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DOI
10.1002/cctc.202101576

Publication date
2021

Document Version
Final published version

Published in
ChemCatChem

ChemCatChem

Research Article
doi.org/10.1002/cctc.202101576

Chemistry Europe
European Chemical Societies Publishing

www.chemcatchem.org

Synthesis of Chiral Amines via a Bi-Enzymatic Cascade Using an Ene-Reductase and Amine Dehydrogenase

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Access to chiral amines with more than one stereocentre remains challenging, although an increasing number of methods are emerging. Here we developed a proof-of-concept bi-enzymatic cascade, consisting of an ene reductase and amine dehydrogenase (AmDH), to afford chiral diastereomerically enriched amines in one pot. The asymmetric reduction of unsaturated ketones and aldehydes by ene reductases from the Old Yellow Enzyme family (OYE) was adapted to reaction conditions for the reductive amination by amine dehydrogenases. By studying the substrate profiles of both reported biocatalysts, thirteen unsaturated carbonyl substrates were assayed against the best duo OYE/AmDH. Low (5%) to high (97%) conversion rates were obtained with enantiomeric and diastereomeric excess of up to 99%. We expect our established bi-enzymatic cascade to allow access to chiral amines with both high enantiomeric and diastereomeric excess from varying alkene substrates depending on the combination of enzymes.

Introduction

Chiral amines are encountered in a myriad of building blocks and are valuable chemicals used in pharmaceutical and fine chemical industries. Besides their synthesis by metal-assisted catalysis or by more sustainable organocatalytic alternatives,[1] biocatalytic methods have been increasingly developed over the last decade.[2] Among the panel of enzymes reported to date for this transformation, amine dehydrogenases (AmDHs; EC 1.4.1) are described to catalyze the reductive aminations of carbonyl-containing compounds to corresponding primary amine products with ammonia as amine source and the 1,4-dihydronicotinamide adenine dinucleotide (NAD(P)H) cofactor. Particularly, native AmDHs have been reported to be active toward various aliphatic aldehydes and ketones to access a variety of amine products with *S*-configuration in case of chiral amines.[3] Their implementation in biocatalytic cascade reactions could allow access to various substituted (chiral) products.

Biocatalytic cascades, *i.e.* the combination of at least two reaction steps in a single reaction vessel without isolation of the intermediates, have been reported for the synthesis of many compounds,[4] allowing to save reagents, time and operational workup steps, with easier access or more appropriate initial substrates. Particularly, the use of two catalysts in *in vitro* linear sequences have been applied for the synthesis of amines. Ramsden *et al.* have described the one-pot combination of reductive aminase (RedAm) from *Aspergillus oryzae* with choline oxidase or carboxylic acid reductase to perform biocatalytic *N*-alkylation of amines through *in situ* generation of aldehydes.[5] Hydrogen-borrowing asymmetric amination was carried out by coupling alcohol dehydrogenase (ADH) and AmDH/RedAm to prepare various (*R*)-amines,[6] including with co-immobilized biocatalysts.[7] By adding another step catalyzed by a P450 monooxygenase, unfunctionalized alkanes can be employed to access amines with isolated enzymes[8] or *E. coli* whole cells.[9]

Ene reductases (EREDs) of the Old Yellow Enzyme family (OYEs; EC 1.6.99.1) catalyze the asymmetric reduction of unsaturated ketones and aldehydes in high conversion and enantiomeric excess, and are promising biocatalysts for industrial applications.[3a] OYEs have already been applied in cascade reactions, especially for the synthesis of carboxylic acids also in a hydrogen-borrowing concept,[10] or in combination with ADH to access α-substituted alcohols.[11] The transformation of enones to amines was accomplished with OYEs and transaminases (TAs) chosen unreactive toward the starting enones. Moderate to high diastereomeric excess of (1*R*,3*S*)-, (1*S*,3*S*)-, (1*R*,3*R*)-1-amino-3-methylcyclohexane and substituted aryl amines were obtained depending of TA from Codexis and wild-type or engineered OYE used.[12]

Here we envisioned a bi-enzymatic cascade combining OYE and native AmDH to obtain a panel of amine compounds, several with various methyl substitutions leading to chiral products (Scheme 1). With the extending substrate scope of AmDHs and the growing number of characterized OYEs, it appeared interesting to prove the viability of such a cascade

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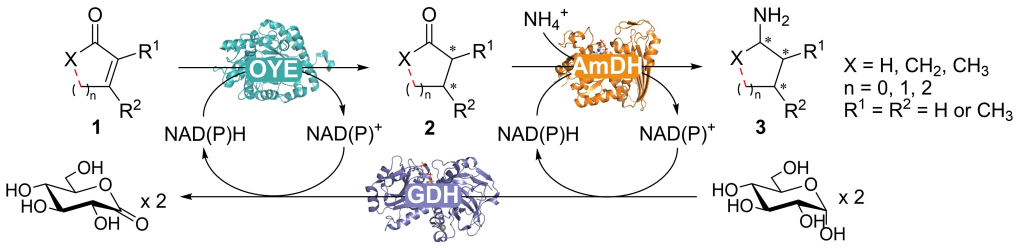
Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.202101576



Scheme 1. Overview of the one-pot bi-enzymatic cascade to obtain chiral amines, from asymmetric reduction catalyzed by an OYE to reductive amination with ammonia and AmDH.

and to apply it on a large number of substrates. This one-pot biocascade circumvents the need for isolation and purification of intermediates while retaining the enantioselectivity of each enzyme. A NAD(P)H cofactor recycling system with the well-established glucose dehydrogenase (GDH) provides the necessary electrons for the system *via* inexpensive glucose. After an extended substrate scope study of both OYEs and AmDHs, we describe here the results obtained with the best duo OYE/AmDH for the biocatalytic conversion of nine common substrates. Several key cascade reactions were conducted at 10 mM scale to demonstrate the viability of this setting.

