A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

# Accepted Article

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**To be cited as:** *ChemBioChem* 10.1002/cbic.201800533

**Link to VoR:** http://dx.doi.org/10.1002/cbic.201800533

A Journal of ChemPubSoc Europe

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10.1002/cbic.201800533

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FULL PAPER

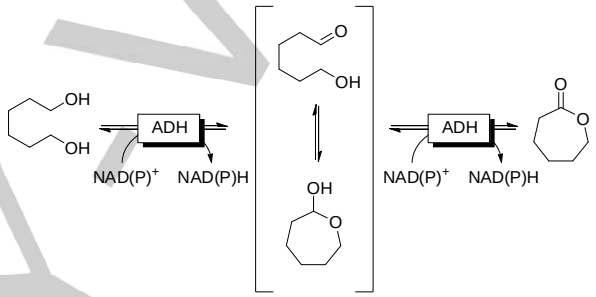
# An alcohol dehydrogenase from the short-chain dehydrogenases/ reductases family of enzymes for the lactonization of 1,6-hexanediol

Choaro D. Dithugoe,[a] Jacqueline van Marwijk,[a] Martha S. Smit,[a] and Diederik J. Opperman*[a]

**Abstract:** Biocatalytic production of lactones, and in particular ε-caprolactone (CL), have gained increasing interest as a greener route to polymer building blocks, especially through the use of Baeyer-Villiger monooxygenases (BVMOs). Despite several advances in the field, BVMOs, however, still suffer several practical limitations. Alcohol dehydrogenase (ADH) mediated lactonization of diols in turn has received far less attention and very few enzymes have been identified for the conversion of diols to lactones, with horse-liver ADH (HLADH) remaining the catalyst of choice. Screening of a diverse panel of ADHs, *Aa*SDR-1, a member of the short-chain dehydrogenase/reductase family, was found to produce ε-caprolactone from 1,6-hexanediol. Moreover, cofactor regeneration by an NADH oxidase eliminated the requirement of co-substrates, yielding water as the sole by-product. Despite lower turnover frequencies as compared to HLADH, higher selectivity was found for the production of CL, with HLADH forming significant amounts of 6-hydroxyhexanoic acid and adipic acid through aldehyde dehydrogenation/oxidation of the gem-diol intermediates. Also, CL yield were shown to be dependent on buffer choice, as structural elucidation of a Tris-adduct confirmed the buffer amine to react with aliphatic aldehydes forming a Schiff-base intermediate which through further ADH oxidation, forms a tricyclic acetal product.

## Introduction

Lactones are important building blocks in organics synthesis, especially of polymers such us polycaprolactones and other polyesters as well as precursors for the synthesis of polyamides such as nylon[1–6]. In recent years, Baeyer-Villiger monooxgyenases (BVMOs) have been studied intensively as a green alternative to current organometallic catalysts for BV oxidation of ketones[7–10]. The practical application of this class of enzymes are however generally limited due to their requirement for reduced cofactors (NADPH) and thus requirement of co-substrates for cofactor regeneration, instability and substrate or product inhibition. An alternative to BVMO catalyzed oxygenation of ketones, is the oxidative lactonization of diols utilizing alcohol dehydrogenases (ADHs; Scheme 1)[11,12] or the laccase/TEMPO system[13].




**Scheme 1.** ADH mediated oxidative lactonization of 1,6-hexanediol to ε-caprolactone.

ADHs catalyzes the interconversion of primary and secondary alcohols to aldehydes and ketones respectively. ADHs are currently divided into three classes based on the protein's monomer chain length[14,15]: medium-chain dehydrogenases/reductases (MDR), short-chain dehydrogenases/reductases (SDR) and long-chain dehydrogenases/reductases (LDR). ADHs from the MDR superfamily of enzymes, which include horse-liver alcohol dehydrogenase from *Equus caballus* (HLADH) and yeast ADHs (YADH) from *Saccharomyces cerevisiae* (*Sc*ADH1), predominantly contain $Zn^{2+}$ within the active site. In contrast, the SDR family of enzymes doesn't contain any metal ions in their active sites.

ADHs (often termed ketoreductases/KREDs) have been extensively studied and used for the production of chiral alcohols through (dynamics) kinetic resolution, enantioconvergence, deracemization and stereoinversion reactions[16,17]. The lactonization of diols using ADHs have in turn received less attention. The earliest examples of ADH-catalyzed lactonization of diols came from the research group of J. Bryan Jones in the late 1970s. Although the oxidation of hydroxy aldehydes to lactones via their hemiacetals were already known at this stage, it was their investigation into the regioselectivity and possible enantioselectivity of HLADH that demonstrated the usefulness of ADHs in the lactonization of diols[18–21]. Much of the early work on ADH mediated lactonization of diols were focused on the synthesis of chiral synthons. Asymmetric oxidation of pro-chiral

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diols were shown to be stereoselective and the oxidation of various bishydroxy cyclopentyl and cyclohexyl substrates demonstrated the selectivity of HLADH to yield pure regiomeric lactones. The production of enantiopure lactones were also demonstrated through the asymmetrization of various acyclic and cyclic *meso*-diols[22–25]. This enantiotopic oxidation was again shown to be absolute, resulting in enantiopure lactones from various exo- and endo-bridged bicyclic diols[26,27].

