] catalyzing the synthesis of chiral amines from pro chiral ketones with a sacrificial amine donor,[6] have become the tools of choice.[7] Enzymatic reductive amination with ammonia of pro chiral ketones into chiral amines is naturally abundant but restricted to the α-keto acid substrates which give chiral α-amino acids by reaction catalyzed by enzymes of the α-amino acid dehydrogenase family.[1, 8] As far as we know, there are very few examples of enzymatic reductive amination by wild-type amine dehydrogenase (AmDH) active towards ketones without carboxylic acid moiety in α or β position. Itoh and coworkers described this activity in *Streptomyces virginiae* on isolated enzyme but with low enantioselectivity,[9] and Wang et al. on whole-cells of *Pseudomonas kilonensis*.[10] But until today, no gene sequence has been identified to encode for these amine dehydrogenases. Facing the importance of this reaction promoted as the holy grail of biocatalysis,[11] hard work has been done to create such amine dehydrogenase by protein engineering. Bommarius and coworkers firstly reported the rational design of amine dehydrogenases[12] (L-AmDH and F-AmDH, respectively from *Bacillus stearothermophilus*[13] and *Bacillus badius*[14]) using α-amino acid dehydrogenase as starting scaffold. These mutants catalyze the reversible reduction of various ketones including hydrophobic ones, through the development of a biphasic aqueous-organic solvent reaction system.[15] Biocatalytic potential of F-AmDH was newly highlighted in a remarkable dual-enzyme hydrogen-borrowing cascade by Turner and coworkers.[16] The same cascade has been described by Xu et al. with a double mutant of leucine dehydrogenase from *Exiguobactertium sibiricum* coupled with a low enantioselective alcohol dehydrogenase from *Streptomyces coelicolor*.[17] Very recently, a phenylalanine dehydrogenase from *Rhodococcus sp.* M4 was engineered into an amine dehydrogenase enabling the highly enantioselective reductive amination of phenylacetone and 4-phenyl-2-butanone.[18] In addition, substantial promises for imine reductase family (IREDs) in this asymmetric reductive amination of ketone with ammonia have also been reported over the last two years[19] as illustrated recently by engineered IREDs.[20]

To match the demand in synthetically useful AmDH, enzymes exhibiting excellent stereoselectivity with related genes are needed. Among established strategies to provide enzymes possessing new or improved properties, exploring protein natural diversity is one of the most attractive.[21] Moreover, having in hands wild-type AmDHs should expend the synthetic scope of amine dehydrogenases and furnish new scaffolds for semi-rational protein engineering.

Experimental

Chemicals, equipment

All reagents were purchased from commercial sources and used without additional purification. 4-aminopentan-2-one, pentan-2-one, 4-oxopentanoic acid, hexan-2 -one, 5-oxohexanoic acid, glucose, glucose-6-phosphate, (*S*)-2-(5-fluoro-2,4-dinitrophenylamino)propanamide (FDAA), NADH, NADPH were purchased from Sigma-Aldrich. 1-fluoro-2,4-dinitrobenzene (DNFB) was purchased from Acros Organic, racemic 4-aminopentanoic acid rac-**9** and 5-aminohexanoic acid rac-**10** from Enamine. Recycling enzyme glucose-6-dehydrogenase (G6PDH) G5885 from *Leuconostoc mesenteroides* and G7877 from *S. cerevisiae*, and formate dehydrogenase (FDH) from *Candida boidinii* were purchased from Sigma-Aldrich. A sample of phosphate dehydrogenase (PTDH) was gratefully given by Pr. Marco Fraaije (University of Groningen). 1g of glucose dehydrogenase (GDH) GDH105 and CDX901 were given by Codexis®. NMR spectra were recorded on a Bruker 300 MHz spectrometer (Evry University, France). Chemical shifts (expressed in ppm) of ^{1}H and ^{13}C NMR spectra were referenced to the solvent peaks δ(H)=3.34 and δ(C)=49.9

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for MeOD-d*4*, δ(H)=4.79 for D_2O. Thin-layer chromatography (TLC) was performed with aluminium-backed sheets with silica gel 60 F254 (Merck). Column chromatography was performed on a CombiFlash® Companion using Redised® Rf cartridges. UHPLC analyses were performed on a UHPLC U3000 RS 1034 bar (Thermo Fisher Scientific, Waltham, USA) equipped with diode array detector DAD3000. HPLC-MS analyses were performed on a U3000 RS 1034 bar (Thermo Fisher Scientific) coupled to a Hybrid triple quadrupole-linear ion trap mass-spectrometer (QTRAP 5500 from ABSciex, Toronto, Canada). Ion exchange column purification was performed on a Dowex®50WX8-200 mesh purchased from Sigma Aldrich. Polarimetry measurements were done at 589 nm on a Unipol L Polarimeter (Schmidt + Haensch) equipped with a glass tube 50 mm.