Results and Discussion

In this study, we focused our effort on the following characterized AmDHs: *Cfus*AmDH from *Cystobacter fuscus*, *Apau*AmDH from *Aminomonas paucivorans*, *Msme*AmDH from *Mycobacterium smegmatis*, *Micro*AmDH from *Microbacterium* sp. MA1, *Chat*AmDH from *Hungatella hathewayi*, and IGCAmDH5 and MATOUAmDH2 from metagenomic data. Their reported substrate scope are mainly aliphatic aldehydes and ketones, linear or cyclized.[13] No or very low activity were described for carbonyl compounds bearing an aromatic group. Therefore, we decided to select unsaturated enones structurally identical or similar to this preliminary list, despite the only few activity data of OYEs for this type of substrates. We extended this list to methyl-substituted unsaturated aldehydes to take advantage of the stereoselectivity of OYEs toward substituted enones and access diastereoisomers *via* the cascade process, resulting in the selection of differently substituted cycloalkenones **1a**–**1f**, aliphatic enones **1g**–**1i**, methyl-substituted unsaturated aldehydes **1j**–**1l** and unsaturated aldehyde **1m** (Figure 1).

We started screening the activity toward substrates **1a**–**1m** with available OYEs from *Thermus scotoductus* (*Ts*OYE),[14] *Saccharomyces cerevisiae* (OYE2),[15] *Bacillus subtilis* (YqjM),[16] *Gluconobacter oxydans* (GluER)[17] and *Geobacillus kaustophilus* (*Gk*OYE).[18] *Ts*OYE was already described to catalyze the reduction of maleimides and cycloalkenones,[19] and was expected to accept substrates such as aliphatic, unsaturated aldehydes and ketones not yet explored but known as OYE substrates.[20] Compared with other OYEs, we initially chose *Ts*OYE as a model enzyme due to its high solvent and thermal stability. We measured the specific activity for the reduction of

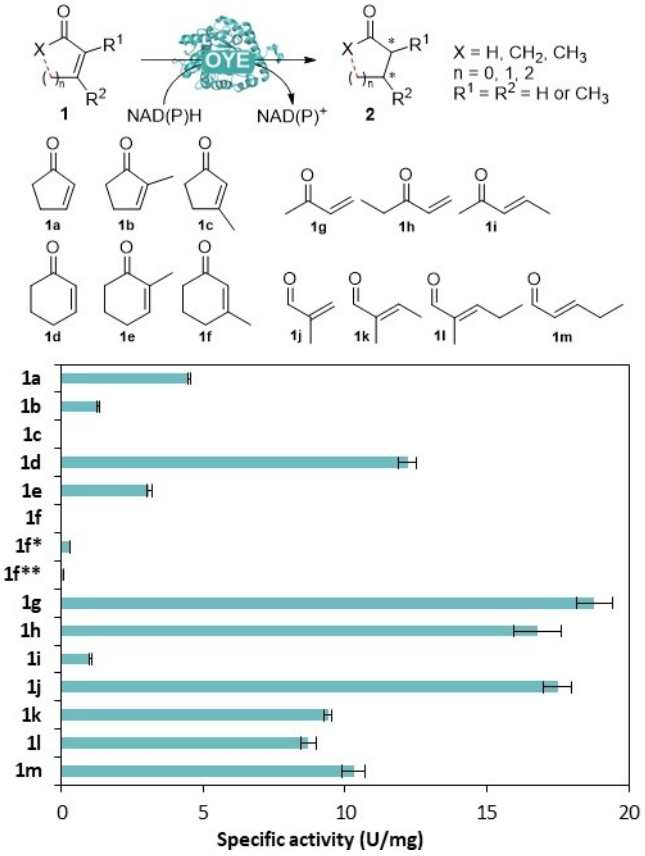


Figure 1. Specific activities of *Ts*OYE for unsaturated ketone and aldehyde substrates **1a**–**1m**. Conditions: 10 mM substrate, 50 mM Tris-HCl buffer pH 8.0, *Ts*OYE, 10.0 U/mL glucose oxidase (GOx), 20 mM glucose, 0.2 mM NADPH. 3-methylcyclohexenone **1f*** was tested with *Ts*OYE-C25D/I67T and **1f**** with OYE2 and 0.2 mM NADH. Average of duplicates.

substrates **1a**–**1m** with *Ts*OYE by spectrophotometry under standard conditions (Figure 1).

The highest specific activity was obtained with 3-buten-2-one **1g** (18.8 U/mg) and decreased with increasing alkyl chain length, with 2-penten-3-one **1h** (16.8 U/mg) and 3-penten-2-one **1i** (1 U/mg), as well as with α-methyl substituents, from *trans*-pentenal **1m** (10.3 U/mg) to *trans*-2-methyl-pentenal **1l** (8.7 U/mg). The activity was lower for cyclic substrates cyclopentenone **1a** and cyclohexenone **1d** compared with linear ones, and the same activity decrease was observed with α- and β-methylated substrates (**1b**, **1c**, **1e** and **1f**). In particular, β-

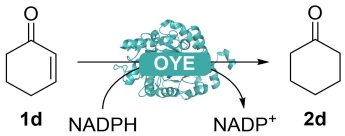
methyl substituted substrates 3-methylcyclopentenone **1 c** and 3-methylcyclohexenone **1 f** afforded no observable activity with *Ts*OYE. Therefore, we also probed the double mutant *Ts*OYE-C25D/I67T[21] (0.30 U/mg) and OYE2 (0.06 U/mg) with 3-methylcyclohexenone **1 f** (Figure 1). For cyclohexenone **1 d**, the highest activities were obtained with *Ts*OYE followed by OYE2, then YqjM (Table 1).[22] In case of enals **1 k** and **1 l**, there specific activities were also tested toward *Gk*OYE and GluER but *Ts*OYE remained the most active one in the tested conditions (Figure S4).

Thus, we established that the selected OYEs had a substrate scope highly compatible with the preliminary data reported for the studied AmDHs, with high activity toward aliphatic unsaturated aldehydes and cyclohexenone, and some notable activity toward the other tested ketones/substituted cycloalkenones.