One of the major drawbacks of the early HLADH catalysed reactions were inefficient cofactor regeneration, leading to various research groups investigating bacteria and yeast species for *in-vivo* lactonization of various diols[28–30]. With advances in cofactor regeneration strategies, the use of purified ADHs has again emerged as a feasible reaction as it alleviates unwanted side product formation. Hollmann and co-workers demonstrated the use of a laccase-mediator system for cofactor regeneration of ADH mediated lactonization of various diols, where only molecular oxygen is required and water is produced as the sole by-product[11] as well as photocatalytic regeneration of $NAD^+$[31]. More recently, a novel convergent cascade was developed, combining both BVMO and ADH strategies with no requirement for external cofactor regeneration enzymes[32–35].

Despite the numerous advances made within the field, the repertoire of available ADHs for the efficient lactonization of diols unfortunately remains limited, with HLADH remaining the ADH of choice. Here we explore a diverse set of ADHs for the lactonization of 1,6-hexanediol as an alternative to HLADH.

## Results and Discussion

### ADH panel selection

In an effort to improve on the current state of the art in ADH-mediated lactonization of 1,6-HD, we screened a panel of ADHs, diverse in structure/mechanism, cofactor dependence and origin. Apart from ADHs already available in our laboratory, ADHs described in literature as having the ability to accept aliphatic alkanols and/or alkanals were selected, as well as ADHs from organisms known to metabolize linear alkanes. These were compared with already known ADHs that efficiently catalyze the formation of CL from 1,6-HD and thus 13 distinct ADHs were included in this study (Table 1).

**Table 1.** Panel of alcohol dehydrogenases (ADHs) evaluated for oxidative lactonization of 1,6-hexanediol to ε-caprolactone

| ADH | Origin | Co-factor preference | Type |
|---|---|---|---|
| HLADH | *Equus caballus* | $NAD^+$ | MDR (Zn) |
| TADH[36] | *Thermus* sp. ATN1 | $NAD^+$ | MDR (Zn) |
| TeSADH[37] | *Thermoanaerobacter ethanolicus* 39E | $NADP^+$ | MDR (Zn) |
| MLADH[38] | *Micrococcus luteus* WIUJH20 | $NAD^+$ | MDR (Zn) |
| CAD (cinnamyl-alcohol DH)[39] | *Eucalyptus gunnii* | $NAD^+$ | MDR (Zn) |
| calA (coniferyl-alcohol DH)[40] | *Pseudomonas* sp. HR199 | $NADP^+$ | SDR |
| *Ac*ADH[41] | *Acinetobacter calcoaceticus* GK2 | $NADP^+$ | MDR (Zn) |
| *Aa*SDR-1 (farnesol DH)[42] | *Aedes aegypti* | $NADP^+$ | SDR |
| *Sc*ADH1 | *Saccharomyces cerevisiae* S288C | $NAD^+$ | MDR (Zn) |
| *Sc*ADH6 | | $NADP^+$ | MDR (Zn) |
| *Ad*ADH1 | *Alcanivorax dieselolei* B-5 | n.d. | MDR (Zn)[a] |
| *Ad*ADH2 | | n.d. | MDR (Zn)[a] |
| *Ad*ADH3 | | n.d. | MDR (Zn)[a] |

n.d. not determined, [a] Predicted based on sequence similarity

HLADH, TeSADH and TADH have previously been reported for their use in the lactonization of 1,6-HD[33]. Apart from its well-known acetaldehyde and ethanol conversion, one of the best studied ADHs, ADH1 from *Saccharomyces cerevisiae*, have also been reported to accept longer chain alkanols, such as hexanol[43]. ADH6 from the same organism has also been shown to be active towards both hexanol and hexanal[44]. The three ADHs from *Alcanivorax dieselolei* (*Ad*ADH1-3) were included as this organism have been reported to have an extensive metabolism for alkane degradation[45]. Similarly, various strains of *Acenitobacter* strains have been described for their *n*-alkane metabolism and to contain ADHs with the ability to oxidize medium-chain alkanols[46,47]. These ADHs together with cinnamyl-alcohol dehydrogenase (CAD) are all Zn-dependent ADHs from the MDR-family of ADHs. We therefore decided to also include ADHs from the SDR-family. The farnesol dehydrogenase (*Aa*SDR-1) *from Aedes aegypti* is known to accept isoprenoid and aliphatic alcohols including octanol, 2-decanol and citronellol[42].

