Two anti-Prelog NAD-Dependent Alcohol Dehydrogenases with Broad Substrate Scope and Excellent Enantioselectivity

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Two *anti*-Prelog NAD-Dependent Alcohol Dehydrogenases with Broad Substrate Scope and Excellent Enantioselectivity

Matteo Damian[a] and Francesco G. Mutti*[a]

Enantiomerically pure alcohols are important to produce active pharmaceutical ingredients, agrochemicals and fine chemicals. Herein, we explored the substrate scope and chemo- and enantioselectivity of two NAD-dependent anti-Prelog alcohol dehydrogenases from *Candida maris* (Cm-ADH) and *Pichia finlandica* (Pf-ADH) in the asymmetric reduction of ketones. The ADHs were tested for the reduction of acetophenone with NADH that was recycled using a formate dehydrogenase and sodium formate. Cm-ADH and Pf-ADH performed best at 30 °C and at around pH 7 and pH 6, respectively. Pf-ADH operated well at 50 mM acetophenone concentration, while Cm-ADH was limited to 10 mM. Regarding the substrate scope, linear-chain alkyl ketones were efficiently reduced (up to 98 % conversion), while branched and cyclic ketones gave lower conversions (up to 60 %). Aryl-aliphatic ketones showed variable levels of conversion (<1–79 %), while α,β-unsaturated and heteroaromatic ketones exhibited good to excellent conversions. In most of the cases, the enantiomeric excess was >99 %. Aliphatic and aryl-aliphatic aldehydes were converted with up to >99 % conversion. A scale-up experiment with Pf-ADH using acetophenone as substrate led to 73 % isolated yield and >99 % *ee* (*R*).

This work contributes to filling the gap in biocatalytic asymmetric synthesis of chiral alcohols by introducing two NAD-dependent ADHs with anti-Prelog selectivity.

Introduction

Synthesis of enantiopure molecules from prochiral substrates is of pivotal importance in chemistry. In particular, enantiomerically pure alcohols are of great interest for the production of active pharmaceutical ingredients, agrochemicals and fine chemicals.[1] Chiral alcohols can be synthesised in laboratory and industrial scale using organocatalysis,[2] metallorganic catalysis,[3] or biocatalysis.[4] In this context, biocatalysis allows for overcoming the common limitations of chemocatalysis in terms of simultaneous chemo-, regio- and stereoselectivity.[5] For instance, kinetic resolution (KR) catalysed by lipases is a common industrial biocatalytic approach to synthesise chiral alcohols; however, only a maximum of 50 % theoretical yield is attainable in this way.[6] This limitation can be circumvented by dynamic kinetic resolution (DKR) that combines the racemisation of the alcohol substrate with a simultaneous enantioselective acylation reaction catalysed by a hydrolase (e.g., a lipase).[1d,7]

Otherwise, the asymmetric reduction of prochiral ketones catalysed by alcohol dehydrogenases (ADHs), also called keto-reductases (KREDs) in organic synthesis, represents a more atom-efficient and sustainable alternative for the synthesis of optically pure alcohols with high yields and enantioselectivity.[8] ADHs require a coenzyme as hydride donor, that is either the nicotinamide adenine dinucleotide (NAD(H)) or its phosphate analogue (NADP(H)). Notably, in a reduction catalysed by alcohol dehydrogenases, there are four possible events in the transfer of the hydride from NAD(P)H to the prochiral ketone. In fact, the nicotinamide moiety of NAD(P)H has two potential hydrogen atoms that can act as the departing hydride: the pro-(*S*) and the pro-(*R*) depending on their position in the heterocyclic ring. Notably, only one of these hydrogen atoms – depending on the inherent 3D structure of the ADH's active site – can serve as the hydride for substrate reduction. Regardless of that, the hydride attack can occur either to the *Re* or the *Si* face of the carbonyl, again depending on the 3D structure of the enzyme and the productive binding mode(s) between enzyme, substrate and coenzyme (Figure 1).

Most of the wild-type ADHs follow the Prelog's rule[9] (see example depicted in Scheme 1) that can be illustrated by considering a typical substrate possessing a bulkier group on one side of the carbonyl moiety (e.g., the phenyl ring in acetophenone) and a small substituent on the other side (e.g., the methyl group in acetophenone). If the Prelog's rule is fulfilled, this means that the *Re* face of the acetophenone is attacked by the hydride, thereby leading to the (*S*)-configured alcohol as depicted in Scheme 1. Conversely, if an ADH yields the (*R*)-configured 1-phenylethan-1-ol, it is classified as anti-Prelog.

