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Review

Artificial Biocatalytic Linear Cascades to Access Hydroxy Acids, Lactones, and α- and β-Amino Acids

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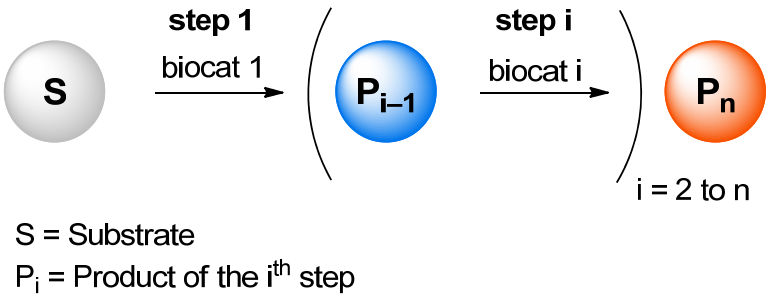
Abstract: α-, β-, and ω-Hydroxy acids, amino acids, and lactones represent common building blocks and intermediates for various target molecules. This review summarizes artificial cascades published during the last 10 years leading to these products. Renewables as well as compounds originating from fossil resources have been employed as starting material. The review provides an inspiration for new cascade designs and may be the basis to design variations of these cascades starting either from alternative substrates or extending them to even more sophisticated products.

Keywords: cascade; biocatalysis; biotransformations; hydroxy acids; lactones and α- and β-amino acids

1. Introduction

Artificial cascades involving biocatalysts have received increased attention recently [1–29], also in combination of enzymes with metal catalysis [30–37]. In this context, a cascade is defined as the combination of a minimum of two chemical reaction steps in a single reaction vessel without isolation of the intermediate(s) [38]. Linear cascade means that the product of one step in the cascade is the substrate of a second subsequent step (Scheme 1) [1ac].



Scheme 1. Linear cascade comprising n steps (n ≥ 2).

In this review, the focus is on biocatalytic cascades of the last 10 years, whereby all steps are performed simultaneously, leading to hydroxy acids, lactones, and α- and β-amino carboxylic acids as final products.

2. Hydroxy Acids

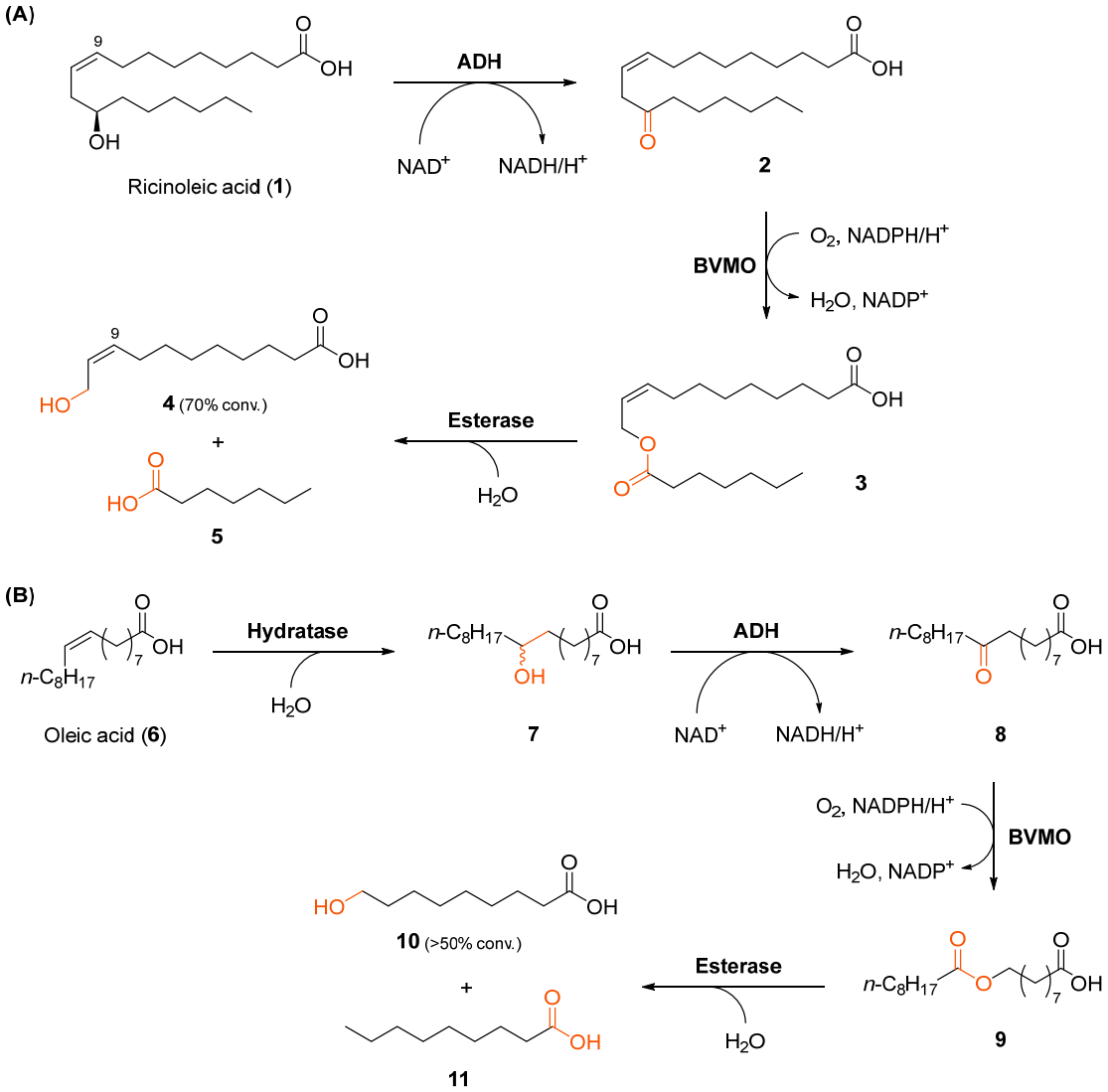
Hydroxy acids play an important role in chemical industry: α-Hydroxycarboxylic acids are part of numerous natural products, such as depsipeptides or aeruginosins, as well as of various

pharmaceuticals [39–41]. Their ω-hydroxy counterparts, on the other hand, are used in the production of a variety of chemical products and intermediates such as polyesters, resins, lubricants, plasticizers, and perfumes [42,43]. Hence, efficient synthetic entries to these compounds are highly desired.

2.1. Preparation of ω-Hydroxycarboxylic Acids from Fatty Acids

The biocatalytic synthesis of ω-hydroxycarboxylic acids has recently been realised starting from ricinoleic acid or oleic acid in a three- or four-step cascade (Scheme 1) [44].

Biotransformations producing the desired hydroxy acids were performed with a conversion ranging from 60% to 70% at 1 mM substrate concentration employing *E. coli* cells co-expressing all the necessary enzymes. Several other fatty acids, such as 5-hydroxydecanoic acid, linoleic acid, and lesquerolic acid, were transformed by this cascade leading to a similar range of conversion. Treating 5 gram per litre olive oil with lipase generated a product mixture containing 11.3 mM oleic acid and 1.2 mM palmitic acid. The subsequent biotransformation was performed as outlined in Scheme 2B, yielding 9-hydroxynonanoic acid (**10**) with 60% conversion.