Enzymes preparation

Choice of candidate enzymes. The collection of candidate enzymes was built up as previously described,[22, 23] using the protein sequence of (*2R*,*4S*)-2,4-diaminopentanoate dehydrogenase (2,4-DAPDH) from *Clostridium sticklandii* (Uniprot entry: E3PY99, related gene *ord*).

Cloning and candidate enzyme production. All steps from primers purchase to cell lysate preparations were carried out as previously described.[23] 20 enzymes were obtained in a 96-microwell plate as cell free extracts.

Purification of enzymes. The enzymes were purified by loading the cell free extract (8 mL) onto a Ni-NTA column (QIAGEN), according to the manufacturer's instructions. Elution buffer was 50 mM Tris pH 7.5, 50 mM NaCl, 250 mM imidazole and 10 % glycerol. The positive fractions were desalted using Amicon ultracel-10K (Merck Millipore) with buffer containing 50 mM phosphate pH 7.5, 50 mM NaCl and 10 % glycerol to obtain purified enzymes at 1.7 to 5 mg/ml (yield: 0.6 to 2 % from crude extract). Large scale purification of AmDH4 was done by heat treatment: cell free extracts (35 mL) were heated at 70°C for 20 min. After centrifugation (12000 rpm, 4°C, 20 min), the supernatant containing the purified enzyme was taken. Protein concentrations were determined by the Bradford method: no significant loss of material (yield: 42%) and activity was observed. The samples were analyzed by SDS-Page using the Invitrogen Nupage system (See Figure S1 and S2). The purified proteins were stored at -80 °C.

HPLC conditions

HPLC-MS analyses. HPLC-MS analyses were conducted on a Zic-pHilic (Merck) column (100 x 4.6 mm; 5 µm) with the following conditions : mixture of $(NH_4)_2CO_3$ 10 mM / MeCN as eluent with a linear gradient (ratio 20/80 to 60/40 in 10 min, then 60/40 during 6 min); flow 0.5 mL.min^{-1}; temperature 40°C; injection volume 10 µL. MS parameters: ion source 5500V, curtain gas 20 a.u., temperature 450 °C, gas1 45 a. u., gas2 60 a.u., CAD – medium; MRM (multiple reaction monitoring) scan type using multiple ion transitions in positive mode (rac-9: 117.9→101.0; 117,9→83.1; rac-10: 132.0→114.8; 132.0→96.9; 132.0→69.0).

UHPLC-UV analyses. UHPLC analyses were performed using a Kinetex® F5 (Phenomenex) column (100 x 2.1 mm; 1.7 µm). Conditions A: mixture of MeCN/H_2O + 0.1% formic acid as eluent with a linear gradient (ratio 30/70 during 2 min, then 30/70 to 80/20 in 2.5 min, then 80/20 during 0.5 min); flow 0.5 mL.min^{-1}; temperature 25 °C; injection volume 1 µL; UV detection at λ = 360 nm. Conditions B: mixture of MeCN/H_2O + 0.1% formic acid as eluent with a linear gradient (ratio 10/90 during 2 min, then 10/90 to 50/50 in 5 min, then 50/50 to 100/0 during 2 min); flow 0.5 mL.min^{-1}; temperature 25 °C; injection volume 1 µL; UV detection at λ = 340 nm.

Library selection for activity

Enzymatic screening assay. All the reactions were conducted at 25°C in 96-microwell plates. Amination reactions: to a reaction mixture (100 µL) containing 46 mM of ketone substrate, 0.5 mM of NADH and 0.5 mM of NADPH in 225 mM NH_4Cl/NH_4OH buffer (pH 9.8) was added 30 µL of cell free extract. Deamination reactions: to a reaction mixture (100 µL) containing 46 mM of ketone substrate, 0.5 mM of NAD and 0.5 mM of NADP in 100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 9.8) was added 30 µL of cell free extract. Absorbance at 340 nm was measured immediately and monitored for 4 h. A background plate was established in the same manner but with a mixture lacking ketone (amine in case of deamination reaction) substrate. Active enzyme corresponds to a well exhibiting a higher slope in the reaction well over the background well.

