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Conversion of alcohols to enantiopure amines through dual enzyme hydrogen-borrowing cascades

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Abstract

α-Chiral amines are key intermediates for the synthesis of a plethora of chemical compounds on industrial scale. Here we present a biocatalytic hydrogen-borrowing amination of primary and secondary alcohols that allows for the efficient and environmentally benign production of enantiopure amines. The method relies on the combination of an alcohol dehydrogenase (ADHs from *Aromatoleum sp., Lactobacillus sp.* and *Bacillus sp.*) enzyme operating in tandem with an amine dehydrogenase (AmDHs engineered from *Bacillus sp.*) to aminate a structurally diverse range of aromatic and aliphatic alcohols (up to 96% conversion and 99% enantiomeric excess). Furthermore, primary alcohols are aminated with high conversion (up to 99%). This redox self-sufficient network possesses high atom efficiency, sourcing nitrogen from ammonium and generating water as the sole by-product.

Amines are amongst the most frequently used chemical intermediates for the production of APIs (active pharmaceutical ingredients), fine chemicals, agrochemicals, polymers, dyestuffs, pigments, emulsifiers and plasticizing agents (1). However, the requisite amines are scarce in nature and their industrial production mainly relies upon the metal-catalysed hydrogenation of enamides (*i.e.* obtained from related ketone precursors), a process requiring transition metal complexes, which are expensive and increasingly unsustainable (2). Moreover, the asymmetric synthesis of amines from ketone precursors requires protection and deprotection steps that generate copious amounts of waste. As a consequence, various chemical processes for the direct conversion of alcohols into amines have been developed during the last decade. The intrinsic advantage of the direct amination of an

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alcohol is that the reagent and the product are in the same oxidation state and therefore, theoretically, additional redox equivalents are not required.However, many of these methods have low efficiency and high environmental impact (*e.g.* Mitsunobu reaction) (3). The amination of simple alcohols such as methanol and ethanol, *via* heterogeneous catalysis, requires harsh conditions (>200 °C) and more structurally diverse alcohols are either converted with extremely low chemoselectivity or not converted at all (4). Furthermore, most of the work in this field involves non-chiral substrates whereas 40% of the commercial optically active drugs are chiral amines (2). Increasingly, biocatalytic methods are applied for the production of optically active amines, *e.g.* the lipase catalysed resolution of racemic mixtures of amines or the ω-transaminase process with a most recent example employing an engineered enzyme applied to the industrial manufacture of the diabetes medication Januvia® (sitagliptin) (5,6,7).

Multi-step chemical reactions in one pot avoid the need for isolation of intermediates and purification steps. This approach leads to economic as well as environmental benefits since time-consuming intermediate work-ups are not required and the use of organic solvents for extraction and purification as well as energy for evaporation and mass transfer is minimised (8). As a consequence, cascade reactions generally possess elevated atom efficiency and potentially lower environmental impact factors (9). The major challenge is to perform cascade reactions wherein an oxidative and a reductive step are running simultaneously. Even more challenging is to carry out a simultaneous interconnected redox neutral cascade wherein the electrons liberated in the first oxidative step are quantitatively consumed in the subsequent reductive step (8,10). This concept is the basis for the hydrogen-borrowing conversion of alcohols (primary or secondary) into amines. The reducing equivalents (*i.e.* hydride) liberated in the first step – the oxidation of the alcohol to the ketone – are directly consumed in the second interconnected step – reductive amination of the ketone. A number of chemo-catalytic hydrogen-borrowing methods have recently been developed using ruthenium as well as iridium catalysts (11,12). Nevertheless, the required reaction conditions (*e.g.* high catalyst and co-catalyst loading, low substrate concentration, moderate chemoselectivity, moderate or total lack of stereoselectivity, the requirement of an excess of substrate, stringent temperature and elevated pressure requirements) complicate the application of these methods on a large scale (13). Another recent hydrogen-borrowing chemical method involves the stoichiometric use of Ellman's enantiopure sulfinamide auxiliary as nitrogen donor in combination with Ru-Macho catalyst (14). Beside the requirement of the expensive chiral auxiliary, the maximum diastereomeric excess was 90%. A reported biocatalytic hydrogen-borrowing amination of alcohols combining three enzymes, namely a ω-transaminase (ωTA), an alcohol dehydrogenase (ADH) and the alanine dehydrogenase from *Bacillus subtilis* (AlaDH), also lacks efficiency due to both the requirement for at least 5 equivalents of L- or D-alanine as the sacrificial amine donor, and also the lower conversion and chemoselectivity for the amination of secondary alcohols (15,16). Another redox neutral biocatalytic cascade was applied for the deracemisation of mandelic acid to enantioenriched L-phenylglycine. However, the method was limited to the conversion of this specific α-hydroxy acid (17).