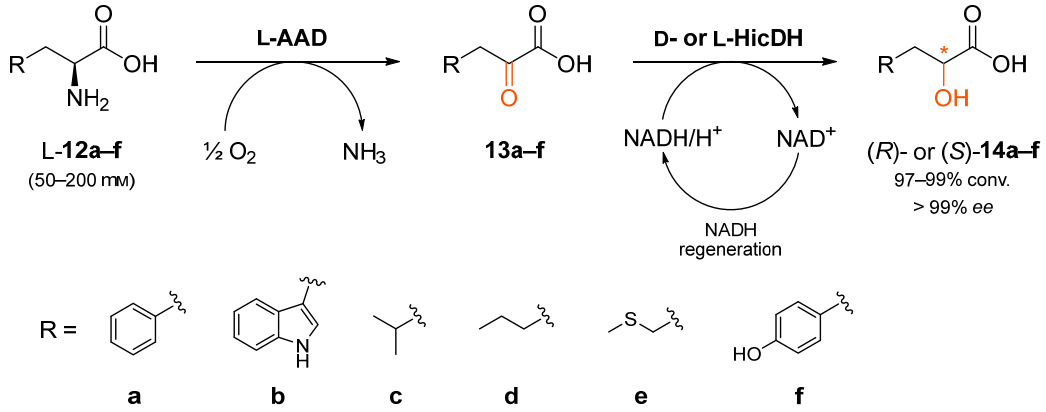


Scheme 2. Cascades to ω-hydroxycarboxylic acids. (**A**) Biotransformation starting from ricinoleic acid (**1**) to yield *n*-heptanoic acid (**5**) and 11-hydroxyundec-9-enoic acid (**4**); (**B**) Four-step cascade starting from oleic acid (**6**) producing *n*-nonanoic acid (**11**) and 9-hydroxynonanoic acid (**10**). Enzyme abbreviations: ADH, alcohol dehydrogenase; BVMO, Baeyer–Villiger monooxygenase.

In a follow-up study [45], the biotransformation conditions and expression protocol were optimised for the preparation of 11-hydroxyundec-9-enoic acid (**4**), which may have potential as an antifungal agent [46]. Employing increased concentrations of ricinoleic acid (**1**; 10 mM) and the biocatalyst (>10 g cell dry-weight per litre), a final concentration of 4.0 g/L 11-hydroxyundec-9-enoic acid (**4**) was achieved.

2.2. Conversion of α-Amino Acids into α-Hydroxy Acids

The synthesis of (*R*)- and (*S*)-α-hydroxy acids has been described starting from the corresponding L-amino acids, applying an L-amino acid deaminase (L-AAD) from *Proteus myxofaciens*, followed by the asymmetric reduction mediated by either L- or D-hydroxyisocaproate dehydrogenases (HicDHs), derived from *Lactobacillus confusus* DSM 20196 and *Lactobacillus paracasei* DSM 20008, respectively (Scheme 3) [47].



Scheme 3. Transformation of L-amino acids into the corresponding α-hydroxy acids in a two-step cascade. Enzyme abbreviations: L-AAD, L-amino acid deaminase; HicDH, α-hydroxyisocaproate dehydrogenase.

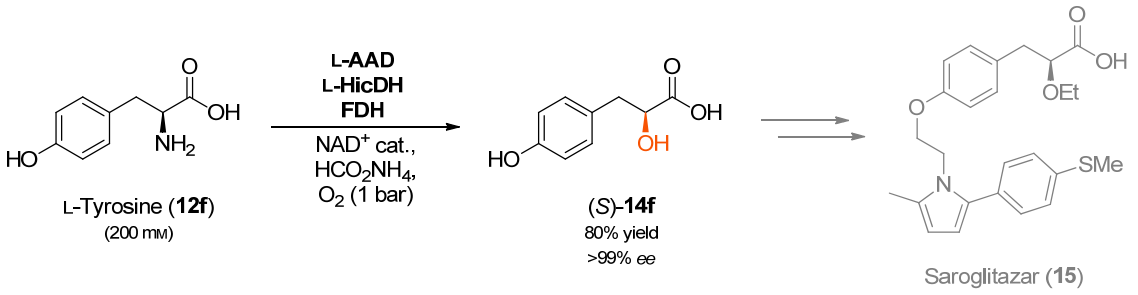
The transformation of all tested amino acids allowed conversions of 97–99% (73–85% isolated yield) within 7 h, even with high substrate loadings (up to 200 mM; see Table 1), furnishing the α-hydroxy acids **14** in optically pure form (>99% *ee*).

Table 1. Transformation of α-amino acids **12** into α-hydroxy acids **14** via a two-step cascade (Scheme 3) [a].

Substrate (mM)	Substrate Conc. (mM)	HicDH	Conv. [b] (%)	*ee* (%)
12a	100	L-Hic	>99 (81)	>99 (*S*)
12a	100	D-Hic	>99 (84)	>99 (*R*)
12b	50	L-Hic	>99 (83)	>99 (*S*)
12b	50	D-Hic	>99 (83)	>99 (*R*)
12c	100	L-Hic	>99 (81)	>99 (*S*)
12c	100	D-Hic	>99 (85)	>99 (*R*)
12d	100	L-Hic	>99 (79)	>99 (*S*)
12d	100	D-Hic	>99 (82)	>99 (*R*)
12e	200	L-Hic	>99 (77)	>99 (*S*)
12e	200	D-Hic	>99 (73)	>99 (*R*)

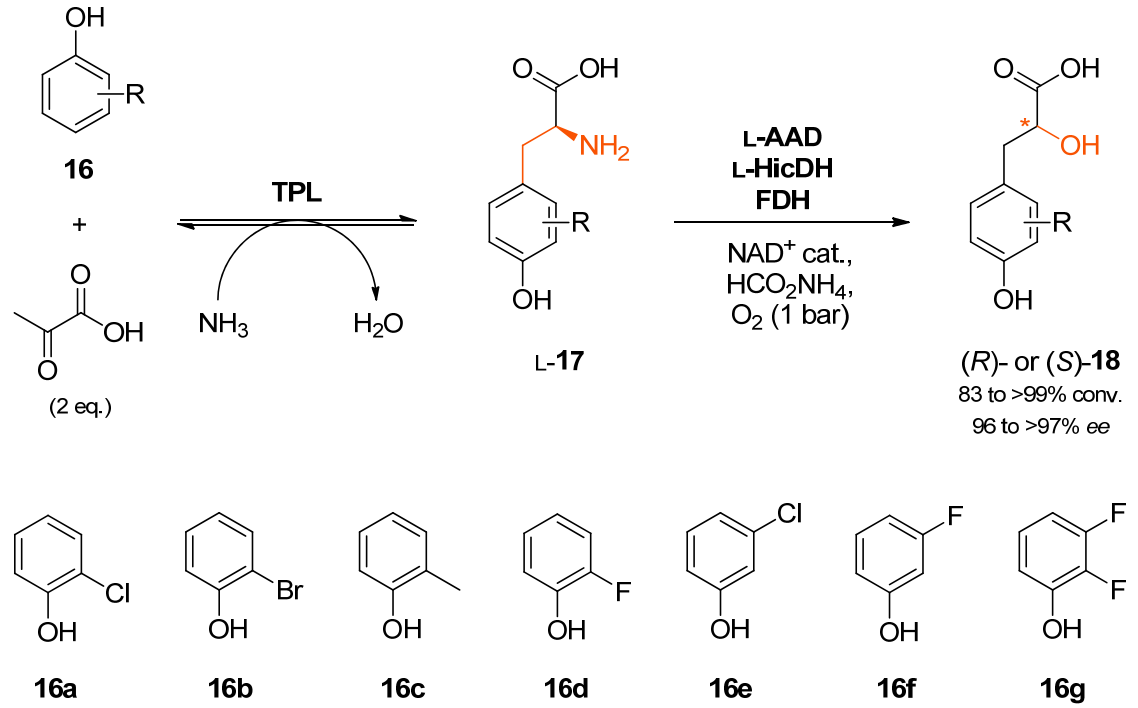
[a] Reaction conditions: Substrate L-**12a–e** (50–200 mM), HCO_2NH_4 (3 eq.), NAD^+ (1 mM), FDH (formate dehydrogenase, 42 U/mmol), L-AAD (15–30 U/mmol), L-Hic (132 U/mmol) or D-Hic (96 U/mmol), 1 bar O_2, 21 °C, 7 h. [b] Isolated yield in parentheses.