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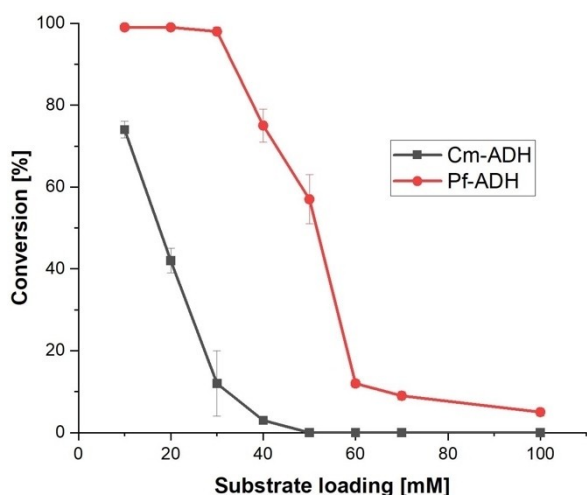European Chemical
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Figure 1. Initial screening conducted on Cm-ADH and Pf-ADH to test their productivity at different loadings of **1 a** (10—100 mM, added as 2.5 M stock solution in DMSO) in Tris-HCl buffer (100 mM, pH 8). Error bars represent the standard deviation obtained from three tests.

As mentioned above, another important distinction between ADHs is the preference for the coenzyme that can be NADH or NADPH. In general, most of the known natural ADHs are classified as Prelog and comprise both NAD- and NADP-dependent enzymes.[8c–e] In contrast, anti-Prelog ADHs are far less ubiquitous in nature and almost all of them are NADP(H) dependent.[8d,10] For instance, a NAD-dependent anti-Prelog pseudoephedrine dehydrogenase from *Arthrobacter* sp. TS-15 was found to be active on 1-phenyl-1,2-propanedione but with high substrate specificity.[11] Another NAD-dependent anti-Prelog ADH was identified in *Leifsonia* sp. strain S749 and applied in cascade reactions.[12] This common NADP(H)-dependence for anti-Prelog ADHs results in a less attractive applicability because of the ca. four-times higher cost of NADP$^+$ compared with NAD$^+$.[13] However, anti-Prelog ADHs are important as they can give access to the complementary alcohol enantiomer (e.g., like the (*R*)-configured one in the case of the reduction of acetophenone as model substrate).[10e,14] For this reason, anti-Prelog ADHs have been engineered in the past to switch the co-enzyme acceptance from NADPH to NADH such as in the

case of the ADH from *Lactobacillus brevis* or other dehydrogenases.[15] The other option is to turn a NAD-dependent Prelog-ADH into an anti-Prelog one.[14f,16] However, this latter approach commonly leads to variants with imperfect stereoselectivity. Although both strategies have led to the generation of synthetically applicable anti-Prelog NAD-dependent ADHs, the toolbox of anti-Prelog ADHs is still insufficient in terms of substrate scope and enantiomerical purity of the final product compared with the most available Prelog-enzyme counterparts. Consequently, the discovery or characterisation of available anti-Prelog NAD-dependent ADHs is still important to fill this gap in biocatalytic asymmetric synthesis of ketones.

Herein, we studied the substrate scope and chemo- and stereoselectivity of two NAD-dependent ADHs from yeasts such as *Candida maris*[17] and *Pichia finlandica*[18] for the reduction of a broad range of structurally diverse ketones. Many of the tested substrates are of interest for API manufacture. In fact, in previous reports from the biochemistry literature, both enzymes exhibited anti-Prelog selectivity towards the ketones that were tested for specific activity. Therefore, a detailed investigation was necessary to determine the optimal reaction conditions and the substrate scope of these ADHs.

Results and Discussion

The ADHs from *Candida maris* (Cm-ADH) and from *Pichia finlandica* (Pf-ADH) were initially tested for the reduction of the acetophenone (**1 a**) to yield the corresponding 1-phenylethan-1-ol (**1 b**). A catalytic amount of NAD$^+$ was added and internally recycled using a formate dehydrogenase from *Candida boidinii* (Cb-FDH) and sodium formate as the hydride source. ADHs and Cb-FDH were overexpressed as His$_6$-tagged enzymes in *E. coli* BL21(DE3) cells and used in purified form.

The first tests were conducted in Tris-HCl buffer (100 mM, pH 8) with **1 a** (10 mM), ADH (25 μM), Cb-FDH (20 μM), NAD$^+$ (0.5 mM) and HCOONa (50 mM) at 30 °C (Scheme 2).