Next we chose cyclohexenone **1 d** as a model to determine reaction conditions for OYEs that would be compatible with AmDH activity, this substrate giving good activity and being one of the least volatile. To design one-pot cascade reactions, reaction conditions must fit to all the enzymes involved in the steps. In case of enzymes working with specific conditions, the other enzymes must tolerate these conditions sufficiently to ensure the success of the cascade without this being to the detriment of the use of too large quantity of catalysts. In our case, a high ammonia concentration is essential for AmDH activity, mainly due to the very high K_M of ammonia. Thus, the main parameter to be studied was the tolerance of OYEs to high ammonia concentrations.

In addition to commonly used buffers, 0.25–1.00 M ammonium formate buffers were tested. At 50 mM, *Ts*OYE gave the highest activity with MOPS-NaOH at pH 7.0 and Tris-HCl at pH 8.0, decreasing from 12.0 to 6.5–7.3 U/mg when increasing the pH to 9.0 (Table 1). This significant activity decrease at pH 9.0 can be ascribed to deprotonation of tyrosine 177,[23] known to play an important role as proton donor in the OYE active site.[14,24] Notably, the specific activity of *Ts*OYE with and without glucose oxidase (GOx) to remove molecular oxygen *in situ* showed no significant difference (see Figure S3). The use of ammonium formate as buffer at 0.25–1.00 M concentration resulted in a significant decrease in activity, all the more important as the concentration increased (Table 1).

Table 1. Specific activity of OYEs for substrate cyclohexenone **1 d**.[a]



OYE	Buffer	[Buffer] [M]	pH	Specific activity [U/mg]
OYE2	Tris-HCl	0.05	8.0	3.3 ± 0.1
YqjM	Tris-HCl	0.05	8.0	2.1 ± 0.0
*Ts*OYE	MOPS-NaOH	0.05	7.0	12.0 ± 0.0
*Ts*OYE	Tris-HCl	0.05	8.0	12.2 ± 0.3
*Ts*OYE	Tris-HCl	0.05	8.5	10.9 ± 0.1
*Ts*OYE	Tris-HCl	0.05	9.0	6.5 ± 0.0
*Ts*OYE	Na_2CO_3	0.05	9.0	7.3 ± 0.3
*Ts*OYE	NH_4HCO_2	0.25	8.0	1.4
*Ts*OYE	NH_4HCO_2	0.5	8.0	0.3
*Ts*OYE	NH_4HCO_2	1.0	8.0	0.1

[a] Conditions: specified buffer, [OYE] (0.05 M buffer: 0.05, 0.73 and 0.65 μM of *Ts*OYE, OYE2 and YqjM respectively; 0.56, 1.38 and 3.96 μM of *Ts*OYE at 0.25, 0.50 and 1.00 M NH_4HCO_2 buffer respectively), 10 U/mL glucose oxidase (GOx), 20 mM glucose, 0.2 mM NADPH, 10 mM cyclohexenone; average of duplicates.

We carried out biocatalytic reactions monitoring conversion after one hour to determine whether these low specific activities observed at 0.25–1.00 M ammonium formate still allowed for conversion while maintaining appropriate amounts of catalysts (< 0.1 mg/mL), with the substrate scope (**1 a**, **1 c**, **1 d**, **1 e**, **1 f**, **1 h**, **1 l**, **1 m**). For better understanding, these reactions were tested in four different buffer conditions (Figure 2). The synthetic cofactor 1-benzyl-1,4-dihydronicotinamide (BNAH) was used as an inexpensive alternative to NADPH to simplify screening, as using BNAH avoids a cofactor recycling system and is stable under basic conditions (Figure S6).[25] Overall, the conversion rates were similar for the buffers used (Tris-HCl or NH_4HCO_2), even if a concentration of at least 50 mM of Tris-HCl is preferred to sufficiently buffer the medium. Nearly complete conversions were obtained for substrates **1 a**, **1 d**, **1 e** and **1 h**, moderate (< 70 %) conversions for substrates **1 l** and **1 m**, and low to no conversions for 3-methyl-2-cyclopenten-1-one **1 c** and 3-methylcyclohexenone **1 f** (Table S4).

Substrate 3-methylcyclohexenone **1 f** was better reduced with the double mutant *Ts*OYE-C25D/I67T (92 % *ee R*) and OYE2 (99 % *ee S*), giving access to both product enantiomers, despite low conversion rates (10–20 %) (see Table S4).[21] 25 mM and 1 M ammonium formate buffer were not tested for **1 f** because of the already low conversions with Tris-HCl buffer pH 8.0.

Interestingly, when we studied this same buffer effect but with NADPH/GDH recycling system planned for the whole cascade, the conversions were drastically affected by the presence of high concentrations of ammonium formate buffer (Figure 2B), which was not the case with BNAH (Figure 2A/B). We hypothesize the GDH may be the limiting factor in these high ionic strength conditions,[23] despite its use in many reduction processes with such buffer.

Based on this buffer study, 1 M ammonium formate buffer was beneficial for the reductive amination step, benign to the OYE conversions but detrimental to the GDH regeneration system. Nevertheless, we decided to proceed with this important prerequisite for the AmDH activity, taking care of reaction times and optimized amount of OYE/GDH to counter this negative effect.

Substrates 2-methylbutenal **1 k** and 2-methylpentenal **1 l** were further screened with three OYEs for conversion and *ee*. As **1 l** was a mixture of both *cis* and *trans* isomers, the conversion was limited to 50 % conversion as *Ts*OYE prefers the *trans* over the *cis* isomer. Thus, we screened the *trans*-**1 k** and *trans*-**1 l** with *Ts*OYE, *Gk*OYE and GluER. With *Gk*OYE and GluER, we obtained a significantly higher *ee* of products 2-methylbutanal **2 k** and 2-methylpentanal **2 l** (Figure 3).