L-Tyrosine (**12f**) was also transformed at high substrate concentrations (200 mM) and on a 100 mg scale to afford the α-hydroxy acid (*S*)-**14f** in 80% isolated yield and >99% *ee* (Scheme 4). The product **14f** is an important building block for various biologically active compounds, such as Saroglitazar (**15**), which is used as a treatment for diabetes Type II [48].



Scheme 4. Multi-enzyme cascade synthesis of (*S*)-**14f**, a building block for the preparation of the antidiabetic drug Saroglitazar (**15**). Enzyme abbreviations: L-AAD, L-amino acid deaminase; HicDH, α-hydroxyisocaproate dehydrogenase; FDH, formate dehydrogenase.

In a subsequent study, an extended version of the cascade was published to access α-hydroxy acids starting from phenol derivatives and pyruvate (Scheme 5) [49].



Scheme 5. Biocatalytic cascade for the synthesis of α-hydroxy acids **18** starting from phenol derivatives **16** and pyruvate. Enzyme abbreviations: TPL, L-tyrosine phenol lyase; L-AAD, L-amino acid deaminase; HicDH, α-hydroxyisocaproate dehydrogenase.

As an additional step in this new reaction setup, the amino acid is formed within the cascade employing a tyrosine phenol lyase (TPL) from *Citrobacter freundii*, which performs a C–C coupling between substituted phenols **16** and pyruvate. The oxidative deamination and subsequent reduction are done by the same enzymes as described before. However, by introducing the additional TPL into the cascade, it has to be performed in sequential mode since small amounts of product **18** cause an inhibition of step one.

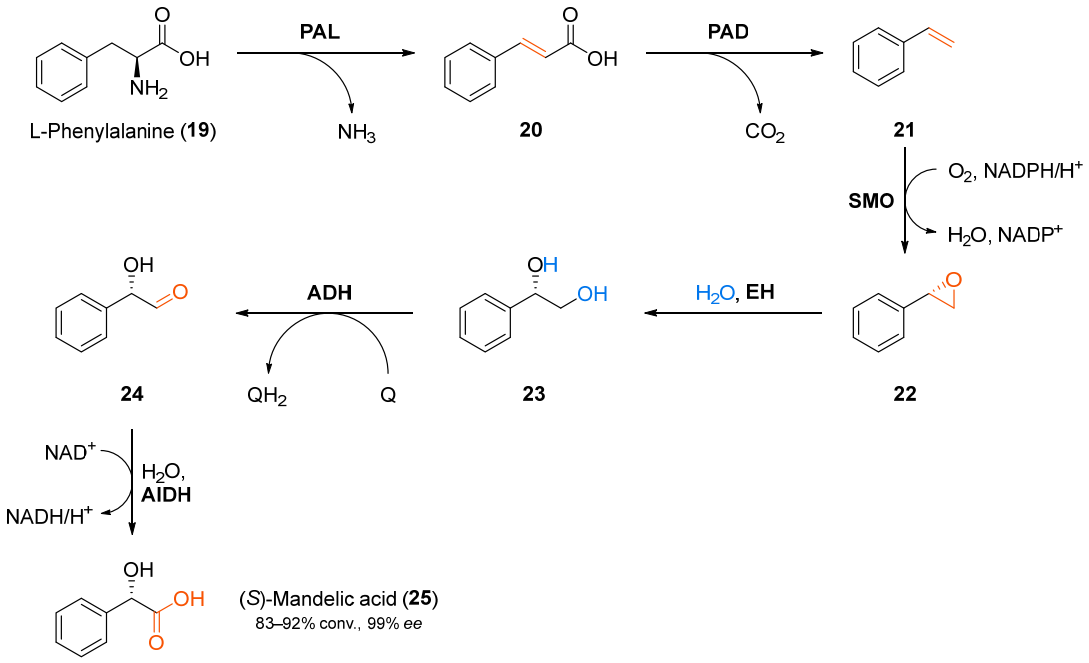
The model compound phenol (**16a**, R = H) was converted in a range of 23–46 mM with high conversions (93–97%) and *ee* (>99%) for both enantiomers. Related phenol substrates were accepted at concentrations up to 92 mM reaching conversions up to >99% and perfect *ee* (>97% by HPLC; Table 2). A preparative reaction transforming 56.6 mg of substrate **16a** afforded the optically pure hydroxy acid (*S*)-**18a** with 97% conversion in 77% isolated yield (*ee* > 97%).

Table 2. Results of cascade transforming phenols to *p*-hydroxyphenyl lactic acids **18** (Scheme 5) [a].

Substrate (mM)	Substrate Conc. (mM)	HicDH	Conv. [b] (%)	*ee* (%)
16a	46	L-HicDH	97 (77)	>97 (*S*)
16a	46	D-HicDH	97 (73)	>97 (*R*)
16b	46	L-HicDH	92 (60)	>97 (*S*)
16b	46	D-HicDH	92 (58)	>97 (*R*)
16c	69	L-HicDH	96 (77)	>97 (*S*)
16c	69	D-HicDH	96 (75)	>97 (*R*)
16d	92	L-HicDH	>99 (80)	>97 (*S*)
16d	92	D-HicDH	>99 (77)	>97 (*R*)
16e	92	L-HicDH	93 (81)	>97 (*S*)
16e	92	D-HicDH	95 (80)	>97 (*R*)
16f	23	L-HicDH	83 (63)	>97 (*S*)
16f	23	D-HicDH	85 (63)	96 (*R*)
16g	46	L-HicDH	96 (80)	>97 (*S*)
16g	46	D-HicDH	95 (85)	>97 (*R*)

[a] Reaction conditions: Substrate **16** (23–92 mM), pyruvate (2 eq.), NH_4Cl (4 eq.), NAD^+ (1 mM), NH_4HCOO (3 eq.), TPL (41 U/mmol), L-AAD (40 U/mmol), D-HicDH (114 U/mmol) or L-HicDH (68 U/mmol), FDH (76 U/mmol), 21 °C, 24 h for C–C coupling, 3 h for oxidative deamination and reduction. [b] Isolated yields in parentheses.

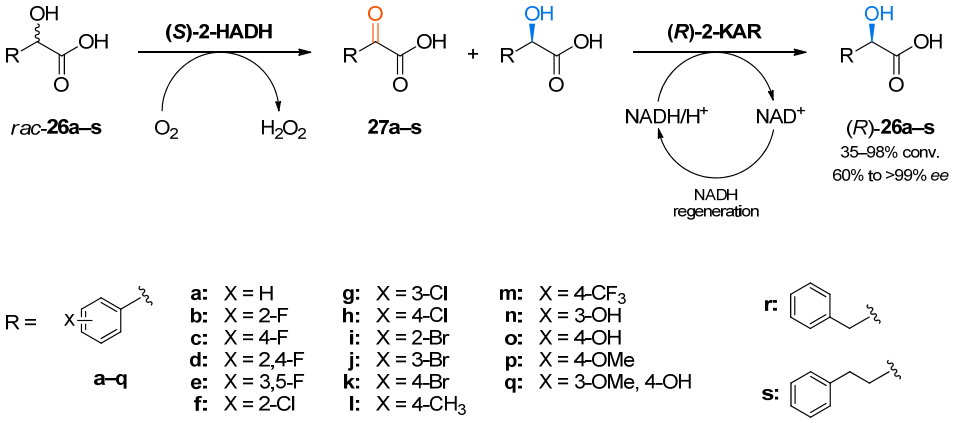
Starting from L-phenylalanine, (*S*)-mandelic acid was produced via a six-step cascade involving ammonia elimination, decarboxylation, epoxidation, epoxide hydrolysis, and oxidation of the primary alcohol to the carboxylic acid (Scheme 6) [50]. Performing the reaction with 120 mM of L-phenylalanine to (*S*)-mandelic acid with *E. coli* cells co-expressing all enzymes in a single strain afforded 83% conversion within 10 h and 92% conversion within 24 h and a perfect *ee* of 99%.