**Cloning and heterologous expression**

The ADHs from *S. cerevisiae* and *A. dieselolei* were PCR amplified from genomic DNA and sub-cloned to the pET28-b(+) vector for heterologous expression in *E. coli*. Our in-house library of ADHs, and those commercially synthesized for this study, were similarly cloned to pET28-b(+) to allow for the addition of an N-terminal hexa-histidine tag. With the exception of CAD and TADH, all the ADHs expressed in relatively high quantities as soluble proteins with molecular weights as expected (Table S1 and Figure S1 in Supporting Information).

**Biotransformations**

The panel of recombinant ADHs were evaluated for their ability to convert 1,6-HD to CL using cell-free extracts (CFE). Initial screening reactions gave low yields (less than 10% conversions) of 1,6-HD to CL, even with HLADH (Figure S2 in Supporting Information). Similar to reports by other research groups, neither the hydroxy-aldehyde nor its cyclic hemiacetal were detected[11,48]. In an effort to improve the reactions, the CFEs were supplemented with additional cofactor [1 mM NAD(P)$^+$] and the concentration of the starting material increased to 20 mM. An improvement in CL production was observed (Figure S3 in Supporting Information) but only 4 of the ADHs yielded CL. In both sets of reactions an unknown product was formed either as a byproduct as seen with HLADH and TADH, or as the sole product with *Sc*ADH1 and *Ad*ADHs. Only trace amounts of products were observed with MLADH, CAD and calA. MLADH is a known secondary alcohol dehydrogenase that can in all probability not accept terminal alcohols. CAD (cinnamyl alcohol dehydrogenase) and calA (coniferyl-alcohol dehydrogenase) are known primary alcohol dehydrogenases but typically function on substrates containing an aryl group.

GC-MS analysis (Figures S6 in Supporting Information) revealed the unknown compound to have a molecular weight of at least 198 g.mol$^{-1}$ but a positive identification could not be made against the available mass spectral libraries such as the NIST database. As *Ad*ADH3 yielded this unknown compound as the major product, with near complete conversion of the 1,6-HD, the reaction was up-scaled for isolation and identification using NMR. Unfortunately the molecular structure of the unknown compound could still not be elucidated. As these reactions were performed using CFE, we assumed that either side-reactions with *E. coli* host enzymes or small molecular weight metabolites were taking place with the hydroxy-aldehyde products from 1,6-HD. We therefore turned to purified enzymes for the biotransformations, with the 4 most promising ADHs selected from the initial screening for CL production (HLADH, TADH, TeSADH, and *Aa*SDR-1) as well as *Ad*ADH3.

To avoid the use of stoichiometric amounts of NAD(P)$^+$ cofactor, we employed an NADH-oxidase for cofactor regeneration. The water forming *Streptococcus mutans* NADH oxidase 2 (*Sm*NOX2) is specific for NADH. Petschacher and co-workers however engineered *Sm*NOX2 as a universal regeneration system to regenerate both NAD$^+$ and NADP$^+$[49]. The *Sm*NOX2_V193R_V194H mutant (Mut10) was shown to accept both NADH$^+$ and NADP$^+$ with kinetic studies revealing similar affinities and catalytic efficiencies for both cofactors. Purified ADHs and *Sm*NOX2_V193R_V194H were therefore used for biotransformations at concentrations of 0.2 and 0.1 mg mL$^{-1}$ respectively. Despite CL yields improving significantly, surprisingly, the unknown compound remained as the major product in all the reactions except for *Aa*SDR-1 (Figure 1).