In terms of nicotinamide cofactor, the selected AmDHs displayed high preference either for the phosphorylated form NADPH (*Cfus*AmDH, MATOUAmDH2, *Msme*AmDH and *Micro*-AmDH) or NADH (*Chat*AmDH, IGCAmDH5 and *Apau*AmDH). The

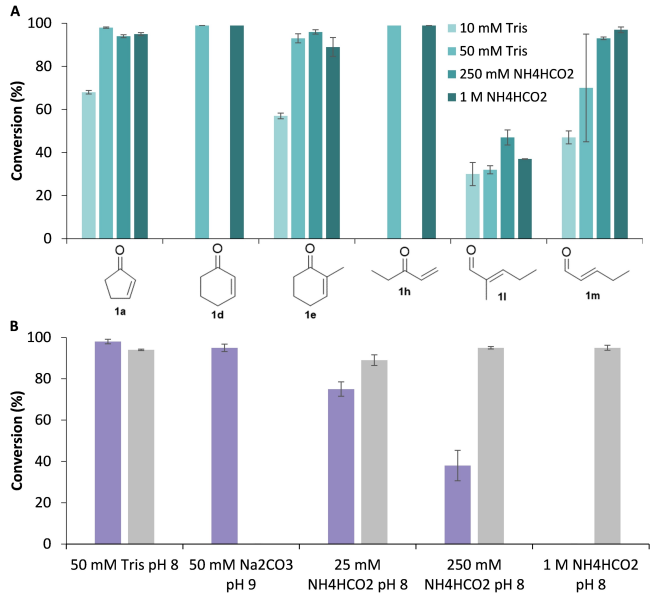


Figure 2. (A) *Ts*OYE-catalyzed reduction of unsaturated ketones and aldehyde substrates **1** after 1 h with BNAH. Conditions: 2 μM *Ts*OYE, 10 mM alkene substrate **1**, 15 mM BNAH, 10% v/v DMSO, 1 mL volume. **1 d** and **1 h** were tested in 50 mM Tris-HCl and 1 M NH_4HCO_2 only. Average of duplicates. **(B)** *Ts*OYE-catalyzed asymmetric reduction of 2-methylcyclohexenone after 1 h using GDH-recycled NADPH (in purple) or BNAH (in grey). Conditions: 1% v/v DMSO, 2 μM *Ts*OYE, 10 mM 2-methylcyclohexenone in different buffer conditions, 1 mL volume. Average of duplicates. **purple**: with GDH cofactor recycling system: 175 U/mL GDH-105, 0.1 mM NADPH, 12 mM glucose. **grey**: with 11 mM BNAH cofactor.

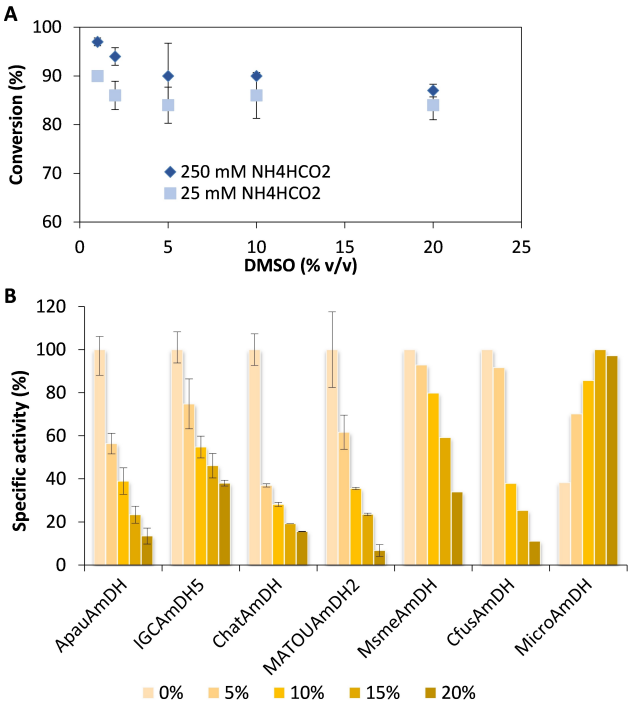


Figure 4. (A) *Ts*OYE-catalyzed reduction of 2-methylcyclohexenone to 2-methylcyclohexanone with 0–20% v/v DMSO after 1 h. Conditions: 2 μM *Ts*OYE, 10 mM substrate, 11 mM BNAH cofactor. Average of duplicates. (**B**) Specific activities of AmDHs for substrate **2 j** in the presence of 0, 5, 10, 15 and 20% v/v DMSO. For each AmDHs, their maximum specific activity was set up at 100%, which corresponds to specific activity without co-solvent for *Apau*AmDH, IGCAmDH5, *Chat*AmDH, MATOUAmDH2, *Msme*AmDH and *Cfus*AmDH, and to specific activity with 15% DMSO for *Micro*AmDH. Conditions: 5 mM substrate (**2 j** for *Apau*AmDH, IGCAmDH5, *Chat*AmDH and **2 d** for MATOUAmDH2, *Msme*AmDH, *Cfus*AmDH and *Micro*AmDH), 0.04–0.10 mg/mL purified AmDH, 0.4 mM NADH (NADPH for MATOUAmDH2) in 1 M ammonium formate buffer pH 9.0 at room temperature (20 °C) (*Apau*AmDH, *Msme*AmDH, *Cfus*AmDH, *Micro*AmDH) or 50 °C (IGCAmDH5, MATOUAmDH2, *Chat*AmDH). Specific activities were calculated based on spectrophotometric assay (see SI for details). Errors bars represent the standard deviation of two independent experiments.

OYEs used in this study are reported to prefer NADPH over NADH, seen for **1 b**, **1 j**–**1 m** (Figure S5), but the use of recycled NADH still provides good conversions.[25] Therefore, the preferred cofactor for AmDH enzymes were chosen in priority.