Scheme 6. Cascade transforming L-phenylalanine to (*S*)-mandelic acid. Enzyme abbreviations: PAL, phenylalanine ammonia lyase; PAD, phenylacrylic acid decarboxylase; SMO, styrene monooxygenase; EH, epoxide hydrolase; ADH, FAD-dependent alcohol dehydrogenase AlkJ from *Pseudomonas putida*; AlDH, aldehyde dehydrogenase.

2.3. Deracemization of α-Hydroxy Acids

Following previous reports on deracemization [1n-t], selected α-hydroxy acids were deracemized in an enantioselective oxidation–stereoselective reduction sequence (Scheme 7, Table 3) [51]. Oxidation was achieved using a flavin-mononucleotide-dependent (*S*)-2-hydroxy acid dehydrogenase [(*S*)-2-HADH] from *Pseudomonas aeruginosa.* For the asymmetric reduction of the intermediate α-keto acid, an NADH-dependent ketoreductase from *Leuconostoc mesenteroides*, was employed, whereby the cofactor was regenerated via GDH (glucose dehydrogenase) from *Exiguobacterium sibiricum*. For the substrates investigated at 20 mM concentration, the *ee* values obtained ranged between 60% and >99%, whereby in most cases optically pure product was obtained with up to 98% conversion.



Scheme 7. Deracemization of α-hydroxy acids by enantioselective oxidation and stereoselective reduction. Enzyme abbreviations: (*S*)-2-HADH, (*S*)-2-hydroxy acid dehydrogenase; (*R*)-2-KAR, (*R*)-2-keto acid reductase.

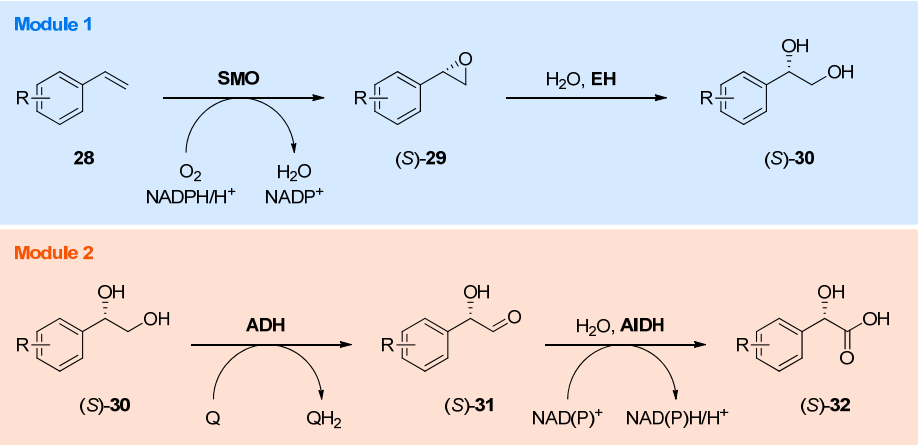
Table 3. Deracemization of α-hydroxy acids by enantioselective oxidation and stereoselective reduction (Scheme 7).

Substrate *rac*-26	Reaction Time (h)	Conv. (%)	*ee* of (*R*)-26 (%)
a	4	95	>99
b	6	95	>99
c	6	93	>99
d	4	95	>99
e	4	96	>99
f	4	97	>99
g	4	98	>99
h	2	95	>99
i	4	96	>99
j	2	95	>99
k	4	96	>99
l	2	96	>99
m	4	95	>99
n	6	36	82
o	6	35	72
p	6	60	>99
q	6	43	>99
r	6	77	63
s	6	58	60

2.4. Styrenes to α-Hydroxy Acids

An alternative route to α-hydroxycarboxylic acids has recently been developed as a modular multi-enzyme system for the functionalisation of styrene derivatives (Scheme 8) [52]. In this

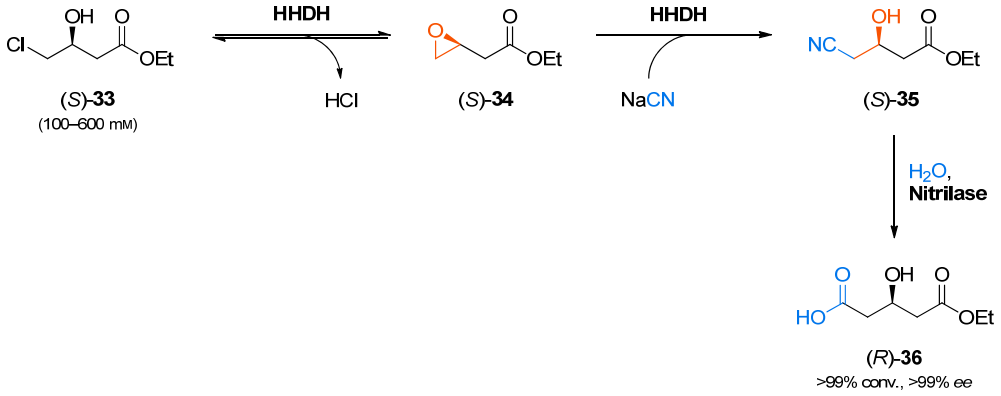
approach, aryl-substituted styrenes **28** were converted into vicinal diols (*S*)-**30** using the enzymes of Module 1, styrene monooxygenase and epoxide hydrolase. Selective oxidation of the primary alcohol moiety to the carboxylic acid was then realised by a second bi-enzymatic module, comprising the membrane-associated alcohol dehydrogenase AlkJ from *Pseudomonas putida* as well as phenylacetaldehyde dehydrogenase from *E. coli*. Eleven different mandelic acid derivatives (*S*)-**32** were thus produced with 69–99% overall conversion and *ee* values ranging from 96% to >99%.



Scheme 8. Modularised multi-enzyme system for the production of 1,2-diols (*S*)-**30** or α-hydroxy acids (*S*)-**32** from styrene derivatives **28**. Abbreviations: SMO, styrene monooxygenase; EH, epoxide hydrolase; ADH, FAD-dependent alcohol dehydrogenase AlkJ from Pseudomonas putida; AlDH, aldehyde dehydrogenase; Q, ubiquinone.

2.5. Access to α β-Hydroxy Dicarboxylic Acid Derivative Ethyl (R)-3-Hydroxyglutarate

The biocatalytic synthesis of ethyl (*R*)-3-hydroxyglutarate (**36**), a key intermediate in the preparation of the cholesterol-lowering drug rosuvastatin, was achieved in a three-step cascade with two enzymes [53]. Ethyl (*S*)-4-chloro-3-hydroxybutyrate (**33**) was employed as starting material, which was converted to the corresponding epoxide by a halohydrin dehalogenase (HHDH) from *Agrobacterium radiobacter* AD1, followed by a ring opening and subsequent hydrolysis to the product, using a nitrilase from *Arabidopsis thaliana* (Scheme 9).