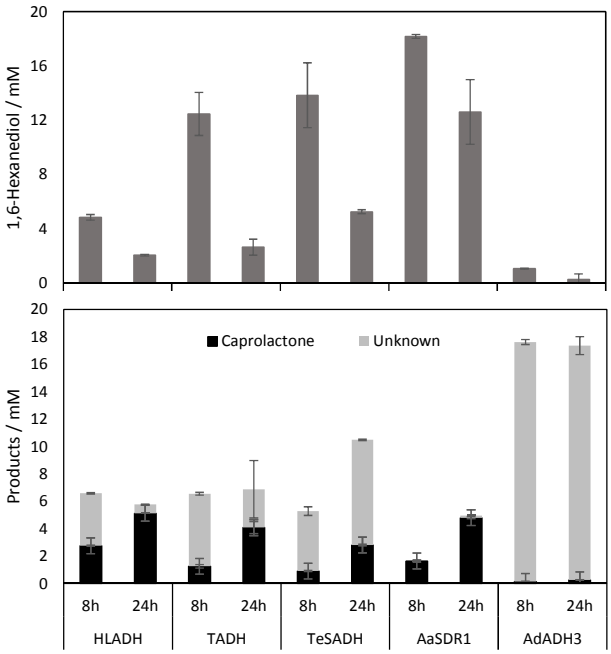



**Figure 1.** Screening of 5 purified ADHs for the synthesis of ε-caprolactone (bottom graph) from 1,6-hexanediol (top graph). Reaction conditions: 1 mL reaction volumes containing 0.2 mg mL$^{-1}$ ADH, 0.1 mg mL$^{-1}$ *Sm*NOX2_V193R_V194H, NAD(P)$^+$ = 1 mM, 1,6-hexanediol = 20 mM, buffer: 50 mM Tris-HCl (pH 8), 30°C, 200 rpm.

**Identification of byproduct formation**

As NMR analysis revealed the unknown compound to contain 10-carbons, we turned our attention to a possible side reaction with the Tris-buffer. Indeed, low molecular weight aldehydes have been shown to react with Tris in aqueous solutions[50]. The free amine groups of Tris at pH 8 could potentially nucleophilically add to the carbonyl carbon of aldehydes formed from 1,6-HD to give the hemiaminal followed by dehydration to a Schiff base. The use of Tris buffers in ADH-mediated lactonization reactions have been extensively reported without the mention of such a Tris-adduct, although these studies were conducted at different pH values. We therefore performed the Tris-buffered reactions at different pH values (6 – 8). At these lower pH values, the amount of unknown byproduct is significantly reduced (pH 7), and no byproduct could be detected at pH 6 (Figure 2). As Tris has a p$K_a$ of approximately 8, we assume that at these lower pH values, the amine group is protonated and thus not a nucleophile. The lowering of the pH

could also promote the cyclization of the hydroxy-aldehyde to the lactol, however no significant improved CL production was observed at these lower pH values with HLADH and indeed decreased CL production with *Aa*SDR-1 (Figure S7 in Supporting Information).

To further understand and confirm our theory, buffers without primary amines (HEPES and Na-phosphate, pH 8) were tested in the biotransformation reactions using HLADH. Gratifyingly, not only did the unknown byproduct decrease, CL production increased significantly, with more than 10 mM CL formed after 24 h in phosphate buffer. Since the purified enzymes were in Tris-buffer, small amounts of Tris were still introduced into the reaction mixtures. Purification of the enzymes (HLADH, *Aa*SDR-1 and *Sm*NOX2_193R194H) in sodium phosphate buffer completely eliminated the formation of the Tris-adduct as byproduct, with yields increasing to approximately 12 mM CL (Figure 2).

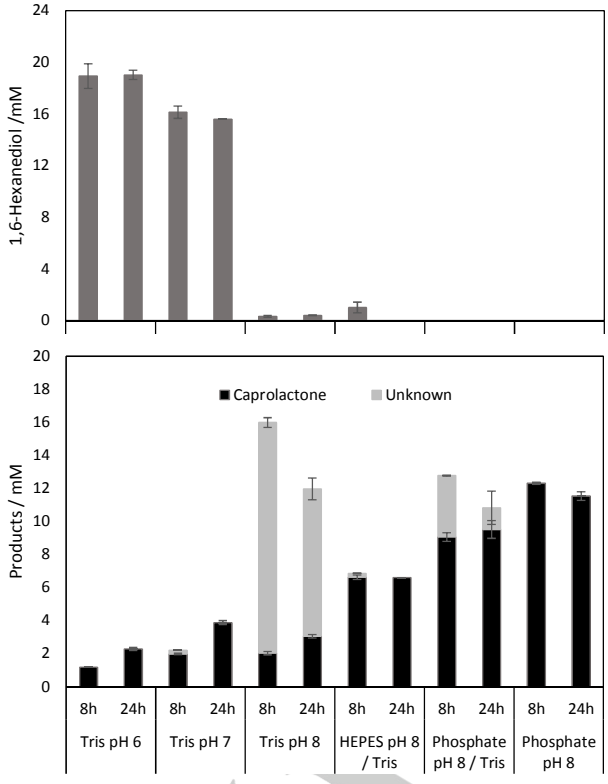
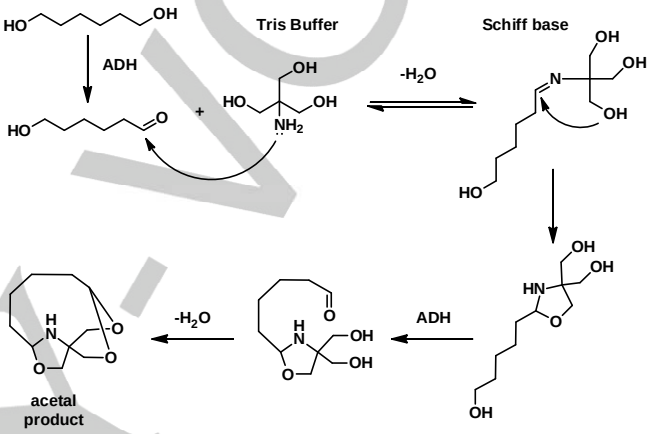