HPLC-MS activity confirmation. To a reaction mixture (50 µL) containing 10 mM of ketone substrate, 4 mM of NADH and 4 mM of NADPH in 225 mM NH_4Cl/NH_4OH buffer (pH 9.8) was added 7.5 µL of cell free extract. The reaction mixture was stirred at 30°C (500 rpm). After the specified period of time (2h or 3 days), an aliquot (4 µL) was diluted in 396 µL of HPLC mobile phase (2/8 $(NH_4)_2CO_3$ 10 mM / MeCN), filtered on 0.22 µm, and analyzed by HPLC-MS following conditions detailed above. A background reaction was performed in the same manner but with a mixture lacking the cell free extract. MS-calibration curves based on MRM responses were established with commercially available racemic amines rac-**9** and rac-**10**.

Substrates synthesis

Synthesis of (*2R*)-4-oxopentanoïc acid (2). (*2R*)-4-oxopentanoïc acid **2** was obtained by hydrogenation followed by water free acidic treatment of benzyl 2-((*tert*-butoxycarbonyl)amino)-4-oxopentanoate synthesized by Alfa Chemica.

Synthesis of 2-amino-5-oxohexanoic acid (rac-3). To a solution of *tert*-butyl 2-((diphenylmethylene)amino)acetate (1.63 mmol, 1.1 equiv., 482 mg) in dried THF (8 mL) was added but-3-en-2-one (1.48 mmol, 1 equiv., 120 µL) followed by cesium carbonate (1.48 mmol, 1 equiv., 482 mg). The mixture was stirred under inert atmosphere for 16 h at room temperature

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and then concentrated under reduced pressure to remove THF. The residue was then taken up in a mixture of water and ethyl acetate and extracted with ethyl acetate (2 x 20 mL). The combined organic layers were washed with brine (10 mL), dried over magnesium sulfate, filtered and concentrated to dryness under reduced pressure. Purification of the product by flash chromatography on silica gel (isocratic mode: petroleum ether/ethyl acetate 90/10) afforded the desired keto ester as a colorless oil; yield: 500 mg (92 %). The ester (0.24 mmol, 1 equiv., 86 mg) was treated with 4M hydrochloric acid in dioxane (200 µL) at room temperature and concentrated to dryness under reduced pressure. After trituration (x 3) with dichloromethane, the resulting solid was dried under reduced pressure to afford the desired 2-amino-5-oxohexanoic acid as hydrochloride salt; yield: 20 mg (48%). ^{1}H NMR (MeOD-*d4*, 300 MHz): δ = 5.18 (m, 1H), 3.24 (m, 2H), 2.72 (m, 1H), 2.58 (d, *J* = 1.59 Hz, 3H), 2.44 (m, 1H); ^{13}C NMR (MeOD-*d4*, 75 MHz): δ = 199.6, 171.8, 69.2, 40.2, 26.3, 19.4) (See Figure S6 and S7).

Activity measurements

Specific activities of the selected enzyme. To a mixture of ketone (100 mM), ammonia buffer (300 mM NH_4Cl/NH_4OH pH 7.2) and NADH (0.2 mM) (or NADPH 0.2 mM if the activity was better in the tested conditions) in a non-thermostated spectrophotometric cell was added an appropriate amount of enzyme AmDH (reaction final volume 100 µL). The initial slope measured at 340 nm determined the specific activity of the enzyme according to Beer-Lambert's law and molar absorptivity of β-NADH (ε = 6,298 M^{-1} cm^{-1}).[24]

Activity versus temperature. A reaction mixture of ketone **6** (100 mM) in ammonia buffer (300 mM NH_4Cl/NH_4OH pH 7.2) heated to the specified temperature in a ThermoMixer® Eppendorf was added to a thermostated spectrophotometric cell containing NADH (0.2 mM) and the enzyme AmDH4 purified by heat treatment (reaction final volume 100 µL). The mixture was rapidly homogenized and the initial slope measured at 340 nm in a temperature controlled UV-spectrophotometer set to the specified temperature. The difference in slope with NADH oxidation with no enzyme at the same temperature determined the specific activity of AmDH4.

Thermostability studies. To a reaction medium containing a solution of a 100 mM solution of 4-oxopentanoic acid (**6)** in 300 mM ammonia buffer (NH_4Cl/NH_4OH pH 7.2) preheated at 60°C during 5 min, was added a solution of 0.2 mM NADH following by 0.5 mg/mL of AmDH4 purified by heat treatment previously incubated for specified time at specified temperature. Residual activity was measured at 60°C at 340 nm as described above.

Enantioselectivity and conversion

The reactions were performed in a 100 µL volume, containing substrate 4-oxopentanoic acid (**6**), specified cosubstrate, cofactor recycling enzyme, cofactor NADH, specified buffer, and specified amount of AmDH at specified temperature and 400 rpm. After a specified period of time, an aliquot (10 µL) was subjected to DNFB derivatization for conversion studies (see example in Figure S3) or FDAA derivatization for *ee* determination. The reactions were compared to blank reactions performed without enzyme and replicated when specified. Conversions were deduced from calibration curves obtained with commercial racemic amine.