Scheme 9. Biocatalytic transformation of ethyl (*S*)-4-chloro-3-hydroxybutyrate (**33**) into the pharmacologically important ethyl (*R*)-3-hydroxyglutarate (**36**). Enzyme abbreviation: HHDH, halohydrin dehalogenase.

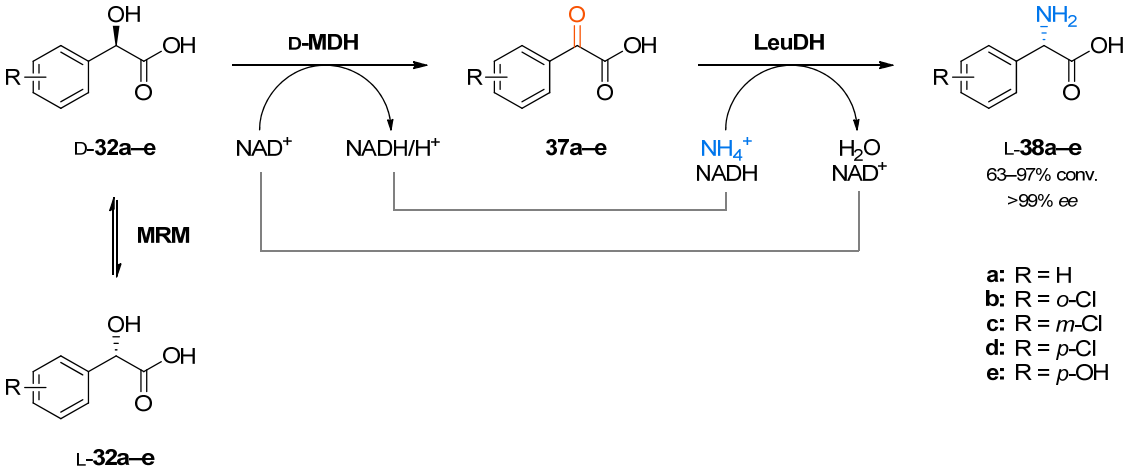
Using *E. coli* cells co-expressing the two required enzymes, up to 300 mM substrate were transformed within 1.5 h. However, at a substrate loading of 600 mM unfortunately only about

20% of the target hydroxy acid was detected after 5 h reaction time while intermediate **35** accumulated. Studying the inhibitory effect of the substrate **33** on the nitrilase, it was shown that above 300 mM a significant decrease in activity was observed. In order to access high product titres, the cascade was performed in a fed-batch mode; thus, 300 mM of substrate was added twice over a period of 2 h. By using this approach, the reaction went to completion within 6 h. Adding 300 mM substrate for a third time, 35% of intermediate **35** remained in the reaction mixture while **33** was completely consumed. Poorly balanced activity levels of the two co-expressed enzymes were identified as the reason for this accumulation of the reaction intermediate as the nitrilase was expressed in significantly lower amounts than the HHDH. Consequently, whole-cell preparations expressing the enzymes individually were used and the ratio of the amount of different cells was adjusted to obtain comparable activity levels for the two enzymes. By employing this setup, 900 mM of compound **33** were completely converted into the final product within 6 h, and (*R*)-**36** was isolated in 84% yield.

3. α- and β-Amino Acids

Natural and non-natural amino acids and their derivatives are pivotal building blocks for agrochemicals, bioactive polypeptide drugs, pharmaceuticals, and polymers [54–57]. Hence, the efficient synthesis of these compounds has received tremendous interest

The synthesis of the non-natural α-amino acid L-phenylglycine and its derivatives has been described using a three-enzyme cascade, starting from racemic mandelic acid. While in an older study only mandelic acid was transformed [58], other mandelic acid derivatives have been investigated more recently [59]. In this redox-neutral cascade that proceeds via 'hydrogen borrowing', an engineered mandelate racemase (MRM) from *Pseudomonas putida*, a D-mandelate dehydrogenase (D-MDH) from *Lactobacillus brevis*, and an L-leucine dehydrogenase (LeuDH) from *Exiguobacterium sibiricum* were selected to produce L-phenyl glycine derivatives **38** in a one-pot simultaneous fashion (Scheme 10).



Scheme 10. Three-step cascade for the conversion of racemic phenyllactic acid derivatives **32** into optically active phenylglycine derivatives **38**. Enzyme abbreviations: MRM, mandelate racemase; D-MDH, D-mandelate dehydrogenase; LeuDH, L-leucine dehydrogenase.

Under optimized conditions, 50 mM of substrate **32a** (R = H) were transformed into the desired product with 97% conversion and a perfect *ee* of >99% after 24 h. When the substrate loading was increased to up to 200 mM, good conversions of more than 95% were observed, whereas at even higher concentrations (500 mM) the conversion was reduced to 63%. To prove the applicability of this cascade, 30.4 g (200 mM) of *rac*-mandelic acid were transformed at a 1 L scale reaction, leading to >96% conversion and an isolated product yield of 86.5%.

Further, mandelic acid derivatives were converted to the corresponding phenylglycine products as well, showing moderate to good conversions (Table 4). Of special interest is product **38b** (R = *o*-Cl), which is a building block for Clopidogrel, an antiplatelet drug.

Table 4. Conversion of mandelic acid derivatives **32** into phenyl glycine derivatives **38** (Scheme 10).

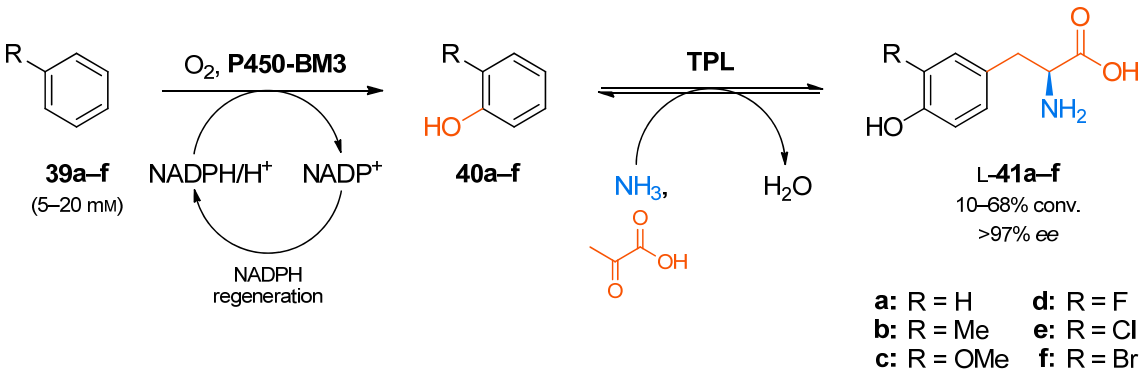
Substrate	Time (h)	Conv. (%)
rac-**32a** (R = H)	12	97
rac-**32b** (R = *o*-Cl)	48	49
rac-**32c** (R = *m*-Cl)	48	90
rac-**32d** (R = *p*-Cl)	24	95
rac-**32e** (R = *p*-OH)	48	66

Reaction conditions: Substrate *rac*-**32** (50 mM), $MgCl_2$ (3 mM), NAD^+ (0.1 mM), MRM (5 U/mL), D-MDH (10 U/mL), LeuDH (10 U/mL), ammonium chloride buffer (2.0 M, pH 9.5), 30 °C, 12–48 h.