**Figure 2.** Effect of pH and buffer composition on the synthesis of ε-caprolactone (bottom graph) from 1,6-hexanediol (top graph) by HLADH. Reaction conditions: 1 mL reaction volumes containing 0.2 mg mL$^{-1}$ ADH, 0.1 mg mL$^{-1}$ *Sm*NOX2_V193R_V194H, NAD(P)$^+$ = 1 mM, 1,6-hexanediol = 20 mM, buffers: 50 mM Tris-HCl (pH 6 - 8), 50 mM HEPES or $NaP_i$ (pH 8) / Tris introduced with enzyme preparation, 50 mM $NaP_i$ (pH 8) from enzyme preparation also in same $NaP_i$ buffer, 30°C, 200 rpm.

As the Schiff base adduct is reversible and our NMR analysis suggested a cyclic or bicyclic compound with ten carbons and no free aldehyde or hydroxyl groups, we suggest that the Tris-adduct cyclizes through a nucleophilic attack of one of the free hydroxyl groups of Tris on the carbon of the C-N double bond. This product can undergo a second round of alcohol oxidation by the ADH, resulting in an acetal tricyclic product after reaction of the aldehyde group with the remaining free hydroxyl groups (Scheme 2). Conceivably this can also occur from the dialdehyde forming the Schiff-base and then cyclizing. The proposed tricyclic Tris-adduct was compared with the NMR data, and indeed, all carbons and protons could be assigned (Figure S8 in Supporting Information). The Tris-adduct however decreased with time, without any additional product identified with GC analysis, suggesting possible further polymerization.




**Scheme 2.** Proposed reaction mechanism of ADH mediated Tris-adduct formation.

When *Ad*ADH3 was again tested in the phosphate buffer, only trace amounts of unknown were detected, however, a depletion of the 1,6-HD was still observed. We speculated that the poor extraction of the hydroxy-aldehyde was below our detection limits and therefore acidified the BRM before extraction. 6-Hydroxy hexanoic acid (6-HHA) was identified as the sole detectable product. As these reactions were performed using purified enzymes, we attribute this aldehyde dehydrogenase/oxidase activity to the aldehyde "dismutase" activity reported for various ADHs. In the absence of cofactor regeneration, ADHs have been shown to both reduce and oxidize various aldehydes to their corresponding alcohols and acids respectively in equimolar amounts. Oxidation of the aldehyde occur via a *gem*-diol formed in aqueous environments. Wuensch and co-workers have however demonstrated that when using catalytic amounts of cofactors, different ADHs can give significantly different ratios of alcohol and acids during time-course reactions[51]. Since our reactions contained excess amounts of *Sm*NOX to ensure cofactor regeneration was not limiting the reactions, only NAD(P)$^+$ is available thus permitting only the *gem*-diol (aldehyde) oxidation reaction.

### *Aa*SDR-1 as an alternative to HLADH

To date, HLADH has served as the prototype ADH for the lactonization of 1,6-HD to CL. Despite HLADH giving rapid

conversion of the 1,6-HD to CL (complete conversion after 8 h) using purified enzyme in the phosphate buffer, the yields of CL was only ca. 60%. Acidification of the HLADH reaction before extraction revealed large amounts of 6-HHA. Although CL have been shown to undergo hydrolysis to 6-HHA, the rates exceeded that observed by Kara and co-workers[33] as well as our tested conditions (Fig 9A in Supporting Information). As our preliminary screening had revealed *Aa*SDR-1 as a promising alternative to HLADH, we compared the two ADHs using time-course reactions. With a higher concentration of ADHs used, HLADH showed complete conversion of the 1,6HD within 4 h, however CL yields within the BRM were only approximately 7.5 mM, with an almost equimolar amount of 6-HHA observed. Further reaction showed the slow hydrolysis of CL and the rapid oxidation of 6-HHA to adipic acid (AA) by HLADH (Figure 3A). Despite the lower reaction rates of *Aa*SDR-1, the same amount of CL was observed after 48 h at only ca. 40% conversion, with substantially lower 6-HHA concentrations and no AA formation (Figure 3B). The initial rate of 6-HHA production suggests that the HLADH has a higher affinity for the *gem*-diol compared to *Aa*SDR-1. Reaction of both the HLADH and *Aa*SDR-1 with CL as starting material further revealed HLADH to further oxidize the 6-HHA hydrolysis product to AA (Figure S9B and C in Supporting Information).