A screening of the substrate loading showed that Pf-ADH gives higher productivity compared with Cm-ADH (Figure 1 and SI Tables S1 and S6). In fact, Cm-ADH could perform the reaction to high yield (73%) only up to a 10 mM concentration
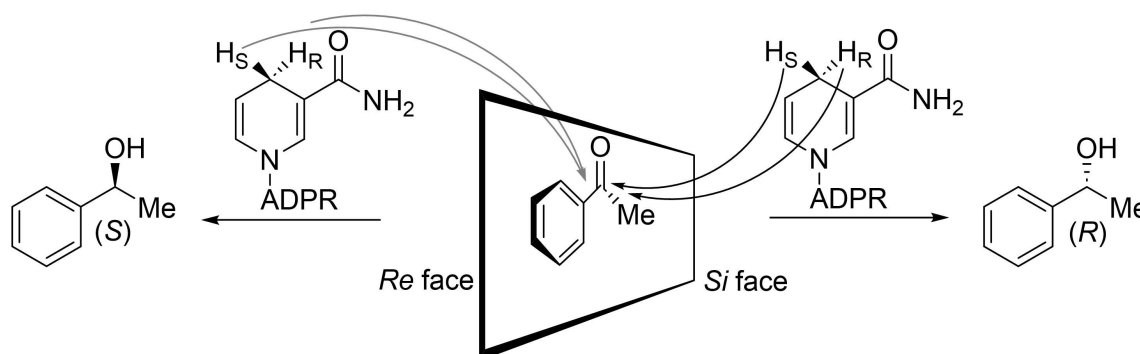
Scheme 1. The four possible events in the selective attack of the hydride from NAD(P)H to the carbonyl substrate in the active site of an ADH.

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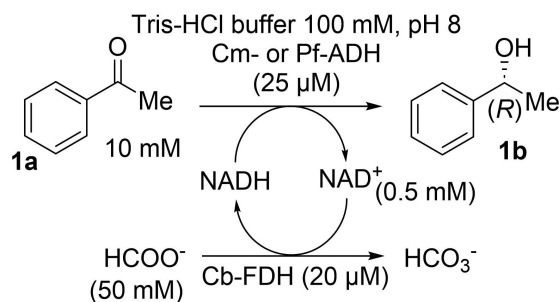
Scheme 2. Asymmetric reduction of acetophenone (**1a**, 10 mM) catalysed by NAD-dependent anti-Prelog ADHs (25 μM) in the presence of a catalytic amount of NAD$^+$ (0.5 mM) recycled with a formate dehydrogenase (Cb-FDH, 20 μM) and sodium formate (50 mM) as the ultimate hydride donor.

of **1a**, while Pf-ADH led to complete conversion at 30 mM of **1a** and yielded a 75% conversion at 40 mM of **1a**.

Next, we investigated the influence of the pH on the catalytic activity for the conversion of **1a** (10 mM for Cm-ADH and 40 mM for Pf-ADH, Figure 2 and SI, Tables S2 and S7). We conducted these experiments using Britton–Robinson (BR) buffer[19] because it enables testing of a broader range of pH without altering substantially the composition of the species in solution, which is not the case with other buffers. In fact, BR buffer has a wide stability between pH 2 and 12.

Cm-ADH and Pf-ADH showed distinct behaviours. While the Cm-ADH led to the best result at pH 7, Pf-ADH showed the best performance around pH 6. Notably, Pf-ADH led to complete conversion of **1a** in the range between pH 5.5 and 6.5; therefore, the substrate loading was increased from 40 to 50 mM for the reactions with Pf-ADH, leading to the 87% conversion at pH 6.0 as the best result.

However, the conversions obtained at pH 8 in BR buffer were significantly different from the conversions obtained in Tris-HCl at the same p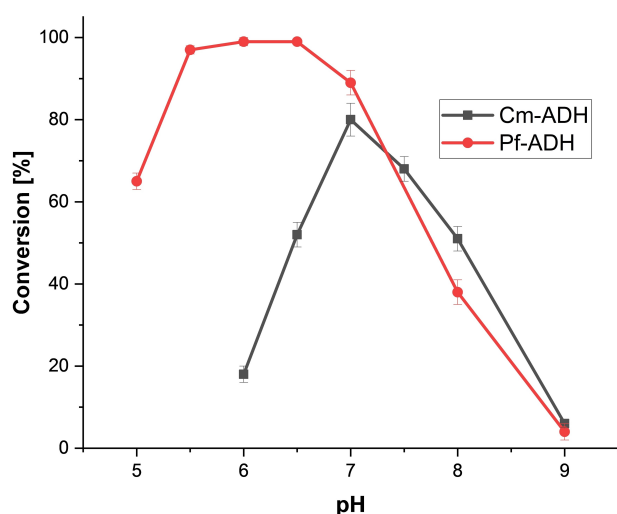H. Therefore, both buffer pH and composition are important to maximise the catalytic performance of the enzymes. For this reason, we tested the reactions with four different buffers (KPi, Tris, MOPS and BR) at pH values around the optimal pH determined in the previous tests (SI, Table S3 and Table S8).

Cm-ADH afforded 89% conversion for the reaction at 10 mM concentration of **1a**, at 30°C in KPi buffer (100 mM pH 7.5) as the best result (TON = 356). In contrast, Pf-ADH yielded 87% conversion at 50 mM concentration of **1a** in BR buffer (60 mM pH 6.0) as the best result (TON = 1740).

Next, we tested the reaction with the optimal conditions but changed the buffer concentration (50, 100 and 150 mM KPi buffer for Cm-ADH and 60, 100, 150, 200 and 250 mM BR buffer for Pf-ADH). For Pf-ADH, increasing the buffer concentration led to lower conversions, while Cm-ADH afforded the highest conversion with 100 mM KPi buffer (SI, Table S4 and Table S9).