An analogous transformation of mandelic acid derivatives into phenylglycine derivatives has been developed as an extension of the above-mentioned modular multi-enzyme system for the functionalisation of ring-substituted styrenes (Scheme 8) [52]. The (*S*)-mandelic acid derivatives **32**, which were ultimately derived from styrenes **28**, were oxidised to oxoacids **37** using hydroxymandelate oxidase from *Streptomyces coelicolor*. Reductive amination of **37** by the branched-chain aminoacid transaminase from *E. coli* using L-glutamate as amino donor and L-glutamate dehydrogenase for regeneration of the latter afforded the L-phenylglycine derivatives **38** (*cf.* Schemes 8 and 10). The overall synthesis sequence starting from styrenes **28** involved eight enzymes (six in a linear sequence) arranged in three modules (each expressed in a separate *E. coli* host) and provided products L-**38** in 16–86% overall yield and excellent optical purity (91% to >99% *ee*).

L-phenylglycine (**38a**) was also obtained in an eight-step cascade starting from L-phenylalanine (**19**), which was transformed to (*S*)-mandelic acid (**25**) as shown in Scheme 6, followed by further conversion into **38a** by the sequence described in the previous paragraph [50]. Transformation of 40 mM of (*S*)-L-phenylalanine with *E. coli* cells co-expressing all required enzymes afforded L-phenylglycine with 85% conversion and 99% *ee* within 24 h. The challenging eight-step biocatalytic cascade was successfully achieved with a single recombinant strain co-expressing 10 enzymes.

To access L-tyrosine and its derivatives substituted on the aromatic ring, a two-step-one-pot cascade was designed consisting of the P450 monooxygenase BM3 from *Bacillus megaterium* and a tyrosine phenyl lyase (TPL) from the microorganism *Citrobacter freundii* (Scheme 11) [60].



Scheme 11. Two-step-one-pot biosynthesis of L-tyrosine derivatives **41** starting from arenes **39**. Enzyme abbreviations: P450-BM3, cytochrome P450 monooxygenase from *Bacillus megaterium*; TPL, L-tyrosine phenol lyase.

Under non-optimized conditions the model substrate **39c** (R = OMe) yielded ~5% of the amino acid product with the majority of intermediate **40c** remaining, proving that both steps can be performed

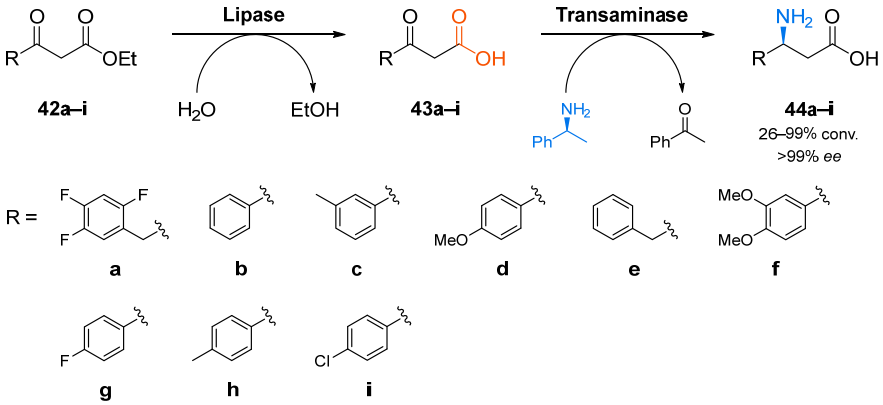
simultaneously. An inhibition study of the cascade revealed that the amount of $NADP^+$ and of the P450 have a substantial effect on the productivity of the TPL enzyme; however, the most critical parameter was found to be the pH drop due to the release of gluconic acid during NADPH regeneration. Furthermore, small amounts (<5%) of the *p*-phenolic side product as well as the *p*-hydroquinone were detected. By changing the buffer to Tris-HCl to maintain a slightly alkaline pH, an improvement in product formation by the factor 1.7 was observed. To investigate the substrate scope of the cascade, six different arenes (**39a**–**f**) were subjected to the biotransformation, showing large variations in conversion and selectivity. The best result in terms of productivity was obtained with substrate **39c**, showing 68% conversion, and with compound **39d** being the best one regarding the selectivity of the reaction (Table 5). *p*-Hydroquinone formation was particularly high for the halogenated substrates, due to the lower pK_a value and therefore better solubility and accessibility in the aqueous phase.

Table 5. Results of the cascade producing L-tyrosine derivatives **41a**–**f** (Scheme 11) [a].

Substrate	Conv. (%)	Selectivity [b] (%)	*ee* (%)
39a (R = H)	14	72	>97
39b (R = Me)	53	85	>97
39c (R = OMe)	68	84	>97
39d (R = F)	10	>99	>97
39e (R = Cl)	28	53	>97
39f (R = Br)	21	65	>97

[a] Reaction conditions: Substrate **39** (5–20 mM), pyruvate (40 mM), D-glucose (100 mM), NH_4Cl (180 mM), $NADP^+$ (0.4 mM), PLP (40 µM), P450-BM3 (2 µM), TPL (3 mg/mL), GDH (12 U/mL), catalase (1200 U/mL), Tris-HCl buffer (100 mM, pH 8.0), dimethylsulfoxide (DMSO) (1% *v*/*v*), room temperature, 6 h. [b] Selectivity is defined as the percentage of L-**41a**–**f** in the final product mixture.

Besides α-amino acids, their β-counterparts have attracted attention since they might be applied in the synthesis of bioactive compounds as well as in the design of hybrid peptides [61–64]. A biosynthetic route accessing various aromatic β-amino acids has been reported using a two-step-one-pot cascade starting from β-keto esters. The first step involves the hydrolysis of the ester by a lipase originating from *Candida rugosa*, followed by the amination of the keto acid by an ω-transaminase (ω-TA) from *Polaromonas* sp. JS666 (Scheme 12) [65].



Scheme 12. Biocatalytic synthesis of β-amino acids **44** starting from β-keto esters **42** using a lipase and a transaminase (TA).

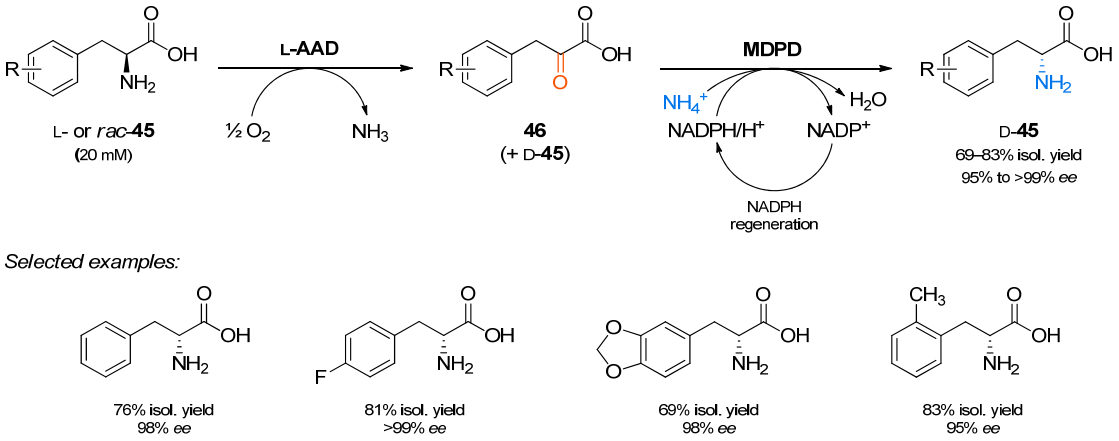
The cascade was optimized with respect to the use of co-solvent, amine donor, and lipase concentration; substrate **42a** was converted under improved conditions with a conversion of 89% and a perfect *ee* of >99% for the (*R*)-enantiomer. Several further β-keto esters were transformed into their corresponding amino acids with conversions ranging between 26 and 99% with excellent *ee* values in all cases (Table 6).