Here we present a highly enantioselective catalytic hydrogen-borrowing amination of primary as well as secondary alcohols that requires only two biocatalysts, namely an alcohol

dehydrogenase (ADH) and an amine dehydrogenase (AmDH) (Fig 1). The redox self-sufficient cycle uses ammonium ion/ammonia as the source of the nitrogen and generates only water as the by-product. The cascade requires only catalytic quantities of a nicotinamide coenzyme that shuttles hydride from the oxidative step to the reductive step. The method has been successfully applied to: *i*) amination of optically active secondary alcohols with inversion of configuration; *ii*) amination of the corresponding enantiomeric secondary alcohols with retention of configuration; *iii*) asymmetric amination of racemic secondary alcohols; *iv*) amination of primary alcohols.

Initially we examined the catalytic activity of the amine dehydrogenase variant that was recently obtained by protein engineering of the wild-type phenylalanine dehydrogenase from *Bacillus badius* (Ph-AmDH) (18,19). The substrate scope of the Ph-AmDH variant K78S – N277L for the conversion of a broad range of ketone substrates has not been reported; only the reductive amination of *para*-fluoro-phenylacetone (**2b**) was previously described using glucose and glucose dehydrogenase (GDH) for cofactor regeneration and very recently three other ketones were also tested (20). Hence the Ph-AmDH variant K78S – N277L was expressed and purified as His-tagged protein. The activity of the enzyme was initially studied using **2b** as the test substrate in ammonium buffer systems with a range of different counterions (chloride, sulfate, acetate, phosphate, borate, citrate, oxalate and formate). The pH was also varied from 4 up to 11.5 depending on the ammonium buffer employed (Fig. S5). The highest catalytic activity was observed at pH 8.2 to 8.8, whereas the optimal buffer was ammonium chloride. In contrast, previous studies with this enzyme were carried out at pH 9.6 (18). Reductive amination of **2b** (20 mM) was carried out at varying concentrations of NH_4Cl/NH_3 buffer at pH 8.7 using GDH/glucose for cofactor regeneration. Quantitative conversion (>99%) was achieved after 12h using a concentration of *ca.* 0.7 M ammonium buffer (Table S1, Fig. S6).

ADHs have been extensively employed in biocatalysis for the interconversion of ketones and alcohols and hence a wealth of data is available for these enzymes (21). Due to the dependence of Ph-AmDH on NAD as cofactor, we searched for suitable stereocomplementary NAD-dependent secondary ADHs that might exhibit high stability and activity towards a wide range of secondary alcohols at pH >8.5 and also tolerance of high concentrations of ammonium ions. The NAD-dependent Prelog ADH from *Aromatoleum aromaticum* (AA-ADH, previously named as denitrifying bacterium strain EbN1, PDB 2EW8 and 2EWM) (22) and an engineered anti-Prelog ADH from *Lactobacillus brevis* (LBv-ADH, (PDB 1ZK4 for the WT enzyme) were selected for this study (23,24,25). Analysis of the crystal structures of the ADHs with bound NAD(P)H as well as previous docking studies (*i.e.* substrate bound to the enzyme) revealed that the active sites of AA-ADH and LBv-ADH possess a very similar amino acid arrangement, but in an inverted conformation. In particular a tyrosine residue (Tyr 93 for AA-ADH, Tyr 189 for LBv-ADH) is crucial for the stereoselectivity as it protrudes into the active site and forces the substrates to bind with the larger group in the opposite direction. This Tyr residue is in a mirror image position in the active site of the two ADHs (22,25). The amination of alcohol substrate (*R*)**-1a** (20 mM) was carried out initially by combining a crude cell extract of LBv-ADH with purified His-tagged Ph-AmDH in the presence of catalytic NAD^+ (1 mM; 5 mol%) and