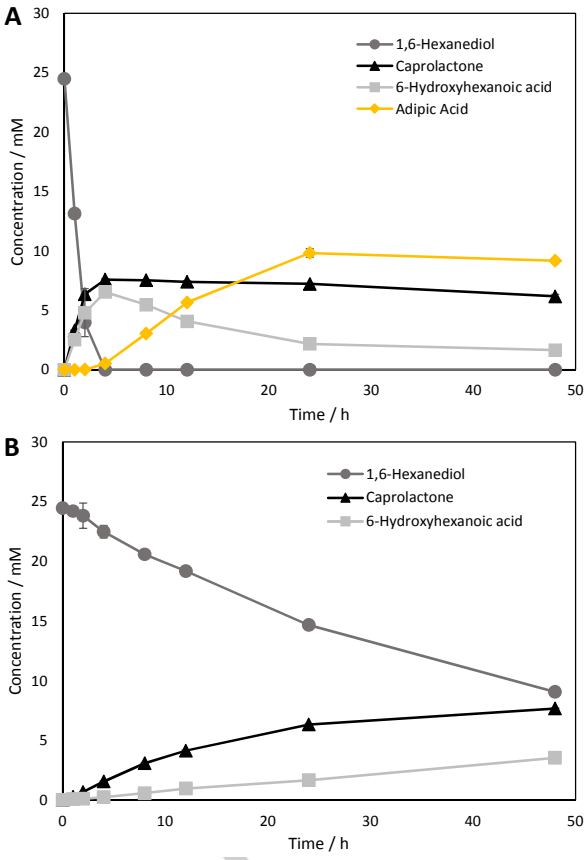



**Figure 3.** Time-resolved conversion of 1,6-hexanediol by HLADH (A) and *Aa*SDR-1 (B). Reaction conditions: 1 mL reaction volumes containing 0.5 mg mL$^{-1}$ ADH, 0.25 mg mL$^{-1}$ *Sm*NOX2_V193R_V194H, NAD(P)$^+$ = 1 mM, 1,6-hexanediol = 25 mM, buffer: 50 mM $NaP_i$ (pH 8), 30°C, 200 rpm.

Yields can potentially be increased using similar strategies as employed in BVMO oxidations for the production of CL: hydrolysis of CL to 6-HHA by the lipase Cal-B from *Candida antarctica*[52] or the polymerization of CL to oligo-ε-CL by Cal-A from the same organism[53].

### Substrate scope

Lastly, the *Aa*SDR-1 was evaluated for its ability to convert aliphatic diols other than 1,6-HD, as well as for its ability for desymmetrization reactions using the prochiral diol, 3-methyl-1,5-pentanediol. Unlike HLADH where the turnover frequencies (TOFs) increase with decreasing chain length, *Aa*SDR-1 showed minimal conversion of 1,4-butanediol and 1,5-pentanediol (<0.3 mM product formation) after 24 h. Product formation from 3-methyl-1,5-pentanediol was similarly low (ca. 1.3 mM after 24 h), but chiral analysis showed the same enantiospecificity with the (*S*)-enantiomer of 4-methyloxane-2-one formed as the major product. During time resolved reactions the lactol of 3-methyl-1,5-pentanediol were only observed with HLADH, suggesting the second ADH-catalyzed step (acceptance of a secondary alcohol) is not the rate-limiting step for *Aa*SDR-1, which could further explain the low Tris-adduct concentration seen in previous experiments.

## Conclusions

*Aa*SDR-1 is an ADH from the short-chain dehydrogenase/reductase family of enzymes able to lactonize 1,6-hexanediol to ε-caprolactone (CL). Despite its lower TOFs compared to HLADH, higher selectivity was found for the production of CL, with HLADH forming significant amounts of the overoxidized products 6-hydroxyhexanoic acid and adipic acid. As these rates exceed the hydrolysis rates of CL, their formation is most likely due to the ability of HLADH to also readily accept the geminal diols of the aldehyde intermediates as substrates. Furthermore, as *Aa*SDR-1 is NADP$^+$ dependent, its use in convergent cascade strategies with NADPH dependent BVMOs could facilitate and improve on these redox balanced, co-substrate free reactions.

Lastly, choice of buffer should be carefully considered during ADH-mediated lactonization reactions, as many commonly used buffers such as Tris contain primary amines which under basic conditions can rapidly react with the aldehyde intermediates, yielding carbinolamines which dehydrate to a Schiff base. These side-products can further polymerize, leading to significant losses in product formation. During the preparation of this manuscript, Kara and co-workers elegantly exploited this property for the production of lactams[54]. Oxidative lactamization was achieved through ADH catalyzed oxidation of amino alcohols with subsequent intramolecular cyclization to the hemiaminal followed by a second ADH oxidation to yield various lactams.