Next, we investigated the influence of the temperature for both ADHs by testing the reactions at 20, 25, 30, 35 and 40°C (SI, Table S5 and Table S10). In general, Pf-ADH catalysed the reaction faster than Cm-ADH under the respective optimised reaction conditions. At 10 mM of **1a**, Cm-ADH showed the highest conversion (55%) after 3 h at 40°C. However, the enzyme proved to be not very stable at 40°C since the highest conversion (89%) was obtained after 22 h at 30°C. In fact, at 25, 30 and 35°C, the conversion was lower after 3 h but it increased after 22 h if compared with the same experiments at 40°C. In summary, the reaction at 30°C was the compromise between activity and stability of Cm-ADH by reaching 89% conversion after 22 h (TON = 356). Pf-ADH was catalytically more active than Cm-ADH since 2 h were required for the reaction to reach the highest conversion in all the tests conducted at 50 mM of **1a**. The best result with Pf-ADH was obtained at 30°C with a conversion of 87% after 22 h (TON = 1740).

Under the best identified reaction conditions, we performed a detailed study in which the progress of the conversion was monitored during the time (Figure 3 and SI, Figure S4, Figure

Figure 2. pH screening for the asymmetric reduction of **1a** catalysed by Cm-ADH (pH 6 to 9) and Pf-ADH (pH 5 to 9) using Britton–Robinson buffer. Error bars represent the standard deviation obtained from three tests.
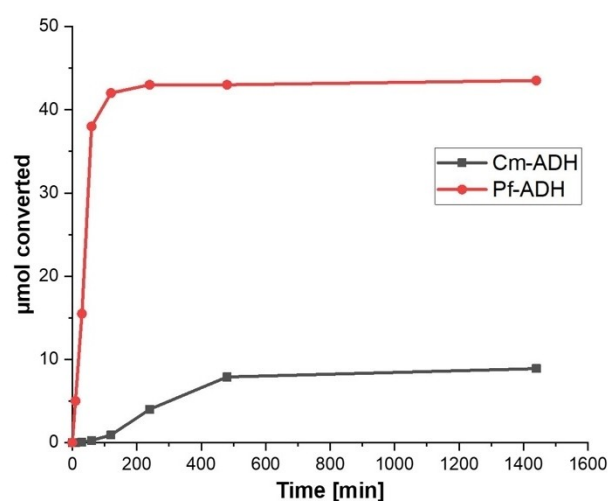

Figure 3. Productivity for the reduction of **1a** (10 mM and 50 mM, respectively) catalysed by Cm-ADH and Pf-ADH depicted as μmol converted vs. time. The reactions were performed under the optimised conditions.

S5). The biotransformation with Cm-ADH plateaued at 89% conversion of **1a** (10 mM) after 10 h, while the reaction catalysed by Pf-ADH was much more rapid, leading to a final conversion of 87% for **1a** (50 mM) after only 2 h.

For Pf-ADH, we investigated whether the reaction stopped at ca. 87% conversion after a certain time because of a deactivation of the ADH or a possible inhibition at a certain product concentration (for details, see SI section 11). Therefore, we pre-incubated Pf-ADH for different times, then added the substrate and studied the progress of reaction afterwards (SI, Table S14). Pre-incubation for 1, 2, 4 or 6 h followed by the start of the reaction gave statistically the same conversion values after 10 min, 30 min, 1 h, 2 h and 22 h reaction time. In contrast, pre-incubation for 24 h followed by the start of the reaction gave a three- to five-times lower conversion at the different time points, compared with the reaction after pre-incubation for 6 h. Pre-incubation for 48 h abolished almost any catalytic activity since 1% conversion was obtained after 10 min reaction time and remained the same afterwards. In another set of experiments, we ran the reaction for 2 h with a first aliquot of substrate and formate salt (50 mM, each), then added a second aliquot of both (another 50 mM, each) and ran the reaction for a further 2 h. The results demonstrated that the second aliquot of substrate was not converted in the next 2 h (SI, Table S15). In the last set of experiments, we ran the reaction for 2 h at 50 mM concentration of **1a** by using a first aliquot of Pf-ADH (25 μM) and Cb-FDH (20 μM); then, other aliquots of both enzymes were added, and the reaction was run for an additional 2 h. In this way, substrate **1a** was quantitatively converted to the **1b**, while the blank experiment only using one aliquot of the enzymes yielded 87% conversion (SI, Table S16). These studies showed that the incomplete conversion was due to enzyme deactivation. We concluded that the catalytic performance of Pf-ADH could be increased by enzyme engineering aimed at increasing its stability at the relevant reaction conditions.