in the presence of buffer systems ranging from pH 7 to 8.7. Although formation of the amine product (*R*)**-3a** was observed, the maximum conversion was 6%. In particular, accumulation of the ketone intermediate **2a** was observed (from 61% to 97%, table S2). However, under these conditions, the concentration of ketone **2a** cannot exceed the concentration of the cofactor NAD^+ (1 mM) and hence the accumulation of high levels of **2a** was attributed to the presence of at least one NAD-oxidase from the host organism (*E. coli*) used for the expression of the ADH as previously observed by other groups (26). The NADH-oxidase competes with the AmDH in the amination step for the oxidation of the NADH, leading to accumulation of **2a** (SM and Fig S3). Therefore, the LBv-ADH was purified by ion exchange chromatography (IEC) and size exclusion chromatography (SEC) and combined again with the purified His-tagged Ph-AmDH for the alcohol amination reaction (**1a** concentration 20 mM, NAD^+ 5 mol%). Under these conditions the concentration of the ketone **2a** at the end of the reaction was between 1.6 and 2.9% (< 5% that is the maximum theoretical value). Unfortunately, the final concentration of the amine product **3a** was also very low (<1%). (Table S3). Replacing LBv-ADH with AA-ADH for the amination of (*S*)**-1a** led to the same results. During the course of these preliminary experiments, we noticed that solutions containing the ADH (LBv-ADH or AA-ADH) together with the His-tagged Ph-AmDH tended to become cloudy after a few minutes of the reaction, generating an enzyme precipitate. However, no precipitation occurred even after more than 24h when the ADHs and the His-tagged Ph-AmDH were separately incubated in the same buffer under the same conditions.AA-ADH and LBv-ADH belong to the family of the short chain dehydrogenases/reductases (SDRs). Both ADHs are homotetramers and possess the characteristic Ser-Tyr-Lys catalytic triad of the SDRs. Additionally, the LBv-ADH possesses two Mg^{2+} sites that are placed at the interphase between the monomeric units and are crucial for its stability (25). Whilst the crystal structure of AA-ADH was reported without an evident metal ion, a high homologue was crystallized in a stable form with additional six divalent cations (27). Furthermore, we noticed that stability of LBv-ADH is significantly improved during purification if Mg^{2}+ is added into the buffer, indicating a reversible dissociation process of the cation from the enzyme (see SM 4). Therefore, we speculated that enzyme precipitation in the dual enzyme cascade might have been caused by coordination of free divalent cations, coming from the ADHs, to the His-terminal tag of the Ph-AmDH. . As a consequence, enzyme aggregation and precipitation occurred. Hence, the His-tag was cleaved from the Ph-AmDH using a highly selective recombinant thrombin (SM and Fig. S7). Incubation of the two purified ADHs with the Ph-AmDH devoid of His-tag resulted in visually more stable systems wherein enzyme aggregation and precipitation was not observed even after 24h.