Table 6. Transformation of β-keto esters **42** into β-amino acids **44** (Scheme 12) [a].

Substrate	Conv. (%)	*ee* (%)
42a	89	>99%
42b	96	>99%
42c	75	>99%
42d	73	>99%
42e	99	>99%
42f	61	>99%
42g	47	>99%
42h	41	>99%
42i	26	>99%

[a] Reaction conditions: Substrate **42** (50 mM), (*S*)-1-phenylethylamine (150 mM), PLP (1 mM), lipase (30 mg/mL), TA (20 mg/mL), Tris-HCl buffer (100 mM, pH 8.0), DMSO (15% *v*/*v*), 37 °C, 24 h.

Phenylalanine and derivatives thereof were deracemized by a combination of *E. coli* whole cells expressing an L-amino acid deaminase and an engineered D-amino acid dehydrogenase to prepare optically pure D-phenylalanines (Scheme 13) [66]. Alternatively, instead of starting from the racemate, optically pure L-enantiomers were used as substrate leading to inversion of the absolute configuration. Various substituted derivatives of the D-amino acids were isolated with good yields (69–83%) and excellent optical purities (95% to >99% *ee*).



Scheme 13. Deracemization cascade for the preparation of D-amino acids **45**. Enzyme abbreviations: L-AAD, L-amino acid deaminase; MDPD, *meso*-diaminopimelate dehydrogenase.

4. Lactones

Lactones—and particularly γ- and δ-lactones—are important flavour and fragrance compounds that are found in various fruits, juices, wines, and spirits [67]. Furthermore, substituted γ-butyrolactones occur as a central motif in many natural products, for instance nephrosteranic acid or arctigenin [68]. ε-Caprolactone, on the other hand, is a valuable chemical for the preparation of biodegradable polymers, such as polycaprolactone [69] and a precursor for ε-caprolactam.

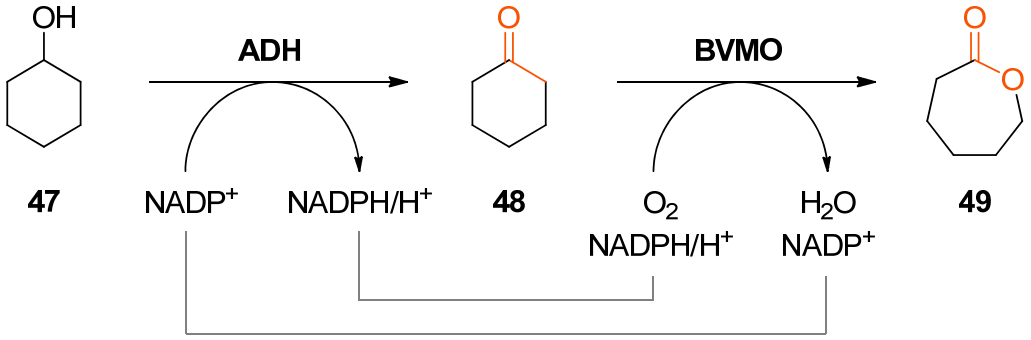
The chemical synthesis of these compounds usually involves a large number of steps, low overall yields, toxic or otherwise hazardous reagents, and impractical reaction conditions, which makes them neither "green" nor atom-economic [70–73]. Hence, biocatalytic cascade transformations have become a useful tool in the preparation of lactones, as they avoid the necessity of time-consuming and yield-reducing isolation and purification of intermediates and reduce waste generation to a minimum.

In most of these multi-enzyme sequences, the lactone is formed in the final step through oxidation of a cyclic ketone by a Baeyer–Villiger monooxygenase (BVMO). However, systems that derive the lactone from an acyclic precursor have also been reported.

4.1. Formation of Lactones by Baeyer–Villiger Monooxygenases in Cascade Systems

The combination of alcohol oxidation by an alcohol dehydrogenase (ADH) and Baeyer–Villiger oxidation of the resulting ketone by a BVMO represents perhaps the simplest biocatalytic cascade system for lactone production. Although this bi-enzymatic reaction represents a double oxidation, it is redox-neutral with respect to the required nicotinamide cofactor, since the Baeyer–Villiger monooxygenase requires NAD(P)H for reducing one oxygen atom of O_2 to water while the other one is incorporated into the substrate. Hence, molecular oxygen is the only stoichiometric co-substrate in the overall process.

The conversion of cyclohexanol (**47**) into ε-caprolactone (**49**) using this cascade concept has been independently reported by two research groups in 2013 (Scheme 14) [74,75].

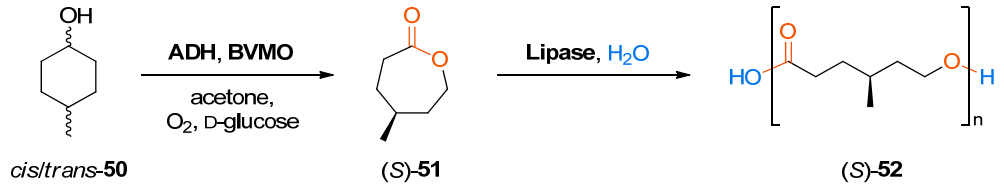


Scheme 14. Transformation of cyclohexanol (**47**) to ε-caprolactone (**49**) using an alcohol dehydrogenase (ADH) and a Baeyer–Villiger monooxygenase (BVMO).

Both studies show that an efficient transformation of cyclohexanol into ε-caprolactone is feasible (94% conversion of 60 mM **47**, 80% conversion of 10 mM **47**); however, both also observed reduced conversions at elevated substrate concentrations which was attributed to inhibition and deactivation of the Baeyer–Villiger monooxygenase. This issue was addressed in subsequent studies carried out as a joint effort of the two involved research groups: [76,77] The ε-caprolactone formed underwent in situ ring-opening oligomerisation enabled by lipase A from *Candida antarctica* (CAL-A), which alleviated product inhibition and produced oligo-ε-caprolactone displaying an average molecular weight of 375 g/mol. It is worth noting that no hydrolysis of **49** to the hydroxycarboxylic acid was observed. In an additional optimisation approach, the authors chose to use a stabilised variant of cyclohexanone monooxygenase (CHMO variant C376L/M400I) [78] and to employ the ADH and the monooxygenase as separate *E. coli* whole-cell preparations. Moreover, despite the theoretical redox-neutral nature of the sequence, acetone and D-glucose (1 equivalent each) were added to improve cofactor regeneration. These reaction conditions enabled the conversion of 200 mM cyclohexanol (**47**) within 48 h going to completion thereby affording a mixture of 75% oligo-ε-caprolactone and 25% of monomeric **49**. Recently, the co-expression of CHMO and ADH in *E. coli* using a Duet™ vector allowed higher conversion values of the substrate **47** in whole-cell biocatalysis compared to an expression of both enzymes from two separate plasmids [79]. Alternatively, fusion of the alcohol dehydrogenase and the BMVO also allowed to engineer the reaction [80]. The same cascade could also be achieved using whole cells of *Geotrichum candidum* CCT 1205 [81].