Procedure for the derivatization with 1-fluoro-2,4-dinitrobenzene (DNFB). To 10 µL of a 10 mM reaction mixture were added 10 µL of a 1 M Na_2CO_3 aqueous solution, 50 µL of a 1 M $NaHCO_3$ aqueous solution and 40 µL of a 5 mM DNFB solution in EtOH. The mixture was stirred (400 rpm) at 65°C for 1h and then quenched by 50 µL of a 1 M HCl aqueous solution, filtered (0.22 µm), and analyzed by UHPLC (condition A).

Procedure for the derivatization with (*S*)-2-(5-fluoro-2,4-dinitrophenylamino)propanamide (FDAA). To 20 µL of a 10 mM reaction mixture were added 8 µL of a 1M $NAHCO_3$ aqueous solution and 20 µL of a 15 mM FDAA solution in EtOH. The mixture was stirred (400 rpm) at 50°C for1h and then quenched by 4 µL of 4 M HCl aqueous solution and centrifuged (10 min, 10000 rpm) to remove proteins. The resulting supernatant (25 µL) was diluted in MeCN (75 µL), filtered (0.22 µm), and analyzed by UHPLC (condition B).

Biocatalytic synthesis of (*4S*)-4-aminopentanoic acid

In a Greiner tube (50 mL) with a screw cap, was poured 9 mL of a media containing 5 M NH_4CO_2H/NH_4Cl buffer (pH 8.5), 0.5 M 4-oxopentanoic acid (6) (basified at pH 8.9), 0.4 mM NADH, 0.5 U of FDH and 9 mg of AmDH4 purified by heat treatment. The reaction mixture was stirred at 400 rpm at 50°C for 24 h then quenched with 6 M HCl aqueous solution to reach pH 1, centrifuged (10 krpm, 20 min). The supernatant was taken and the pellet was dissolved in water and centrifuged (10 krpm, 20 min) a second time. Supernatants were combined and lyophilized overnight. The freeze-dried product was dissolved in 0.1 M HCl aqueous solution and purified by ion exchange chromatography using a Dowex® 50WX8-200 mesh resin previously activated by hydrochloric acid 1M (non-linear gradient elution (4 column volumes): 0.1 M HCl, H_2O until neutral pH, and NH_4OH 3.3%). Positive fractions (TLC, ninhydrine relevation) were combined and lyophilized to afford (*4S*)-4-aminopentanoic acid as a white solid (466 mg, 88% yield) with ee ≥ 99.5 % as determined by UHPLC after FDAA derivatization (Figure S5). *tR* (**9**) =2.30 min (condition A), *tR* [(*S*)-**9**] = 4.79 min, *tR* [(*R*)-**9**] = 5.09 min (conditions B), $[\alpha]_D^{15.4}$ = -11.3° (c = 30,1 mg/ml, H_2O) {lit. for opposite enantiomer (*4R*)-4-aminopentanoic acid: $[\alpha]_D^{22.0}$ = +13.6° (c = 1.05, H_2O)[25] }, ^{1}H NMR (300 MHz, D_2O) δ = 3.34 – 3.19 (m, 1H), 2.20 (ddd, J = 7.8, 7.1, 3.1 Hz, 2H), 1.91 – 1.60 (m, 2H), 1.20 (d, J = 6.6 Hz, 3H) {identical to literature[25]}; ^{13}C NMR (75 MHz, D_2O) δ = 181.9, 47.9, 33.8, 30.9, 17.7 (See Figure S8 and S9).

Results and discussion

Identifying natural Amide dehydrogenase (AmDH)

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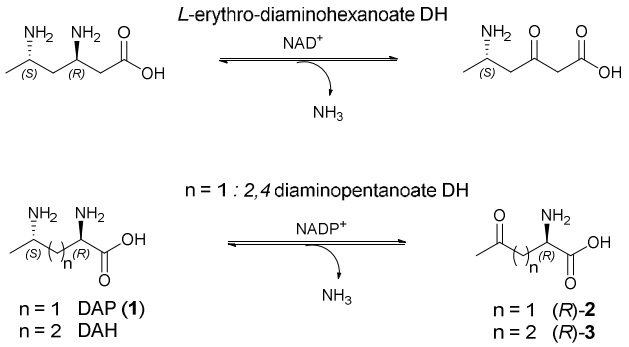
To track genes coding for AmDHs among the natural prokaryotic biodiversity, we first inventory all the reactions transforming unfunctionalized ketones into amines with ammonia using NAD(P)H as cofactor, by exploring metabolic databases.[26] All the reactions on α-keto acids were excluded from the request, removing so any α-amino acid dehydrogenase from the search. We identified L-erythro-3,5 diaminohexanoate dehydrogenase (3,5-DAHDH) involved in lysine fermentation pathway[27] and the (*2R,4S*)-2,4-diaminopentanoate dehydrogenase (2,4-DAPDH) involved in ornithine-fermenting bacteria[28, 29] assumed to be also active towards 2,5-diaminohexanoate DAH (Scheme 1).[30]