We proceeded with investigating the impact of DMSO for both type of enzymes to have a preliminary state of potential OYEs-AmDHs cascade reactions at high substrate loadings, which usually required addition of co-solvent for solubilization. For OYEs, 1 to 20% v/v DMSO were tested with *Ts*OYE toward **1 d**. These amounts of DMSO have a low impact on the conversions, as the conversion at 20% v/v was 85% compared to 97% at 1% v/v DMSO (Figure 4A). For AmDHs, their tolerance to DMSO was studied by measuring specific activity for substrate **2 j** (Figure 4B). All the studied AmDHs maintained at least 50% of their maximum activity at 5% v/v DMSO, except for *Chat*AmDH, for which a higher decrease was observed (37%

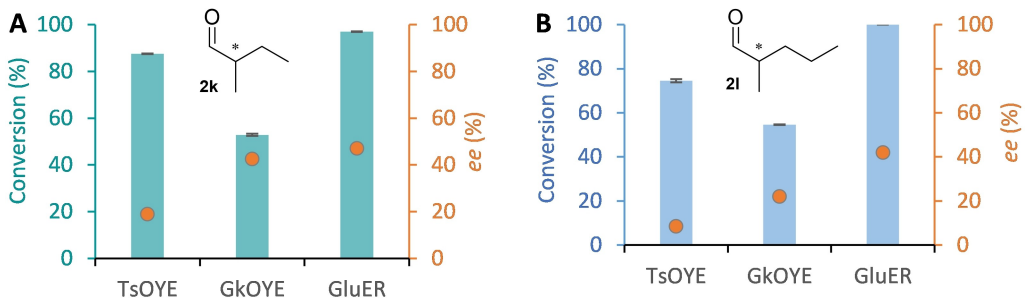


Figure 3. Conversion of unsaturated aldehydes **1 k** and **1 l** by *Ts*OYE, *Gk*OYE and GluER after 1 h with BNAH. Brown circles show *ee*. Conditions: 1 μM *Ts*OYE or *Gk*OYE or GluER, 10 mM substrate **1**, 15 mM BNAH, 10% v/v DMSO, 1 mL volume in 50 mM Tris-HCl buffer pH 8.0. **(A)** *trans*-2-methylbutenal **1 k**; **(B)** *trans*-2-methylpentenal **1 l**.

of maximum specific activity at 5% v/v DMSO). AmDHs *Msme*AmDH, *Cfus*AmDH and *Micro*AmDH gave the highest resistance to DMSO at 5% v/v DMSO with more than 70% activity retained. Above 10% v/v DMSO, *Msme*AmDH, IGCAmDH5 and *Micro*AmDH were the more tolerant enzymes with even higher activity in presence of DMSO for *Micro*AmDH.

Based on these results, 1% v/v DMSO could be used to test each substrate in the cascade at 10 mM without high negative impact on conversions. Higher percentages than 1% v/v of this co-solvent could surely be used for further scaled up reactions at higher substrate concentrations, especially with *Micro*AmDH for AmDH and *Ts*OYE for OYEs, subject to prior stability tests under 5–20% v/v DMSO.

All of the AmDHs used in this study were previously characterized in term of substrate profile, however these data were in majority specific activities, not conversions rates, and did not cover all the set of substrates defined for this work. AmDHs *Cfus*AmDH, ChatAmDH, IGCAmDH5, *Apau*AmDH, MATOUAmDH2, *Msme*AmDH and *Micro*AmDH were therefore tested toward aldehydes and ketones **2a**–**2m** in addition to the corresponding unsaturated compounds **1a**–**1m** for reductive amination in the conditions defined for OYEs in cascade reaction.

Each series of substrate was tested with the enzyme(s) showing the highest specific activities in previous reported results toward exactly or the more structurally similar substrate. From this screening, selected enzymes for each series of compounds were the enzymes showing the higher amount of amines toward the carbonyl form while exhibiting no or very low activity toward the unsaturated carbonyl to avoid any side product of unsaturated amine **4a**–**4m** (see Figure S8–S10 for details). This screening was carried out in 96-well plate in 100 µL total final volume at 0.5 mg/mL of purified enzyme and amine amounts were estimated using calibration curves and ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) assay (see SI). Despite some inconsistencies due to screening in 96-well plate with uncontrolled evaporation of some aldehydes, results were viable to compare the enzymes for one substrate and so to select the more appropriate enzyme(s) for the cascade reaction. These selected enzymes are highlighted in yellow for each substrate **a**–**m** in (Table 2).

We proceeded to perform the full cascade reactions with both selected OYEs and AmDHs based on their initial individual screening for each substrates considered (Table 3). Each combination OYE-AmDHs-unsaturated substrate **1** was chosen to obtain the highest enantioselectivity and conversion (see SI). The reactions were carried out with the preliminary conditions defined for the cascade *i.e.* ammonium formate 1 M pH 8.0 with 1% v/v DMSO, nicotinamide cofactor preferred by the AmDH and glucose/GDH cofactor recycling system. The concentration of amine products **3a**–**3m** were quantified with GC calibration curves using standards when commercially available (Table 3, see SI), enantiomeric (*ee*) and diastereomeric excess (*de*) were determined by derivatization (see SI).

Starting from 10 mM substrate, the cascades with the cyclic pentenones (**1a**–**1c**) showed low to moderate amine concentrations (0.49 to 3.20 mM), which is in concordance with the low to moderate activity of *Ts*OYE and AmDHs toward these substrates. In the case of **3b**, very low selectivity was measured, probably due to racemization of **2b** in the reaction mixture. With OYE2 instead of *Ts*OYE we still obtained **3c** in low quantity (0.49 mM, (*1S*) = 99% *de* and (*3S*) = 99% *de*). The cyclic hexenones (**1d**–**1f**) gave reasonable conversions (3.4 to 4.9 mM), in concordance with the conversions rates of 30–83% for both *Ts*OYE toward **1e**–**1f** and *Msme*AmDH/*Micro*AmDH toward **2e**/**2f**,[3a] respectively. Although these conversions remain low, the high diastereoisomeric excess obtained for (1*S*,2*R*)-**3e** ((1*S*) = 99% *de* (2*R*) = 93% *de*) and (1*S*,3*R*)-**3f** ((1*S*) = 99% *de* and (3*R*) = 99% *de*) are remarkable and demonstrate the asset of this cascade (Figure S12). Without the first step, amines **3e** and **3f** were previously reported to be obtained with much worse diastereoisomeric excess, respectively (2*R*) = 36% *de* and (3*S*) = 4% *de* with **2e**/*Msme*AmDH and **3e**/*Micro*AmDH.[3a] Thus, the imperfect stereopreference of AmDHs toward α/β-substituted ketones can be compensated by the high stereoselectivity of *Ts*OYE C25D/I67T, and the subsequent stereochemical control of the amine center is brought by the AmDH. Starting from substituted enones and performing this cascade is so a good strategy to access α/β substituted primary