To further extend the applicability of both ADHs, the reaction was tested using lyophilised whole cells overexpressing the ADHs under the optimised conditions. Cm-ADH led to 32% conversion, while Pf-ADH yielded a better result with a 65% conversion. In both cases, the conversion remained almost the same regardless of the amount of whole cells (SI, Table S11 and Table S12). Finally, we also tested the recycling of the coenzyme using the couple-substrate approach by using isopropanol as the hydride source; thus, the isopropyl alcohol was oxidised to acetone as the coproduct. Cm-ADH catalysed the reduction of **1a** using isopropanol (13–131 eq.) as a sacrificial hydride donor (SI, Table S13), leading to similar results as those obtained by applying the couple-enzyme approach (i.e., Cb-FDH with formate). In contrast, Pf-ADH did not lead to any product, thereby showing that it cannot accept isopropanol.

Finally, we explored the applicability of both enzymes in organic synthesis by testing them on a panel of methyl-aryl ketones containing electron-donating (EDG, such as methoxy and methyl) or electron-withdrawing (EWG, such as halogens) groups on the ring. It is important to note that the results reported in Table 1 were obtained at 10 mM and 50 mM

substrate concentration for Cm-ADH and Pf-ADH, respectively, since the latter ADH well tolerated the higher substrate concentration of **1a** (Figure 3). In general, we noticed that the presence of a substituent group on the *ortho*-position of the ring led to low or no conversion (**5a**, **8a** and **11a**, Table 1, entries 5, 8 and 11). This behaviour is very likely due to the increased steric hindrance in proximity to the carbonyl moiety that hampers the formation of a productive ternary complex between enzyme's active site, cofactor and substrate. In fact, only the *ortho*-fluoro acetophenone (**14a**) was reduced with an excellent conversion by Cm-ADH (84% conversion), most likely due to the similar size (i.e., van der Waals radius) between the hydrogen and fluorine as substituent. However, Pf-ADH had a much lower capability to reduce **14a** (6% conversion), possibly showing that the small volume difference between fluorine and hydrogen is already significant for this ADH (Table 1, entry 14).

Substitution in *meta*- or in *para*-position did not show a specific trend. For instance, *para*-hydroxy-acetophenone (**2a**) gave higher conversions than the *meta*-substituted analogue (**3a**) with both Cm-ADH and Pf-ADH (Table 1, entries 2 and 3). The behaviour was the opposite with *para*-chloro-acetophenone (**6a**) and the *meta*-substituted analogue (**7a**), and with *para*-methyl-acetophenone (**9a**) and *meta*-substituted analogue (**10a**) (Table 1, entries 6, 7, 9 and 10). As mentioned, *para*- and *meta*- substitution with a fluoride led to very similar conversions (Table 1, entries 12 and 13).

Next, we tested more bulky-bulky ketones, namely substrates having an increased steric hindrance on the other side of the carbonyl moiety. Substrates with a hydroxyl or a chloro-substituent on the methyl group led to conversion of 97% or >99% (**15a** and **16a**, Table 1, entries 15 and 16). However, substrate **17a** with an ethyl chain connected to the carbonyl carbon atom led to no conversion. Finally, 1-phenylpropane-1,2-dione (**18a**) was reduced by both ADHs but at its carbonyl group in the benzyl position. In this case, the non-perfect enantiomeric excess (87% or 95% (*S*)) could be attributed to partial racemisation due to keto-enol tautomeric equilibrium.

Regarding the stereoselective outcome of the other reactions, most of the substrates were converted with >99% *ee* by both enzymes, with the main exceptions for both ADHs being substrates **2a** and **14a**. Cm-ADH led to the worst *ee* with *meta*-hydroxy substituted acetophenone (**3a**, 38% *ee* (*R*) or *meta*-methyl substituted acetophenone (**10a**, 69% *ee* (*R*)), while Pf-ADH always yielded the product with *ee* of 90% or higher.

Subsequently, we explored substrate scope on different ketone families, comprising linear, branched and cyclic alkyl ketones, as well as alkyl aromatic, homo benzyl, α,β-unsaturated and heteroaromatic ketones.

Linear-chain saturated alkyl ketones (**19–21a**) were reduced with different yields ranging from 22% to 96% for Cm-ADH and from 47% to 98% for Pf-ADH (Table 2, entries 1–3). In each case, the reduction occurred with excellent enantioselectivity (>99% *ee* (*R*)). On the other hand, branched and cyclic alkyl ketones (**22–27a**) were converted with a lower yield, namely up to 60% for Cm-ADH and to 59% for Pf-ADH with **22a**. Cyclohex-2-en-1-one (**25a**) and the more sterically demanding substrate **27a** were not converted at all. The enantiomeric

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Table 1. Conversion and enantiomeric excess values obtained for the reduction of benzyl ketones catalysed by purified Cm-ADH and Pf-ADH. Experimental conditions: 1 mL final volume in Eppendorf tubes; buffer: KPi for Cm-ADH (100 mm, pH 7.5) and BR for Pf-ADH (60 mm, pH 6.0); T: 30 °C; reaction time: 22 h; agitation orbital shaker (170 rpm); [substrate]: 10 mM for Cm-ADH and 50 mM for Pf-ADH; [NAD$^+$]: 0.5 mM.