Scheme 1 Reactions catalyzed by L-erythro-3,5 diaminohexanoate DH and 2,4-diaminopentanoate DH

We decided to focus our attention on enzymes active towards ketones without α or β carboxylic acid moiety, all the more so due to the synthesis of various β-amino acids recently reported by Zhu et al. with variants of 3,5-DAHDH from *Candidatus* Cloacamonas acidaminovorans, a bacterium found in an anaerobic digesters.[31] The protein sequence of 2,4-DAPDH from *Clostridium sticklandii* (UniprotKB identifier: E3PY99) was used in a sequence-driven approach[23, 32] to build a collection of 2,4-DAP dehydrogenase similar enzymes.

169 proteins sharing at least 30 % identity over at least 80 % of the length with E3PY99 were collected. Proteins have been clusterized based on sequence identity (≥ 95%) and one representant per cluster, for which genomic DNA was available in Genoscope strain collection, was chosen. From the 26 candidates selected to be representative of their sequence diversity, 20 genes were successfully cloned in an expression vector. PCR failure occurred for the remaining 6 genes for which GC content (<30 % or >65 %) prevent efficient gene amplification. After overexpression of recombinant genes in *Escherichia coli* strain BL21, cells were lyzed and the protein quantified (see Supporting Information). These enzymes were then screened as cell-free extracts against various substrates. To approach proper amine dehydrogenase substrates, a set of structural variants of the substrates of 2,4-DAPDH (**2** and **3**) with deletion of the amino and/or carboxylic moiety have been considered (Fig. 1). AmDH activity was assayed in the forward (reductive amination) or reverse (oxidative deamination) direction by spectrophotometric NAD(P)H-monitoring or HPLC-MS (Table 1).

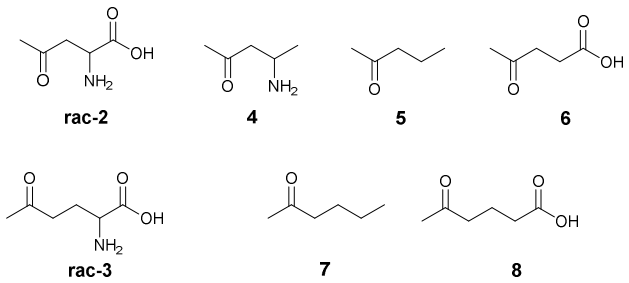


Fig. 1 Compounds selected for the screening of AmDH candidates

Table 1 Spectrophotometric and HPLC-MS screening assay results

Entry	Name	UniprotKB identifier	Organism	Activity towards rac-**2**[a]	Activity towards **6**[b]		Activity towards **8**[b]	
				UV	UV	MS[c]	UV	MS[d]
1	AmDH1	A6LLG5	*Thermosipho melanesiensis*[e]	+	*nd*		*nd*	
2	AmDH2	A7HNJ8	*Fervidobacterium nodosum*[e]	+	+	+++	*nd*	*nd*
3	AmDH3	A8MGL6	*Alkaliphilus oremlandii*	+	*nd*		*nd*	
4	AmDH4	A9BHL2	*Petrotoga mobilis*[e]	+	+	+++	*nd*	++
5	AmDH5	E3PY99	*Clostridium sticklandii*	+	+	+++	*nd*	++
6	AmDH6	B8D005	*Halothermothrix orenii*[e]	+	*nd*		*nd*	
7	AmDH7	D2Z5Z0	*Dethiosulfovibrio peptidovorans*	+	+	+++	+	
8	AmDH8	D7DCF5	*Staphylothermus hellenicus*[e]	+	+	+++	+	++
9	AmDH9	D9RY14	*Thermosediminibacter oceani*[e]	+	+	++	+	*nd*

+: detected; ++: detection of ≥ 0.001 mM of product; +++: detection of ≥ 0.01 mM of product; *nd*: not detected; empty cells: not tested; [a] tested in oxidative deamination reaction; [b] tested in reductive amination reaction; [c] MS detection of 4-aminopentanoic acid **9** after 2h; [d] MS detection of 5-aminohexanoic acid **10** after 3 days; [e] thermophilic organism.