The hydrogen-borrowing cascade was then repeated by combining AA-ADH with the Ph-AmDH (devoid of His-tag) in ammonium chloride buffer at pH 8.7. AA-ADH is selective for (*S*)**-1a** whereas Ph-AmDH shows (*R*)-selectivity in the reduction of the intermediate **2a**; hence the overall cascade was expected to proceed with inversion of configuration. The reaction was tested at various concentrations of ammonium ions in order to ascertain the impact on the conversion. Under the following reaction conditions [(*S*)**-1a** = 20 mM, NAD^+ 1 mM and NH_4^+/NH_3 2 M], the conversion of alcohol to amine reached 85% after 24 h with an ee of >99% (*R*) (Table S4). Monitoring the progress of the reaction revealed a

maximum conversion in excess of 93% after 3 d (Fig. 2 and Tab. S5). Increasing the concentration of ammonia up to 4 M, led to a slight increase in conversion (95%, Tab. 1 entry 1 and Tab. S6-8). Addition of further aliquots of AA-ADH, Ph-AmDH and NAD^+ after 2d gave no further increase in conversion, indicating that the thermodynamic equilibrium had been reached. To improve the catalytic efficiency of the cascade, the concentration of the NAD^+ was reduced 5-fold to 0.2 mM (1 mol%), which resulted in a slight drop in conversion to 76% (Table S5 and Fig. S9,10).

Surprisingly, when the same reaction conditions were applied to the amination of (*R*)**-1a**, (20 mM) using LBv-ADH with the Ph-AmDH (minus His-tag), the conversion to amine was <4% (Table S9). We speculated that the instability of the LBv-ADH in ammonium chloride buffer at pH 8.7 might be the origin of the low conversion and hence lower pH values were investigated. For ammonium chloride buffers, pH values <8.5 cannot be attained and hence ammonium formate buffer was investigated at various pH values (28). At pH 8 to 8.5, the amination of (*R*)**-1a** (20 mM) was achieved in 93% conversion and >99% ee (Table 1, entry 15). The cascade was then run by combining both the stereocomplementary ADHs with the Ph-AmDH in one pot for the asymmetric amination of racemic **1a**, affording (*R*)**-3a** in >99% ee and 81% conversion (Table 1, entry 29).

The hydrogen-borrowing cascade was initially tested on a limited number of 1-phenyl-2-propanol derivatives **1a-1e** (Table 1) for amination with inversion of configuration (entries 1-5), retention of configuration (entries 15-19) and asymmetric amination of racemic alcohols (entry 29-33). Conversion varied from moderate to excellent, whereas the ee was excellent in almost all cases.

Whilst ADHs generally possess broad substrate specificity, the Ph-AmDH accepts solely phenylacetone and phenylacetaldehyde derivatives with elevated turnover numbers. Nevertheless, the generation of chimeric enzymes through domain shuffling from different parents can rapidly lead to new enzymes with increased activity or different and extended substrate specificity. The amino acid sequence of a stable chimeric AmDH (Ch1-AmDH) has been recently published, although its substrate scope and stereoselectivity have not been elucidated (29). Thus the Ch1-AmDH devoid of His-tag was combined with the previously selected ADHs for the amination of a much broader panel of alcohols **1f-1s**. Aromatic substrates **1f-1h** bearing the phenyl ring in the α- (Table 1, entries 6, 20, 34) and β- position (Table 1, entries 7,8,21,22,35,36), relative to the secondary alcohol, as well as phenylethanol derivatives **1i-1n** with substituents in *ortho-, meta-* and *para-* positions (Table 1, entries 9-14, 23-28, 37-42) were aminated with 99% ee (*R*) and conversions ranging from moderate to high. The only exception was alcohol 1g that was aminated with lower enantioselectivity (82 or 83% ee: Table 1, entries 7, 21, 35). For this particular substrate, the progress of ee was monitored as a function of time (Table S14 and Fig. S12). The enantiomeric excess of the amine **3g** remained constant during the time demonstrating that longer incubation times are not detrimental to the stereoselective outcome of the process. All the aliphatic secondary alcohols **1o-1s** examined (medium, long and branched chain) were aminated with perfect ee and high conversions up to 96% (Table 2).