Entry	Sub.	Cm-ADH Conv. [%] (TON)	ee [%]	Pf-ADH Conv. [%] (TON)	ee [%]	Entry	Sub.	Cm-ADH Conv. [%] (TON)	ee [%]	Pf-ADH Conv. [%] (TON)	ee [%]
1	1a	89±4 (356)	>99 (R)	87±4 (1740)	>99 (R)	10	10a	50±4 (220)	69 (R)	70±3 (1400)	>99 (R)
2	2a	66±7 (264)	96 (R)	89±3[a] (1780)	93 (R)	11	11a	n.c.	n.d.	n.c.	n.d.
3	3a	38±2[a] (152)	38 (R)	6±2[a] (120)	>99 (R)	12	12a	92±3 (368)	>99 (R)	53±7 (1060)	>99 (R)
4	4a	19±1 (76)	>99 (R)	48±7 (960)	>99 (R)	13	13a	94±3 (376)	>99 (R)	62±2 (1240)	>99 (R)
5	5a	2±1 (8)	n.d.	<1 (20)	n.d.	14	14a	84±5 (336)	87 (R)	6±1 (120)	92 (R)
6	6a	59±9 (236)	>99 (R)	52±1 (1040)	99 (R)	15	15a	97±1[a] (388)	85 (S)[b]	97±1 (1940)	>99[b] (S)
7	7a	66±7 (264)	>99 (R)	71±2 (1420)	>99 (R)	16	16a	>99 (396)	n.d.	>99 (1980)	n.d.[d]
8	8a	3±1 (12)	n.d.	n.c.	n.d.	17	17a	n.c.	n.d.	n.c.	n.d.
9	9a	31±5 (124)	>99 (R)	33±2 (660)	90 (R)	18	18a[c]	48±1 (192)	87 (S)[b]	12±3 (240)	95[b] (S)

[a] by-product detected deriving from the elimination of the hydroxy group and leading to the styrene derivative: **2a** Pf-ADH ratio product/byproduct 77:23, **3a** Cm-ADH ratio product/byproduct 45:55, **3a** Pf-ADH ratio product/byproduct 12:88; [b] change due to switch of CIP priority; [c] the carbonyl where the reduction occurs is highlighted with a red circle; [d] the two enantiomers could not be separated by chromatography; n.c. = no conversion detected; n.d. = not determined due to too low conversion.

excess was excellent for products **23b** and **24b** (>99% ee (R)) and poor for **22b** (10% ee (R) from Cm-ADH and 35% ee (R) for Pf-ADH). Variable levels of conversions were obtained with aryl-aliphatic (**28–33a**) and structurally diverse α,β-unsaturated ketones (**34–36a**). Aromatic ketones **28a** and **29a** gave higher conversions with Pf-ADH (68% and 79% conversion, respectively) than Cm-ADH, despite the five-times higher substrate concentration used again with the former ADH (50 mM vs. 10 mM). 1-Phenoxypropan-2-one (**30a**) led to the highest conversions of 57% with Cm-ADH and >99% with Pf-ADH. Phenylacetone (**31a**) and the more sterically demanding 1-phenylbutan-2-one (**32a**) and β-tetralone (**33a**) gave little (max

9%) or no conversion. In this regard, the behaviour of the ADHs changed with different ketone substrates possessing an ethyl chain connected to the carbonyl carbon atom. As previously observed, propiophenone (**17a**) and 1-phenylbutan-2-one (**32a**) were converted minimally or not at all, whereas 1-phenylpentan-3-one (**28a**) gave 68% conversion with Pf-ADH.

The α,β-unsaturated ketones were all converted with 34–50% conversions for Cm-ADH and 58–63% conversion for Pf-ADH. When it was feasible to measure, because of the detection limit in case of low conversions, the ee was >99% in most of the cases. The exceptions were with ketones **31a**, **32a** and **35a**

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Table 2. Conversion and enantiomeric excess values obtained for the reduction of other families of ketones catalysed by Cm-ADH and Pf-ADH. Experimental conditions: 1 mL final volume in Eppendorf tubes; buffer: KPi for Cm-ADH (100 mM, pH 7.5) and BR for Pf-ADH (60 mM, pH 6.0); T: 30 °C; reaction time: 22 h; agitation orbital shaker (170 rpm); [substrate]: 10 mM for Cm-ADH and 50 mM for Pf-ADH; [NAD$^+$]: 0.5 mM.