Table 2. AmDH screening for the reductive amination of saturated carbonyl substrates **2**.[a]

AmDH	[Corresponding amines **3a**–**m**] [mM] **2a**	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**2i**	**2j**[d]	**2k**	**2l**[e]	**2m**[e]
*Cfus*AmDH[b]	3.4		3.4							*nd*	3.4	1.8	3.4
*Chat*AmDH[c]										*nd*	8.2	0.4	0.1
IGCAmDH5[c]										*nd*	3.4	0.6	0.1
*Apau*AmDH[c]										*nd*	10.0	0.6	1.9
MATOUAmDH2[b]	4.1	3.4	0.7	3.5	1.2	3.4	2.3	0.1	3.4	*nd*	5.2	2.2	0.1
*Msme*AmDH[b]		0.8			2.2	3.4	2.3	1.2	1.2				
*Micro*AmDH[b]	2.9	3.4	2.4	4.3	3.7	4.5		1.5		*nd*			0.9

[a] Amounts of amine (mM) obtained with each enzyme toward tested ketones/aldehydes are reported. Colors indicate the favorite enzyme (**purple**), good candidate (**orange**) and other potential candidates (**grey**) for the cascade. Conditions: 0.5 mg/mL AmDH, 10 mM substrate, [b] 0.2 mM NADP or [c] 0.2 mM NAD, 3 U/mL GDH105, 24 mM glucose, 5 mM $CaCl_2$, 1 M NH_4HCO_2 pH 8.0, 24 h at 30 °C, volume = 100 µL in 96-well plate. The amounts of amine **3** were deduced from calibrations curves after derivatization of reaction mixtures with benzoyl chloride and UHPLC-MS analysis. Reported results are average of duplicates. See SI and Figures S8 for results with enones **1**. [d] isobutylamine **3j** was not detected probably due to evaporation of this volatile compound; previously reported results allowed us to select the preferred AmDH for this substrate (data not shown); [e] these conversions are underestimated due to evaporation of the compounds; blank boxes = not tested; *nd* = not detected.

Table 3. Overview of proposed bi-enzymatic cascade OYE-AmDH combinations per substrate.[a]

1	ER	2	AmDH	3	Cofactor	Expected configuration	[Amine] [mM]	*ee* [%]	*de* [%]
1a	*Ts*OYE	**2a**	MATOUAmDH2	**3a**	NADPH	–	3.2	–	–
1b	*Ts*OYE	**2b**	*Msme*AmDH	**3b**	NADPH	(*S*, *R*)	2.7	31	31
1c	OYE2	**2c**	*Micro*AmDH	**3c**	NADPH	(*S*, *S*)	0.5	99	99
1d	*Ts*OYE	**2d**	MATOUAmDH2	**3d**	NADPH	–	4.6	–	–
1e	*Ts*OYE	**2e**	*Msme*AmDH	**3e**	NADPH	(*S*,*R*)	3.4	99	93
1f	*Ts*OYE C25D/I67T	**2f**	*Micro*AmDH	**3f**	NADPH	(*S*,*R*)	4.9	99	99
1g	*Ts*OYE	**2g**	*Msme*AmDH	**3g**	NADPH	(*S*)	7.5	92	–
1h	*Ts*OYE	**2h**	*Micro*AmDH	**3h**	NADPH	–	3.5	–	–
1i	*Ts*OYE	**2i**	*Msme*AmDH	**3i**	NADPH	(*S*)	1.5	99	–
1j	*Ts*OYE	**2j**	*Micro*AmDH	**3j**	NADPH	–	2.4	–	–
1k	GluER	**2k**	*Apau*AmDH	**3k**	NADH	(*R*)	10.0	99	–
1l	*Ts*OYE	**2l**	MATOUAmDH2	**3l**	NADPH	(*R*)	4.2	48	–
1m	*Ts*OYE	**2m**	MATOUAmDH2	**3m**	NADPH	–	0.7	–	–

[a] Conditions: 1 M NH_4HCO_2 buffer pH 8.0, 10 mM substrate, 24 mM glucose, 0.2 mM NAD(P)H, 3 U/mL GDH-105, 10 μM *Ts*OYE or 20 μM OYE2, 0.5 mg/mL AmDH, 1 mL volume. Amine products obtained after 24-hour one-pot bi-enzymatic cascade reaction. Conditions: 1 mL volume containing 1 M NH_4HCO_2 buffer pH 8.0, 3 U/mL GDH-105, 24 mM glucose, 0.2 mM $NAD(P)^+$, 0.5 mg/mL AmDH indicated in Table 3, 10 μM *Ts*OYE or *Ts*OYE-C25D/I67T for **3f**, or 3 μM GluER for **3k**), or 20 μM OYE2 for **3c**, and 10 mM alkene substrate **1**, 1% v/v DMSO. See SI for more results

chiral amines with high controlled chirality. We also produced the opposite enantiomer **3f** by using OYE2 instead of *Ts*OYE C25D/I67T (2.4 mM, >99% *de* for (1*S*,3*S*)-**3f**) The precedent results reported for the synthesis of **3f** by coupling OYE1 and TAs gave a diastereomeric excess *de* of 98% to 99% for (1*S*,3*S*)-**3f** at 78% and 62% conversion with ATA-113 and ATA-237, respectively. In this former study, higher amounts of enzyme were used for the second step (2 mg/mL) and longer reaction time (36 h vs 24 h), but lower amount of OYE were required (60 μg/mL) as OYE2 was active toward **1f** in contrast with *Ts*OYE.