The hydrogen-borrowing amination is an extremely efficient and valuable method for the generation of optically active amines from alcohols. However, achiral terminal primary amines are also in high demand by the chemical industry, especially for the production of polymers and plasticising agents (1). To demonstrate the broad applicability of the methodology, the amination of different primary alcohols was accomplished by combining the primary hT-ADH from *Bacillus stearothermophilus* (30) with either the Ch1-AmDH (Table 3, entry 1-6) or the Ph-AmDH (Table 3, entry 7). Quantitative conversion to the amine product was obtained with alcohols **1u–1x**.

Finally the bioamination of five representative substrates - one for each structural category reported in figure 1 - was carried out on a preparative scale, demonstrating therefore the practical application of the methodology. Starting from (*S*)**-1a** as the alcohol substrate, the conversion into the amine product (*R*)**-3a** reached 93% after 48h. The work-up consisted of extraction of the unreacted alcohol and ketone intermediate under acid conditions followed by the extraction of the amine product under basic conditions (see SM for details). The isolated yield of pure (*R*)**-3a** was 85% (99% ee). Following the same protocol, substrates (*S*)**-1g**, (*S*)**-1i**, (*S*)**-1q** and **1u** were converted to the corresponding amines with 89%, 31%, 95% and >99% conversion, respectively. The isolated yields of pure (*R*)**-3g**, (*R*)**-3i**, (*R*)**-3q** and 3u were 78%, 30%, 91% and 91%, respectively. The enantiomeric excesses remained the same as for the experiments on analytical scale.

In summary, we have developed a dual enzyme hydrogen borrowing process that enables the asymmetric amination of a broad range of secondary alcohols to afford the corresponding (*R*)-configured amines in high enantiomeric excess. Furthermore, amination of primary alcohols proceeded in up to quantitative conversion. The biocatalytic system utilizes ammonia as the simplest amine donor and generates water as the sole innocuous by-product. Ongoing studies are currently aimed at extending the substrate scope of the cascade through further protein engineering of AmDHs capable of aminating a wide range of more complex alcohols with elevated stereoselectivity. Whilst only enantiopure (*R*)-configured amines have been generated until to date, the engineering of stereocomplementary AmDHs (*S*-selective) starting from D-amino acid dehydrogenases as scaffolds will complement the scope of our hydrogen borrowing process. Finally, the use of lower concentrations of ammonia may be possible by the addition of further enzymes to derivatise the amine products and hence provide a thermodynamic driving force for the amination step.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One Sentence Summary

A two-enzyme, redox self-sufficient, stereoselective biocatalytic process is described for the conversion of alcohols to amines.