Entry	Sub.	Cm-ADH Conv. [%] TON	ee [%]	Pf-ADH Conv. [%] TON	ee [%]	Entry	Sub.	Cm-ADH Conv. [%] TON	ee [%]	Pf-ADH Conv. [%] TON	ee or de[a] [%]
1	**19a**	96±3 (384)	>99 (R)	98±1 (1960)	>99 (R)	16	**34a**	50±1 (200)	>99 (R)	63±2 (1260)	>99 (R)
2	**20a**	22±2 (88)	>99 (R)	47±3 (940)	>99 (R)	17	**35a**	34±6 (136)	91 (R)	59±1 (1180)	>99 (R)
3	**21a**	37±4 (148)	>99 (R)	68±2 (1360)	>99 (R)	18	**36a**	37±5 (148)	>99 (R)	58±2 (1160)	>99 (R)
4	**22a**[a]	60±7 (240)	10 (R)	59±6 (1180)	35 (R)	19	**37a**	72±6 (288)	>99 (R)	86±4 (1720)	>99 (R)
5	**23a**	58±6 (232)	>99 (R)	25±2 (500)	>99 (R)	20	**38a**	32±3 (128)	23 (R)	62±1 (1240)	54 (R)
6	**24a**[a]	38±2 (152)	>99 (R)	4±1 (80)	99 (R)	21	**39a**	60±4 (240)	92 (R)	28±4 (560)	>99 (R)
7	**25a**	n.c.	n.d.	5±1 (100)	n.d.	22	**40a**	n.c.	n.d.	n.c.	n.d.
8	**26a**[a]	16±3 (64)	n.d.	3±1 (60)	n.d.	23	**41a**	>99 (396)	>99 (R)	92±1 (1840)	99 (R)
9	**27a**	n.c.	n.d.	n.c.	n.d.	24	**42a**	86±1 (344)	>99 (R)	82±1 (1640)	>99 (R)
10	**28a**	4±1 (16)	>99 (R)	68±5 (1360)	>99 (R)	25	**43a**	95±2 (380)	>99 (R)	90±2 (1800)	>99 (R)
11	**29a**	27±3 (108)	>99 (R)	79±1 (1580)	>99 (R)	26	**44a**	54±1 (216)	n.a.	51±1 (1020)	n.a.

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Table 2. continued

19a 20a 21a 22a 23a

24a 25a 26a 27a 28a

29a 30a 31a 32a 33a

34a 35a 36a 37a 38a

39a 40a 41a 42a 43a

44a 45a 46a 47a 48a

Entry	Sub.	Cm-ADH Conv. [%] TON	ee [%]	Pf-ADH Conv. [%] TON	ee [%]	Entry	Sub.	Cm-ADH Conv. [%] TON	ee [%]	Pf-ADH Conv. [%] TON	ee or de[a] [%]
12	30a	57±8 (228)	>99 (R)	>99 (1980)	>99 (R)	27	45a[a]	95±3 (380)	6 (S)[b]	47±8 (940)	4 (S)[b]
13	31a	9±2 (36)	78 (R)	3 (60)	>99 (R)	28	46a	>99 (386)	n.a.	78±5 (1560)	n.a.
14	32a	2±1 (8)	rac	<1 (20)	90 (R)	29	47a	n.c.	n.a.	n.c.	n.a.
15	33a	n.c.	n.d.	n.c.	n.d.	30	48a	75±1 (300)	n.a.	77±2 (1540)	n.a.

[a] substrates **22a**, **24a**, **26a** and **45a** were used as racemic mixture; in these cases, values are de (diastereomeric excess); [b] chirality due to partial dynamic kinetic resolution of α-substituted aldehyde; n.c.=no conversion detected; n.d.=not determined due to too low conversion; n.a.=not applicable as the product is not chiral.

with Cm-ADH (78% ee (R), racemic and 91% ee (R), respectively) and ketone **32a** with Pf-ADH (90% ee (R)).

Heteroaromatic ketones (**37–43a**) afforded good to excellent conversions (32–>99% conversion with Cm-ADH and 28–92% conversion with Pf-ADH) and excellent enantioselectivity in most of the cases (>99% ee). Regarding the enantioselectivity of the reaction, the exception was 2-acetyl-5-methylfuran (**38a**) that yielded **38b** with 23% ee (R) and 54% ee (R) for Cm-ADH and Pf-ADH, respectively. The other exception was the

conversion of 2-acetyl-thiophene (**39a**) catalysed by Cm-ADH that afforded **39b** with 92% ee (R), while Pf-ADH again showed high enantioselectivity (>99% ee (R)).

Finally, we tested whether Cm-ADH and Pf-ADH are strictly secondary ADHs or can also produce primary alcohols. In fact, aldehydes are commonly less accepted substrates by secondary ADHs with the most notable exception being the horse liver ADH.[20] To our delight, both Cm-ADH and Pf-ADH performed the reduction of aliphatic aldehydes such as *n*-hexanal (**44a**)

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and 2-methyl-pentanal (**45a**) with conversions ranging from 47% to 95%. The heteroaromatic thiophene-2-carbaldehyde (**46a**) and the homoaromatic 3-phenylpropanal (**48a**) were also well converted with conversions from 75% to >99%. Only phenylacetaldehyde (**47a**) was not converted by any of the ADHs. This observation agrees with the previously reported reactivity with ketones since the analogous ketone **31a** was also converted with very low conversion (3–9%), while ketone **29a** (structurally related to aldehyde **48a**) gave better results (up to 79% conversion with Pf-ADH).