The linear aliphatic unsaturated ketones (**1g**–**1i**) were good to excellent substrates for *Ts*OYE, but poor to moderate for the AmDH chosen, namely *Msme*AmDH and *Micro*AmDH. Nevertheless, 2-aminobutane **3g** was observed with high conversion (75%) and *ee* (92%). The pentylamine **3i** was quantified to 1.5 mM, this low amine amount being certainly due to the already poor conversion obtained with *Msme*AmDH for this substrate at more appropriate pH 9.5 (27%).[3a] We observed an excellent *ee* of 99% for linear aliphatic amines such as 2-aminopentane **3i**, in accordance with the high *ee* reported with the tested AmDH.[3a] Even if the 3-aminopentane **3h** was obtained in only 35%, this result is highly of interest. Indeed,

native enzymes performing the intrinsic or apparent reductive amination (RedAms, AmDHs, TA) are not, or very poorly, active toward substrates harboring larger substituent than methyl for the smaller substituted group. *Micro*AmDH is so a promising enzyme for reductive amination of ethyl-substituted ketones, the same for its use coupled with OYEs on corresponding enones.

Concerning aldehydes, since GluER displayed a higher selectivity for 2-methylbutanal **1k** compared with *Ts*OYE (Figure 3), we performed the cascade with GluER for this substrate, affording full conversion with 99% *ee* (Table 3). We ascribe the excellent *ee* obtained here compared with Figure 2 to the *in situ* conversion of the reduced enal to the corresponding amine, avoiding racemization. Both *Apau*AmDH and GluER led to full conversion on isolated steps. The larger unsaturated aldehydes 2-methylpentenal **1l** and pentenal **1m** were good substrates for GluER and *Ts*OYE, but poor for the AmDHs chosen, thus explaining the lower calculated conversions (42% and 7% respectively). Furthermore, the selectivity obtained for 2-methylpentylamine **3l** was low, also probably because of racemization of intermediate **2l** in the reaction mixture.

No formation of the unsaturated amines **4** was observed in these cascade reactions, which confirms the high chemoselectivity of the AmDHs toward saturated carbonyl substrates **2** versus unsaturated carbonyl substrates **1**, while benefiting from the much higher activity of OYEs compared with AmDHs toward enones and enones/enals **1**. Thus, the EREDs/AmDHs combination can be run in one pot cascade reactions without requiring a sequential procedure.

Interestingly, presence of small amount of alcohol in reactions with substrates **1d**, **1e**, **1i** and **1k**, was observed (data not shown). Formation of alcohol **5d** was quantified by GC-MS to facilitate peak attribution due to partial co-elution. The effective amount of **5d** due to MATOUAmDH2 was less than 0.14 mM in NH_4HCO_2 buffer pH 8.0. At pH 10.0, this amount was similar in reaction mixture containing the enzyme compared to the one without any AmDH (0.17–0.21 mM) (see SI for more details). To minimize the direct reduction of ketone to alcohol while maintaining sufficient activity of OYEs, pH 9.0 was preferred over pH 8.0 for the reaction scale-up and increased the concentration of *Ts*OYE from 2 to 10 μM to compensate its lower activity at this pH (*vide infra*). The presence or absence of alcohol was also dependent on the substrate, certainly due to varying distances and orientations between the carbon atom of the ketone/aldehyde in case of direct reduction or of the iminium intermediate in case of reductive amination. This point was not further studied in this work but was recently described by Mutti and co-workers.[26]

Finally, for a small scale-up synthesis we selected the best substrate 2-methylbutanal **1k** in combination with *Apau*AmDH and GluER. The bi-enzymatic cascade was carried out with 50 mM substrate **1k** in a total volume of 20 mL and afforded 21% conversion (10% isolated yield), after 24 hours, to 2-methylbutylamine **3k** with 99% *ee*. We ascribe the low conversion to non-optimized conditions with the increased substrate concentration. The pure chiral amine was isolated as a hydrochloride salt as previously described, demonstrating the applicability of this bi-enzymatic cascade (see SI).[3a]

Conclusion

In this study, we established a bi-enzymatic cascade with OYE and AmDH enzymes to produce various substituted chiral amines. We explored the capacity of the cascade by determining the reaction conditions of each step, and the enantiopurity, the diastereomeric excess and conversion from substrate to amine. We designed an efficient bi-enzymatic cascade capable of producing chiral amines with high enantioselectivity and diastereomeric excess (both 99%). The lack of or very low activity of AmDHs toward enones is of great advantage to perform an easy protocol of one pot cascade in simultaneous mode. Before considering chiral amine production on a larger scale, there is still room for improvement, as most cascades resulted in amine concentrations of 5 mM from 10 mM substrate. Nevertheless, the combination of these types of enzymes shows potential to produce chiral amines efficiently.

Despite reduced performance of OYEs in reaction medium rich in ammonia required for the AmDH step, high optical purity of amine can be isolated, as exemplified with the synthesis of (2*R*)-2-methylbutanamine obtained with 99% *ee*. These results can give rise to extended amine products by using other members of OYEs and AmDHs families, still in expansion.

This cascade is all the more interesting as the substrates enones are substituted to access to diastereomerically enriched amines. Nevertheless, this can be also beneficial in the case of non-substituted ones. Indeed, enones can be more easily accessible than their reduced form ketones, depending on commercial resources or synthetic schemes. Enones are indeed products of key chemical reactions extensively used in synthetic strategies such as dehydration of aldol or Wittig-Horner reactions.

The portfolio of AmDHs is growing,[13] thus enabling the access of both amine chirality by using either (*S*)-selective AmDHs such as the ones used in this study, or (*R*)-selective AmDHs such as engineered-AADHs and ε-deaminating L-lysine dehydrogenase.[13] This cascade is therefore a real alternative to the already described EREDs/TA cascade and can be extended to structurally diverse amines, again thanks to the wide substrate scope of both OYEs and NAD(P)H-dependent enzymes performing reductive amination.