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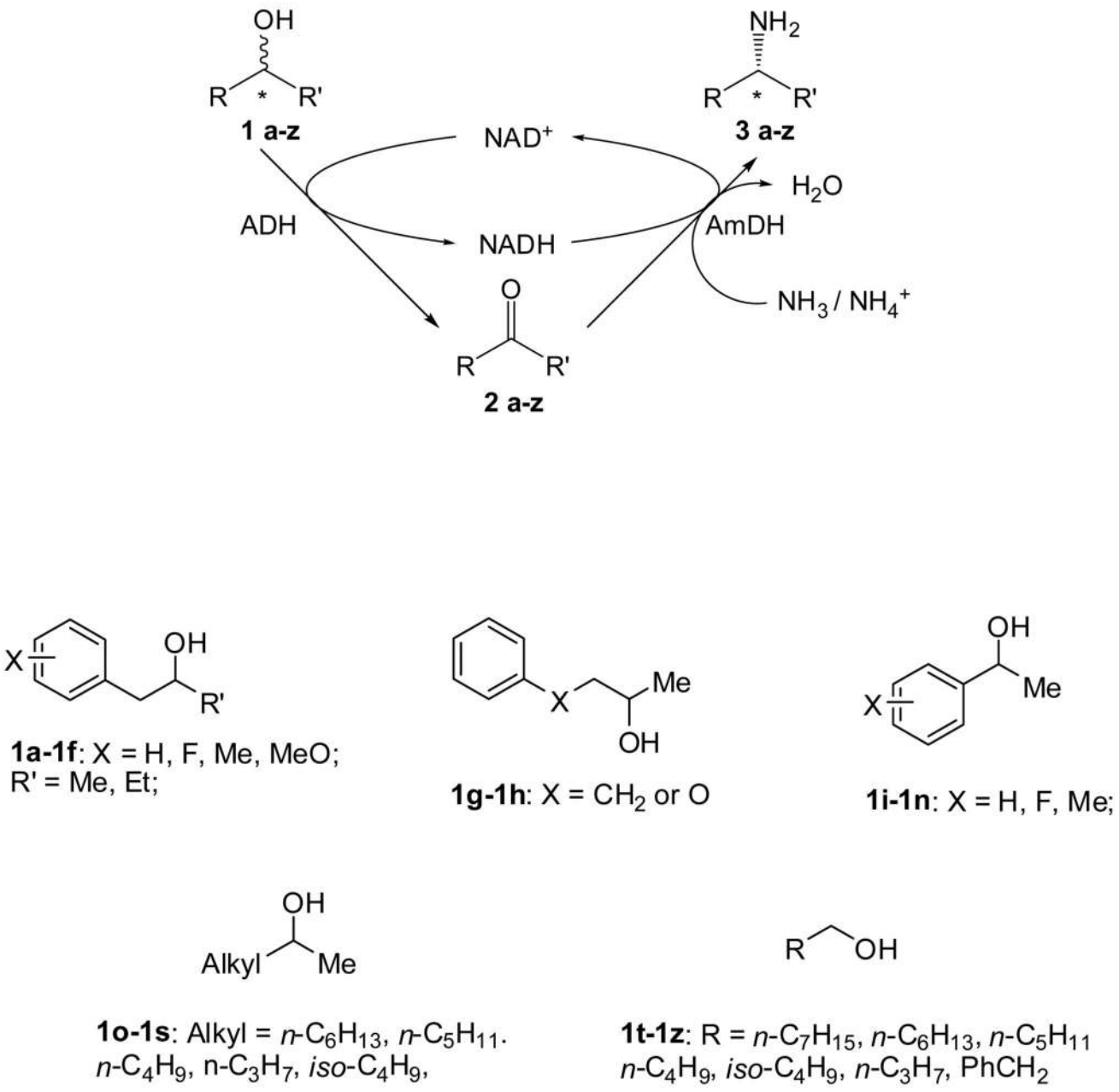

Figure 1.

Two-enzyme cascade for the hydrogen-borrowing amination of alcohols. In the first oxidative step, the Prelog AA-ADH and the anti-Prelog LBv-ADH were applied for the oxidation of the (*S*)- and (*R*)-configured alcohol substrates, respectively. The AmDHs used in this study afforded the (*R*)-configured amines in the second reductive step. Alcohol substrates explored in this study are shown below the schematic catalytic cycle.