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Three compounds were found to be substrates. Nine enzymes demonstrated activity toward ketone **2**, the natural substrate of 2,4-DAPDH.[28] More interestingly, six of them were found active towards the 4-oxopentanoic acid (**6**), which was confirmed by MS analysis (Table 1, entries 2, 4, 5, 7-9), and among the latter three exhibit slight activity towards the homologous 5-oxohexanoic acid (Table 1, entries 4, 5, 8). Being active towards non-α/β keto acids, these enzymes can be described as the first wild-type ω-amine dehydrogenases.

Considering these very promising results, the six enzymes AmDH2, AmDH4, AmDH5, AmDH7, AmDH8, AmDH9 cloned with a polyhistidine tag were purified by nickel affinity chromatography for further studies. Preliminary experiments showed a very high enantioselectivity (*ee* ≥ 95 %) towards ketone **6** for the six purified enzymes (data not shown). Their specific activities were determined by measuring the NAD(P)H absorbance in the reductive amination reaction (Table 2). The activities on ketone **6** were 1000 fold lower than the one on assumed natural substrate **1** (65 U/mg prot. for AmDH5). Among the three enzymes AmDH2,[29] AmDH4 and AmDH5 showing the highest specific activities on **6**, we decided to focus on AmDH4 from the thermophilic bacteria *Petrotoga mobilis* strain DSM 10674. This thermophilic feature usually offers interesting properties of stabilities for subsequent implementation in industrial processes.[33] Moreover, AmDH4 showed activity towards the substrate analogue **8** unlike AmDH2, suggesting a better substrate promiscuity.

Table 2 Specific activity of purified enzymes towards 4-oxopentanoic acid (**6**)

Name	Specific activity (mU/mg prot)[a]
AmDH4	51,9±4.8
AmDH7	2.9±0.1
AmDH8	2.9±0.6
AmDH9	3.1±0.2
AmDH5	34.1±2.6
AmDH2	70.9±15.6

[a] Reaction conditions: 300 mM NH_4Cl/NH_4OH buffer, pH 7.2, 200 µM NAD(P)H, 100 mM **6** at ambient temperature (triplicate).

Temperature studies with AmDH4

The enzyme AmDH4 was easily purified by heat treatment without notable loss of material and activity. Its coenzyme specificity was examined by measuring enzymatic activity towards ketone **6** and either NADH or NADPH as the coenzyme. The highest activity in tested conditions was obtained with NADH with an activity 10 times higher than the one with NADPH. The enzyme seems to have a narrow substrate scope as its activity drop to 5.6 mU/mg of enzyme towards the homolog 5-oxohexanoic acid. No activity could be detected on acetophenone, cyclohexanone, penta-2-one or 2-aminopentan-2-one. A profile of temperature versus activity towards 4-ketopentanoic acid (**6**) revealed that AmDH4 is hardly active at 20 °C and starts to exhibit good activity at 60 °C and even above as better activity was found at 90 °C (Fig. 2). Its thermal optimum activity could not be reached due to limitations of our UV-VIS spectrophotometer. At this high temperature, its specific activity was 8 fold higher than at 20 °C, reaching an activity of 0,7 U per milligram of protein, a value close to the one of the chimeric cfL1-AmDH (1,7 U mg-1 for p-fluorophenylacetone).[34]

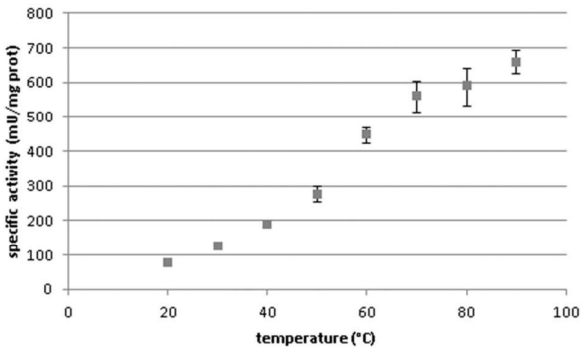

Fig. 2 Profile of activity versus temperature for AmDH4 on 4-oxopentanoic acid (**6**). Reaction conditions: 300 mM NH_4Cl/NH_4OH buffer, pH 7.2, 200 µM NADH, 100 mM **6**. Error bars represent the standard deviation of three independent experiments.