Experimental Section

General information: All chemicals were obtained from commercial suppliers Sigma-Aldrich, Acros Organics, Alfa Aesar, TCI Europe and abcr GmbH with the highest purity available and used as received. Glucose dehydrogenase GDH-105 was generously donated by Codexis®. UV-vis absorbance was measured with a Cary 60 spectrophotometer. Compound analyses were carried out on Shimadzu GC-2010 gas chromatographs (Shimadzu, Japan) with an AOC-20i Auto injector and equipped with a flame ionization detector (FID), using nitrogen or helium as the carrier gas. GC-MS

analyses were performed on a GC (ThermoFisher Focus GC) coupled to a single-quadrupole mass spectrometer (ThermoFisher DSQ II). The instrument was equipped with a non-polar 30 m × 0.25 mm × 0.25 um DB-5MS column (Agilent) and split/splitless injector. Carrier gas was helium at a constant flow rate of 1 mL/min. Injection and transfer line temperature were set up at 200 °C and 250 °C respectively. MS detection was performed in electronic impact ionization, positive mode, ion source 220 °C, detector voltage 70 eV, full scan mode. Glucose dehydrogenase GDH-105 was generously donated by Codexis®. UHPLC-MS analyses were performed on a UHPLC U3000 RS 1034 bar system (Thermo Fisher Scientific, Waltham, USA) equipped with a DAD3000 diode array detector and a MSQ Plus™ Single Quadrupole Mass Spectrometer with electrospray SI in positive mode (cone voltage = 75 V, probe temperature set up at 450 °C). The UHPLC column was a Kinetex® F5 (Phenomenex) column (100 × 2.1 mm; 1.7 μm)

Example of asymmetric reduction reactions with *Ts*OYE: The reactions were performed in 50 mM Tris-HCl buffer, pH 8.0 in a final volume of 1.0 mL: 10 mM substrate **1**, with either: 0.1 mM NADPH, 11 mM glucose, 5 mg/ml GDH or 11 mM BNAH; 2 μM *Ts*OYE, 1% v/v DMSO; in a thermomixer at 900 rpm and 30 °C for 1 h on a Thermomixer (Eppendorf). Extraction was carried out with 0.5 mL EtOAc, dried over $MgSO_4$ and analyzed by GC-FID.

Example of reductive amination reactions with AmDHs: The selected AmDHs (*Cfus*AmDH, *Chat*AmDH, IGCAmDH5, *Apau*AmDH, MATOUAMDH2, *Msme*AmDH, *Micro*AmDH) were tested toward ketones **2a**–**m** and enones **1a**–**m** in 96-well plate with cofactor regeneration system using UHPLC-MS monitoring. To a reaction mixture (total volume 100 μL) containing 10 mM substrate (10 mM of ketone **2** + 10 mM of enone **1** in case of mixture of substrate), 0.2 mM $NAD(P)^+$, 24 mM glucose, 5 mM $CaCl_2$, 3 U/mL glucose dehydrogenase (GDH-105) in 1 M NH_4HCO_2 buffer pH 8.0, was added 0.5 mg/mL of purified AmDH. The 96-well plate was covered with a lid and left for reaction 24 h at 30 °C in a thermocontrolled oven. Reaction mixtures were analyzed by UHPLC-MS after derivatization with benzoyl chloride. Background reactions were performed in the same manner but with mixtures lacking the substrate or the purified AmDH.

Example of bi-enzymatic cascade reaction: Cascade reactions were performed in 1 M NH_4HCO_2 buffer (pH 8.0, 2 M NH_4OH to adjust the pH) in a total volume of 1 mL: 10 mM substrate **1**, 0.2 mM NAD(P)H, 3 U/mL GDH-105, 24 mM glucose, 0.5 mg/mL AmDH, 3–25 μM OYE, 1% v/v DMSO; in a thermomixer at 400 rpm and 30 °C. After 24 h 1 mL of 10 M NaOH was added and the mixture vortexed, followed by extraction with two times 0.5 mL EtOAc, dried over $MgSO_4$ and analyzed by GC-FID. Derivatisation with Ac_2O was carried out when necessary for *ee* determination on a chiral column.[27]

Author participation

C.E.P. conceived the original idea. C.E.P. and C.V.-V. developed and supervised the project. E.P.J.J., A.F.-J., O.M. carried out the experiments. E.P.J.J., A.F.-J., C.V.V. and C.E.P. analyzed and processed the data. E.P.J.J., C.E.P. and C.V.-V. wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

Acknowledgements

C.E.P. acknowledges an NWO Veni grant (no 722.015.011). R.J.C. Oosten, L. Koekkoek-van der Weel and M.J.F. Strampraad are gratefully acknowledged for their technical assistance. C.V.V acknowledges Rudy Bayakimissa for initial experiments on this project.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Biocatalysis · biocascades · Old Yellow Enzymes · asymmetric reduction · reductive amination

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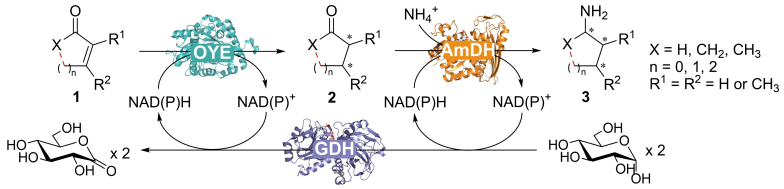
Manuscript received: October 15, 2021
Revised manuscript received: December 2, 2021
Accepted manuscript online: December 3, 2021
Version of record online: ■■, ■■■

RESEARCH ARTICLE



Dynamic duo: The combination of Old Yellow Enzymes and amine dehydrogenases gives access to chiral amines. By combining these enzymes with a cofactor recycling system into a cascade, inexpensive unsaturated aldehydes and ketones were converted into chiral amines with up to two stereocenters and high enantiopurity.

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