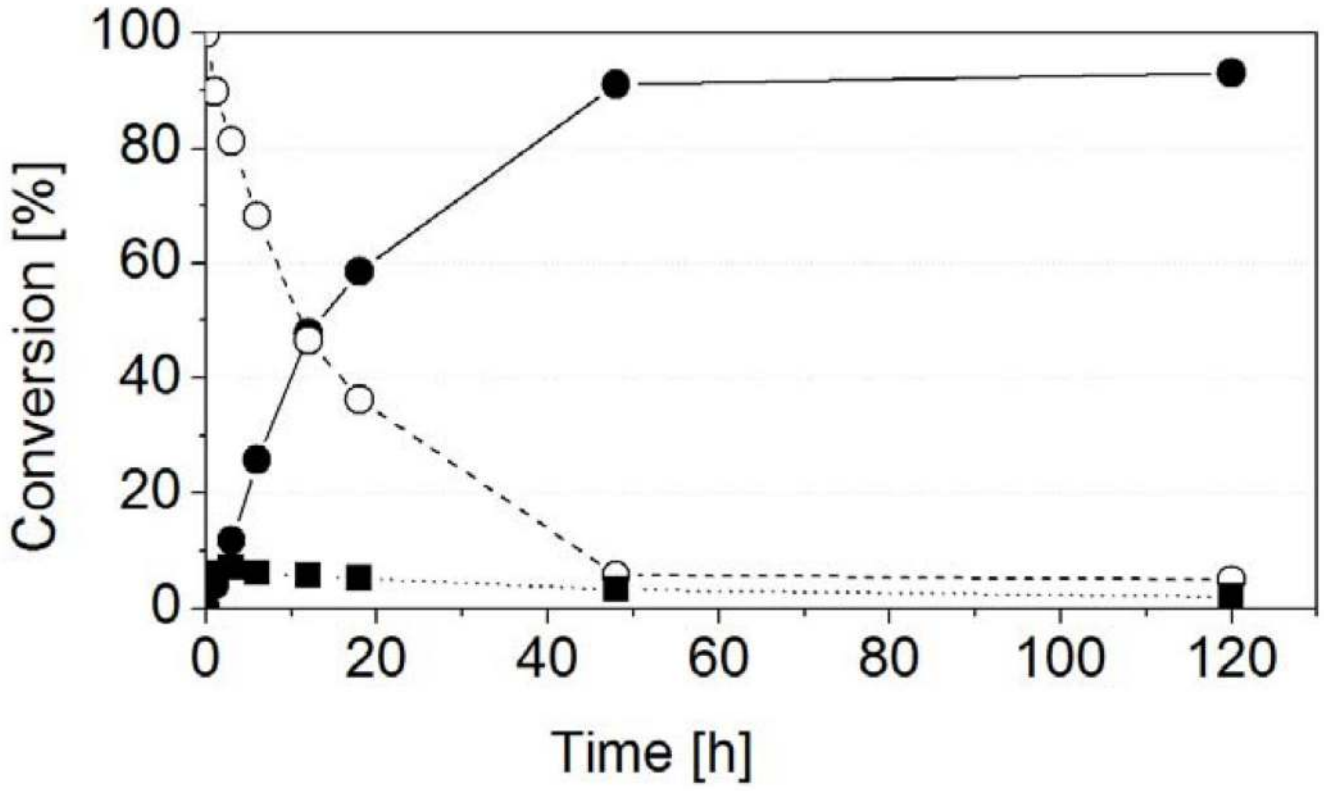

Fig. 2.

Kinetics of asymmetric hydrogen-borrowing biocatalytic amination. The reaction of (*S*)-**1a** (20 mM) gives inverted (*R*)-**3a** using AA-ADH and Ph-AmDH with catalytic NAD^+ (1 mM; 5 mol%). Concentrations of the amine product (solid line, black circles), ketone intermediate (dotted line, black squares) and alcohol substrate (dashed line, white circles) were monitored over time. As expected, the concentration of the ketone intermediate **2a** remains constant and below the concentration of the nicotinamide coenzyme. For details, see SM paragraph S12.

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Table 1

Asymmetric hydrogen-borrowing amination of enantiopure aromatic secondary alcohols **1a-1n**. Reactions were carried out at 30 °C for 48 h. For experimental details see SM paragraph S17.