To estimate the thermostability of AmDH4, the enzyme was incubated at various temperatures (30 °C, 50 °C, 60 °C, 70 °C and 80 °C) during different periods of time and the residual activity towards compound **6** was assayed at 60 °C (Fig. 3). The half-life of the enzyme was estimated to be 65 h at 60 °C and 30 min at 80 °C. Most notably, this enzyme was stable several days at 30 °C as its activity was still around 90 % of the initial activity after 14 days (data not shown).

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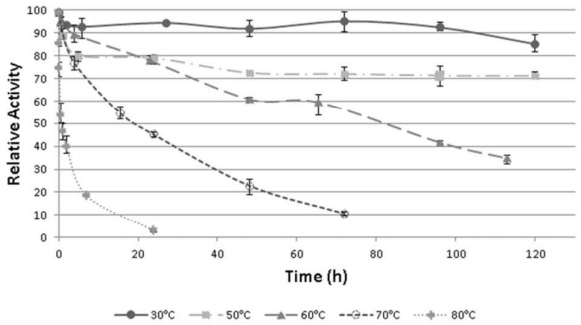
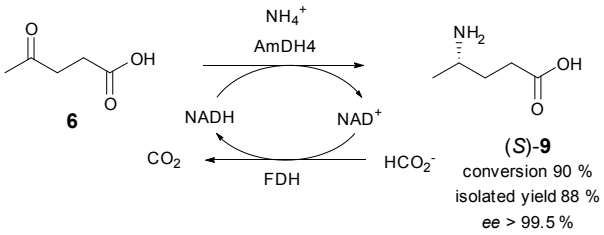


Fig. 3 Thermostability of AmDH4. Residual activity measured at 60 °C at the indicated time points after incubation of AmDH4 at various temperatures. Reaction conditions: 100 mM ketone **6**, 200 µM NADH in 300 mM NH_4Cl/NH_4OH buffer, pH 7.2 Error bars reflect the variation of three independent experiments.

Conversion with cofactor recycle system

Conditions to perform the biocatalytic conversion of 4-ketopentanoic acid (**6**) to optically pure 4-aminopentanoic acid were optimized step by step and monitored by UHPLC-UV after DNFB derivatization (Fig. 4). As expected, one equivalent of NADH did not allow complete conversion due to thermodynamic reaction equilibrium even at the optimized temperature of 50 °C, where a conversion of around 40 % has nonetheless been reached. To drive the reaction to completion without providing large excess of expensive coenzyme, different NADH-recycling systems[35] (phosphite/PTDH, glucose-6-phosphate/G6PDH, glucose/GDH, formate/FDH) were tested at various temperatures, revealing glucose/GDH and formate/FDH as the best systems with an optimum temperature of 60 °C and 50 °C respectively (Supporting Information Figure S4). Higher temperatures near optimum temperature of the enzyme activity do not allow obtaining better conversions due to denaturation of the enzyme of the recycling system and / or start of impaired cofactor stability. Thermostable systems for the regeneration of cofactor, including synthetic nicotinamide mimic,[36] are required to increase the benefit of the thermoactivity of the selected enzyme. Improvements with the formate/FDH recycling system regarding buffer ($Na_2CO_3/NaHCO_3$, NH_4Cl/NH_3 or NH_4HCO_2), pH and buffer/ammonia concentration revealed optimum conditions at a pH of 8.5 in 5 M ammonium formate, with the advantage for the latter of being both buffer and co-substrate. Increasing the initial ketone concentration affected the conversion but still enabled the nearly complete conversion of 0.5 M of ketone **6** by using a somewhat larger concentration of enzyme (1 mg/ml) to perform the reaction over 24 h. The enantiomeric excess determined by UHPLC-UV after FDAA derivatization was up to 99.5 % as the minor enantiomer could not be detected (Supporting Information Figure S5).



Scheme 2 Synthesis of (*4S*)-4-aminopentanoic acid ((*S*)-9) catalyzed by AmDH4 with FDH cofactor regeneration system.

Semi-preparative scale

463 mg (88 % yield) of the amine **9** were isolated from a 9 mL / 0.5 M-scale reductive amination reaction after simple purification on strong acidic cation resin Dowex 50WX8®(Scheme 2). Its structure was confirmed by NMR analysis and its stereochemistry determined by polarimetry to be (*S*), as is the case for the metabolic substrate of 2,4 DAPDH.