To demonstrate the applicability of the best performing anti-Prelog ADH from this study, namely Pf-ADH, we performed a scale-up of the reaction at 500 mg-scale with acetophenone (**1a**, 50 mM) as substrate. Using the optimised condition but Pf-ADH as crude lysate instead of purified form for improved applicability, we obtained the product (R)-**1b** with 75% conversion, 73% isolated yield after work-up and >99% ee (for details, see SI section 7).

Conclusions

In summary, we characterised the substrate scope and the selectivity of two NAD-dependent anti-Prelog ADHs from *Candida maris* and *Pichia finlandica*, which were previously disclosed but never investigated for their applicability in organic synthesis and wide substrate scope.

Wild-type and NAD-dependent anti-Prelog ADHs are rare, a fact that makes these enzymes in demand and attractive from a synthetic and economical perspective. In fact, the use of the non-phosphorylated form of the nicotinamide adenine dinucleotide coenzyme is advantageous compared with the phosphorylated one due to the lower production cost.

In this work, we proved the applicability of both ADHs on many structurally diverse ketones, thus filling some gaps in the substrate scope and/or level of enantioselectivity of few known anti-Prelog NAD-dependent ADHs.

The applicability was also demonstrated on a preparative scale. We performed the scale-up of the biotransformation by focusing on the ADH that displayed the highest productivity. We tested the reaction on 500 mg of acetophenone **1a** using the crude lysate of Pf-ADH, obtaining 75% conversion and 73% isolated yield. More studies are in progress to further improve the applicability by extending the lifetime of Pf-ADH. For instance, this can be done by immobilisation of the enzyme, using flow technologies and performing enzyme engineering to increase stability.

Experimental Section

Optimised procedure for the biocatalytic reduction of ketones in analytical scale: In an Eppendorf tube (2 mL), HCOONa (50 mM), NAD$^+$ (0.5 mM), Cb-FDH (20 μM) and ADH (Pf-ADH or Cm-ADH; 25 μM) were added in the buffer (KPi 100 mM, pH 7.5 for Cm-ADH or Britton–Robinson 60 mM, pH 6.0 for Pf-ADH) up to a final volume of 1 mL. The substrate (10 mM for Cm-ADH or 50 mM for Pf-ADH) was added from a DMSO stock solution 2.5 M as last. The reaction

was incubated at 30 °C, 170 rpm for 22 h on an orbital shaker. The reaction was quenched with KOH 10 M (100 μL) and extracted with ethyl acetate (500 μL×2). The organic layer was dried over MgSO$_4$ and analysed by GC-FID with an Agilent DB-1701 30 m column. For analytical details, see SI, section 13. The enantiomeric excess was determined by GC-FID with an Agilent Chirasil-DEX-CB 30 m column upon derivatisation of the samples with acetic anhydride and 4-dimethylaminopyridine. For details and analytics, see SI, section 4 and 13.

Procedure for scale-up at 500 mg-scale: Lyophilised crude lysate of Pf-ADH (0.340 g, dry weight) was suspended in a 250 mL bottle containing Britton–Robinson buffer (60 mM, pH 6.0) and incubated for 15 min, at 170 rpm and 30 °C in an orbital shaker. Next, HCOONa (50 mM), NAD$^+$ (0.5 mM) and Cb-FDH (20 μM) were added. The substrate **1a** was added as last from a DMSO stock solution (50 mM). The final volume was 83.2 mL. The mixture was incubated for 22 h, at 170 rpm and 30 °C in an orbital shaker. At the end of the reaction, the aqueous phase was saturated with brine (20 mL), extracted with ethyl acetate (30 mL×3), dried over MgSO$_4$ and analysed by GC-FID as for the reaction in analytical scale. The conversion was 75%. Afterwards, the reaction mixture was purified over silica gel (eluent: petroleum ether / ethyl acetate; gradient from 9:1, v v^{-1} to 8:2, v v^{-1}) yielding 373 mg of pure (R)-1b (yield of 73%) in optically pure form (ee >99% (R)). For NMR characterisation, see SI, section 7.

Supporting Information

Supporting information contains materials and methods (section S1), procedures for enzyme expression and purification (section S3), general procedures (section S4), optimisation of the reaction conditions with Cm-ADH and Pf-ADH (sections S5 and S6), scale-up reaction (section S7), reaction-time study (section S8), experiments with lyophilised whole cells (section S9), experiments with NADH recycling using isopropanol (section S10), stability tests with Pf-ADH (section S11), testing at higher formate salt concentrations (section S12), and analytics and representative GC chromatograms (section S13).

Additional references cited within the Supporting Information.[15b,21]

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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