## Experimental Section

### Growth of micro-organisms

*Saccharomyces cerevisiae* S288C was cultured in YPD broth (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ peptone and 20 g $L^{-1}$ dextrose) at 25°C with shaking. *Alcanivorax dieselolei* B-5 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and cultured in Difco Marine broth 2216 at 28°C with shaking. *E. coli* strains were routinely cultured in LB medium (10 g $L^{-1}$ tryptone, 5 g $L^{-1}$ yeast extract and 5 g $L^{-1}$ NaCl) at 37°C with shaking.

### Construction of expression plasmids

The ADHs from *S. cerevisiae* (*Sc*ADH1 and *Sc*ADH6) and *A. dieselolei* (*Ad*ADH1, *Ad*ADH2 and *Ad*ADH3) were PCR amplified from genomic DNA (gDNA). gDNA was isolated from *S. cerevisiae* according to the method by Harju and co-workers[55] and from *A. dieselolei* using the ZR Fungal/Bacterial DNA MicroPrep kit (Zymoresearch). PCR reaction mixtures (50 µl) consisted of 1X KOD Hot Start Polymerase buffer, $MgSO_4$ (1.5 mM), deoxynucleoside triphosphates (0.2 mM each), KOD Hot Start DNA polymerase (1 U), gDNA (25 ng), and both the forward and the reverse primers (Table S2 in Supporting Information; 0.3 µM each). The PCR was performed with an initial denaturing step at 95°C for 2 min, followed by 30 cycles of denaturing at 95°C (20 s), annealing at 53-62°C (Table S1; 10 s), and elongation at 70°C (40 s), with a final extension at 70°C for 10 min. The amplicons were excised from an agarose gel and purified using a GeneJET Gel Extraction Kit (Thermo Scientific). Products were phosphorylated and ligated into pSMART (Lucigen) and transformed into *E. coli* TOP10 competent cells (Invitrogen). Positive transformants were selected for on LB-agar plates containing 30 µg.$ml^{-1}$ kanamycin. Plasmid DNA was isolated using a GeneJET Plasmid miniprep Kit (Thermo Scientific) and inserts verified through DNA sequencing. For expression, the ORFs were sub-cloned in pET28b(+) (Novagen) using *Nde*I and *Xho*I. TADH from *Thermus* sp. ATN1 was kindly provided by Dr. Frank Hollmann (TUDelft, Netherlands) in pET22 where after it was sub-cloned to pET28b(+) using *Nde*I and *Nco*I. All other ADHs were synthesized without codon optimization by GenScript with sub-cloning to pET28b(+) using *Nde*I and *Xho*I (*Eco*RI for MLADH). TeSADH was provided in pUC57 and sub-cloned to pET28b(+) using *Nde*I and *Avr*II (*Nhe*I compatible sticky ends on plasmid). *Sm*NOX2_V193R_V194H was likewise synthesized and cloned into pET28b(+) by GenScript using *Nde*I and *Xho*I.

### Heterologous expression and Cell Free Extract (CFE) preparation

The pET28 constructs were transformed into *E. coli* BL21-Gold(DE3) cells (Agilent Technologies), and expression performed in auto-induction media (ZYP5052) containing 30 mg $L^{-1}$ kanamycin at 25°C for 24 h (200 rpm). Cells were harvested by centrifugation (7 000 *xg*, 10 min, $4^0$C) and suspended in 50 mM Tris-HCl buffer pH 8 (1 g wet weight per 5 mL buffer). Cells were broken using a Constant cell disruption system (Constant Systems) at 30 kPsi. CFEs were obtained by removing unbroken cells and debris through centrifugation (7000 *xg*, 10 min, $4^0$C). Protein expression was evaluated on SDS-PAGE along with PageRuler Prestained Protein Ladder (ThermoScientific) and stained with Coomassie brilliant blue R-250.