Amination of aromatic chiral secondary alcohols 1a-1n with inversion of configuration				**Amination of aromatic chiral secondary alcohols 1a-1n with retention of configuration**				**Asymmetric amination of aromatic racemic secondary alcohols 1a-1n**			
Entry	**Substrate**	**Conv [%]**	**ee [%]**	**Entry**	**Substrate**	**Conv. [%]**	**ee [%]**	**Entry**	**Substrate**	**Conv. [%]**	**Ee [%]**
1	(*S*)-**1a**	95	>99(*R*)	15	(*R*)-**1a**	93	>99(*R*)	29	Rac-**1a**	81	>99(*R*)
2	(*S*)-**1b**	93	>99(*R*)	16	(*R*)-**1b**	36	>99(*R*)	30	Rac-**1b**	66	>99(*R*)
3	(*S*)-**1c**	55	97(*R*)	17	(*R*)-**1c**	27	97(*R*)	31	Rac-**1c**	47	97(*R*)
4	(*S*)-**1d**	65	>99(*R*)	18	(*R*)-**1d**	24	>99(*R*)	32	Rac-**1d**	78	>99(*R*)
5	(*S*)-**1e**	31	82(*R*)	19	(*R*)-**1e**	14	82(*R*)	33	Rac-**1e**	30	82(*R*)
6	(*S*)-**1f**	80	>99(*R*)	20	(*R*)-**1f**	85	>99(*R*)	34	Rac-**1f**	87	>99(*R*)
7	(*S*)-**1g**	92	82(*R*)	21	(*R*)-**1g**	92	83(*R*)	35	Rac-**1g**	92	83(*R*)
8	(*S*)-**1h**	96	>99(*R*)	22	(*R*)-**1h**	94	>99(*R*)	36	Rac-**1h**	84	>99(*R*)
9	(*S*)-**1i**	17	>99(*R*)	23	(*R*)-**1i**	30	>99(*R*)	37	Rac-1i	16	>99(*R*)
10	(*S*)-**1j**	14	>99(*R*)	24	(*R*)-**1j**	17	>99(*R*)	38	Rac-**1j**	16	>99(*R*)
11	(*S*)-**1k**	26	>99(*R*)	25	(*R*)-**1k**	33	>99(*R*)	39	Rac-**1k**	20	>99(*R*)
12	(*S*)-**1l**	12	>99(*R*)	26	(*R*)-**1l**	18	>99(*R*)	40	Rac-**1l**	12	>99(*R*)
13	(*S*)-**1m**	14	>99(*R*)	27	(*R*)-**1m**	27	>99(*R*)	41	Rac-**1m**	19	>99(*R*)
14	(*S*)-**1n**	7	>99(*R*)	28	(*R*)-**1n**	14	>99(*R*)	42	Rac-**1n**	9	>99(*R*)

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Table 2

Asymmetric hydrogen-borrowing amination of aliphatic secondary alcohols **1o-1s**. Reactions were carried out at 30 °C for 48 h. For experimental details see SM paragraph S17.

Amination of aliphatic chiral secondary alcohols with inversion of configuration				**Amination of aliphatic chiral secondary alcohols with retention of configuration**				**Asymmetric amination of aliphatic racemic secondary alcohols**			
Entry	**Substrate**	**Conv. [%]**	**ee [%]**	**Entry**	**Substrate**	**Conv. [%]**	**ee [%]**	**Entry**	**Substrate**	**Conv. [%]**	**ee [%]**
1	(*S*)-**1o**	94	99(*R*)	6	(*R*)-**1o**	91	>99(*R*)	11	Rac-**1o**	93	99(*R*)
2	(*S*)-**1p**	95	99(*R*)	7	(*R*)-**1p**	79	99(*R*)	12	Rac-**1p**	96	99(*R*)
3	(*S*)-**1q**	95	>99(*R*)	8	(*R*)-**1q**	83	>99(*R*)	13	Rac-**1q**	95	>99(*R*)
4	(*S*)-**1r**	74	>99(*R*)	9	(*R*)-**1r**	73	>99(*R*)	14	Rac-**1r**	66	>99(*R*)
5	(*S*)-**1s**	88	99(*R*)	10	(*R*)-**1s**	80	99(*R*)	15	Rac-**1s**	80	>99(*R*)

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Table 3

Hydrogen-borrowing amination of primary alcohols **1t-1z**. Reactions were carried out at 30 °C for 48 h. For experimental details see SM paragraph S17.

Entry	Substrate	Conv. [%]
1	**1t**	8
2	**1u**	99
3	**1v**	99
4	**1w**	99
5	**1x**	99
6	**1y**	61
7	**1z**	10