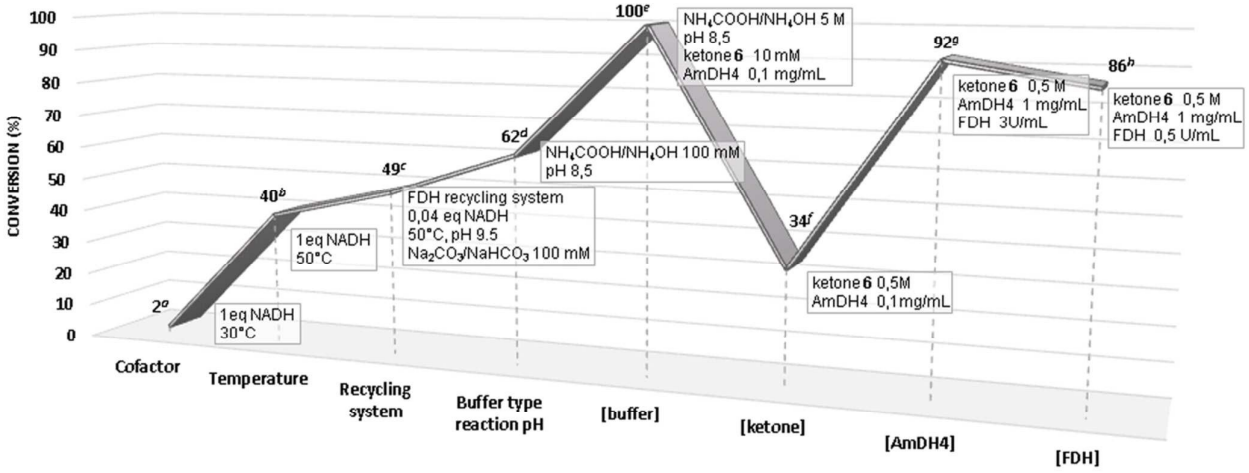


Fig. 4 Biocatalytic reaction improvement. [a] **6** 10 mM, NADH 10 mM, NH_4Cl 200 mM, AmDH4 0.1 mg/mL, $NaHCO_3/Na_2CO_3$ buffer 100 mM pH 9.5, 30 °C. [b] idem as conditions *a* at 50 °C. [c] **6** 10 mM, NADH 0.4 mM, NH_4Cl 200 mM, AmDH4 0.1 mg/mL, $NaHCO_3/Na_2CO_3$ buffer 100 mM pH 9.5,

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HCOONa 20 mM, FDH 3 U/mL, 50 °C. [d] **6** 10 mM, NADH 0.4 mM, AmDH4 0.1 mg/mL, NH_4CO_2H/NH_4OH buffer 200 mM pH 8.5, FDH 3 U/mL, 50 °C. [e] idem as conditions *d* with 5 M buffer. [f] idem as conditions *e* with substrate **6** 0.5 M. [g] idem as conditions *f* with AmDH4 1 mg/mL. [h] idem as conditions *g* with FDH 0.5 U/mL. Acidic substrat **6** was previously basified at studied pH. All reactions were performed on 100 µL reaction volume at 400 rpm in 24h.

Conclusions

In summary, NAD(P)H-proper amine dehydrogenase have been identified among the biodiversity and particularly within the thermophilic bacteria *Petrotoga mobilis* strain DSM 10674. From the latter, AmDH4 was found active towards the 4-oxopentanoic acid, a γ-ketocarboxylic acid. Optimization of the reaction conditions including temperature and cofactor regeneration system enable the sustainable high concentrated production of (*4S*)-4-aminopentanoic acid with high yield (88 %) and excellent enantiomeric excess (> 99.5 %). The robust and easily purifiable enzyme AmDH4 offers a simple access to this valuable optically pure primary amine, already used as building block for the synthesis of therapeutic molecule.[25, 37] This is the first example of a wild-type enzyme with related gene catalyzing the reductive amination of ketone without carboxylic acid moiety in α or β positions. Its robustness makes it a good scaffold for directed evolution, offering significant potential for enzyme improvement.[38] Facing the importance of such reaction in chiral amine synthesis, this discovery will certainly contribute to expending the synthetic scope of amine dehydrogenase-catalyzed reductive amination in industry. Other natural enzymes with broader substrate range are currently sought in our laboratory to expand this unique class of biocatalysts for the synthesis of primary chiral amines.

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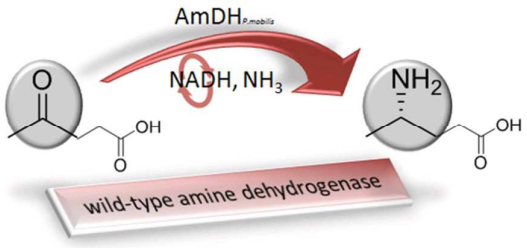

Biocatalytic potential of a new wild type amine dehydrogenase used in an enzyme-catalyzed synthesis of an enantiomerically pure primary amine