### Protein purification

CFEs of the *E. coli* expressed HLADH, TADH, TeSADH, *Aa*SDR-1, *Ad*ADH3 and *Sm*NOX2_V193R_V194H proteins were subjected to ultracentrifugation (100 000 *xg*, 90 min, 4°C). Proteins were purified using Ni-affinity chromatography using gravity-flow Ni-NTA Superflow columns (Qiagen). The columns were equilibrated using 10 mL equilibration buffer (50 mM Tris-HCl, 0.5 M NaCl and 20 mM Imidazole, pH 7.4) where after the clear lysates obtained from ultracentrifugation were loaded onto the columns and allowed to bind. Unbound proteins were removed by washing with 20 ml of equilibration buffer with bound proteins eluted using 3 ml elution buffer (50 mM Tris-HCl, 0.5 M NaCl and 0.5 M Imidazole, pH 7.4). Alternatively, large scale purifications were performed using 5 mL HisTrap FF Ni-affinity columns (GE Healthcare) with elution using a linear gradient of increasing imidazole concentration. The elution fraction were concentrated to approximately 2 mL using 30 000 Da MWCO ultrafiltration spin columns (Millipore) and desalted using gravity flow PD-10 columns (GE Healthcare) equilibrated with 10 mM Tris-HCl (pH 8) containing 20 mM NaCl. Protein concentrations were determined using the ThermoScientific Pierce bicinchoninic acid (BCA) Protein Assay Kit according to the manufacturer's instructions, with bovine serum albumin (BSA) as standard.

### Biotransformations

Crude cell free extracts (CFE) of the 13 ADHs, as well as CFE from *E. coli* transformed with empty pET28b(+) as a negative control, were used for biotransformation reactions. The 1 mL biotransformation reactions were performed in capped 40 mL amber vials and contained 1,6-hexanediol (10 mM), 500 µL CFE and 500 µL of 50 mM Tris-HCl buffer (pH 8). Vials were incubated at 30ºC with shaking at 200 rpm. Samples were taken at 8 h and 24 h to evaluate production of ε-caprolactone. Samples were extracted with a 1:1 ratio of ethyl acetate containing 2 mM 1-undecanol as internal standard. For quantification of 6-hydroxyhexanoic acid and adipic acid, the BRM was acidified using 50 µL 5M HCl before extracting with a 1:1 ratio of ethyl acetate containing 2 mM 1-undecanol as internal standard.

Products were analysed and quantified against authentic standards using GC-FID with separation on a FactorFour VF-5ms column (60 m x 0.32 mm x 0.28 µm, Agilent). 6-Hydroxyhexanoic acid concentrations were estimated based on the response from adipic acid, and the unknown adduct based on that of ε-caprolactone. Samples with unknown products were subjected to GC-MS analysis using the same separation. Chiral analysis were performed on a Chiraldex G-TA column (30 m x 0.25 mm x 0.12µm, Astec) using the reported products from HLADH for identification.

Biotransformations using purified enzymes were similarly performed with reaction mixtures typically containing (unless otherwise stated) 20 mM 1,6-hexanediol, 0.2 mg $mL^{-1}$ ADH and 0.1 mg $mL^{-1}$ *Sm*NOX2_V193R_V194H supplemented with 1 mM $NAD^+$ (HLADH and TADH) or 1 mM $NADP^+$ (TeSADH and *Aa*SDR-1). In later reactions, the buffer was changed from 50 mM Tris-HCl to 50 mM sodium phosphate buffer (pH 8).

### NMR conformation of Tris-adduct

Conversion of 1,6-hexanediol by purified *Ad*ADH3 was monitored by GC-(MS) until complete conversion was observed. Samples were extracted using ethyl acetate and dried under vacuum where after it was dissolved in deuterated acetone or chloroform for NMR analysis. NMR spectra were recorded on an Avance II Bruker (600 MHz) spectrometer.

## Acknowledgements

The authors gratefully acknowledge Mr Sarel Marais for GC-FID/MS analysis and Dr Linette Twigge (Department of Chemistry, UFS) for NMR analysis. This research was supported through a Central Research Fund grant from the Faculty of Natural and Agricultural Sciences (UFS). Mr C.D. Dithugoe thank the National

Research Foundation (NRF, South Africa) for financial support during his M.Sc.

**Keywords:** ε-caprolactone • diols • lactones • alcohol dehydrogenase • short-chain dehydrogenase/reductase

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10.1002/cbic.201800533

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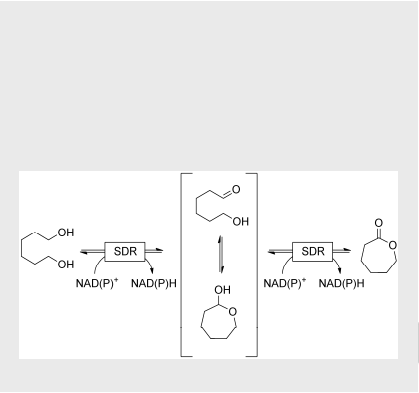
A short-chain dehydrogenase/reductase catalyses the oxidative lactonization of 1,6-hexanediol to ε-caprolactone, with only water as by-product when using NADH-oxidase for co-factor regeneration. ε-Caprolactone yields are significantly affected by buffers containing primary amines, resulting in irreversible adducts with aldehyde intermediates.

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***Page No. – Page No.***

**An alcohol dehydrogenase from the short-chain dehydrogenases/ reductases family of enzymes for the lactonization of 1,6-hexanediol**