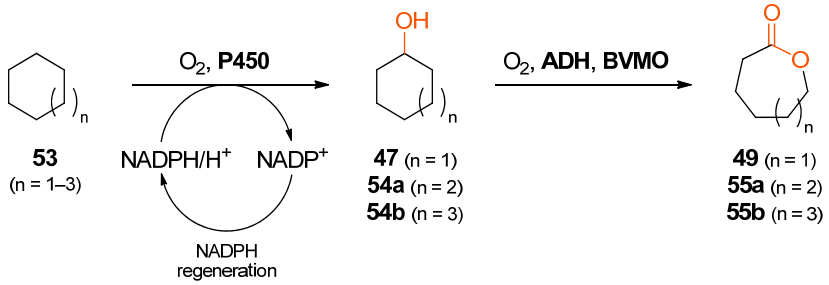
The cascade mentioned above has also been extended by a rhodium-catalyzed hydrogenation step that converts phenol into cyclohexanol (**47**), which is then employed in the biocatalytic cascade without purification [82].

The formation and in situ oligomerisation of a chiral lactone, (*S*)-4-methyl-ε-caprolactone (**51**), from 4-methylcyclohexanol (used as a mixture of *cis* and *trans* isomers) via the same biocatalytic cascade was also demonstrated (Scheme 15) [83].



Scheme 15. Conversion of 4-methylcyclohexanol (**50**) into oligo-(*S*)-4-methyl-ε-caprolactone (**52**) using an alcohol dehydrogenase (ADH), a Baeyer–Villiger monooxygenase (BVMO), and a lipase.

When the alcohol-to-lactone cascade system is extended upstream by a hydroxylation step, lactones can be obtained directly from cycloalkanes. The formation of ε-caprolactone (**49**), ζ-enantholactone (**55a**, n = 2) and η-caprylolactone (**55b**, n = 3) from cyclohexane, cycloheptane, and cyclooctane, respectively, according to this concept has been demonstrated using a variant of the P450 monooxygenase BM3 from *Bacillus megaterium* for introducing the alcohol functionality in the first step (Scheme 16) [84]. The subsequent oxidation to the ketone was performed by an ADH from either a *Thermus* sp. or from *Thermoanaerobacter ethanolicus*, whereby the latter gave the better results in terms of conversion. The final oxidation to the desired lactone was achieved using a stabilised variant of cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871.



Scheme 16. Biocatalytic synthesis of lactones from cycloalkanes using a P450 monooxygenase (P450), an alcohol dehydrogenase (ADH), and a Baeyer–Villiger monooxygenase (BVMO).

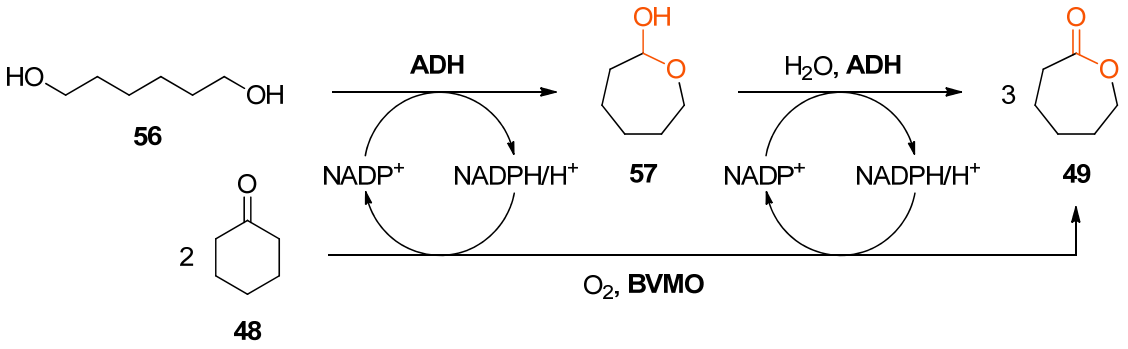
Although the use of a "designer cell" co-expressing all three enzymes did allow production of the desired lactones, yields were generally low (less than 1 mM from 149–185 mM cycloalkane). Therefore, a cascade was tested instead in which the biocatalysts were added as separate cell-free extracts and in which cofactor regeneration was realised by endogenous *E. coli* enzymes and the simple addition of D-glucose and glycerol. Using this reaction setup, 3.2 mM of ζ-enantholactone (**55b**) were formed from cycloheptane within 20 h. The addition of an external cofactor recycling system, consisting of sodium formate and a formate dehydrogenase (FDH), increased the final concentration of **55b** to 10.6 mM, and raising the biocatalyst amounts by a factor of 3.75 resulted in the formation of 23.3 mM **55b**. Table 7 summarizes the results obtained with all three investigated substrates.

Table 7. Results of the biocatalytic synthesis of lactones from cycloalkanes (Scheme 16) [a].

Substrate	TTN [b]	Product Concentrations (mM)		
		Alcohol	Ketone	Lactone
Cyclohexane	822	0	0	5.18
Cycloheptane	4185	0.48	2.63	23.25
Cyclooctane	1017	0.27	2.40	3.74

[a] Reaction conditions: Substrate **53** (149–185 mM), P450 (6.3 µM), ADH (cell-free extract of 150 mg wet-cell weight per mL), CHMO (cell-free extract of 75 mg wet-cell weight per mL), FDH (cell-free extract of 75 mg wet-cell weight per mL), D-glucose (100 mM), glycerol (100 mM), sodium formate (100 mM), NAD^+ (100 µM), $NADP^+$ (100 µM), Tris-HCl buffer (200 mM, pH 8.0), 30 °C, 24 h. [b] Total turnover number: molar amount of products formed per molar amount of P450.

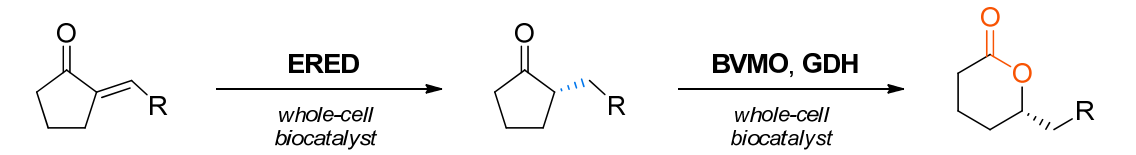
Interestingly, alcohol dehydrogenases and BVMOs have been combined for lactone synthesis also in a second, entirely different way: The Baeyer–Villiger oxidation of cyclohexanone (**48**) to ε-caprolactone (**49**) catalysed by cyclohexanone monooxygenase has been coupled with the double oxidation of hexane-1,6-diol (**56**) to **49** (via the intermediate lactol **57**) catalysed by the ADH from *Thermoanaerobacter ethanolicus* [85]. The resulting convergent cascade system is redox-neutral with respect to the nicotinamide cofactor and theoretically produces three molecules of ε-caprolactone (**49**) from two molecules of **48** and one molecule of **56** (Scheme 17).



Scheme 17. Convergent cascade synthesis of ε-caprolactone (**49**) from 1,6-hexanediol (**56**) and cyclohexanone (**48**). Enzyme abbreviations: ADH, alcohol dehydrogenase; BVMO, Baeyer –Villiger monooxygenase.

However, in practice the formation of three equivalents of ε-caprolactone (**49**) was never observed despite complete depletion of the starting materials due to undesired hydrolysis and oligomerisation of **49** under the reaction conditions. Nevertheless, up to 18.3 mM of **49** were formed from 20 mM of **48** and 10 mM of **56** within a reaction time of 72 h, corresponding to an analytical yield of 61%.

When the asymmetric reduction of prochiral α,β-unsaturated ketones catalysed by ene-reductases is combined with the oxidation by a Baeyer–Villiger monooxygenase catalysed by BVMOs, chiral lactones are obtained. This concept has first been put into practice in a biocatalytic synthesis of 5-alkyl-substituted δ-valerolactones **60** (Scheme 18), which are flavour and fragrance compounds [86].



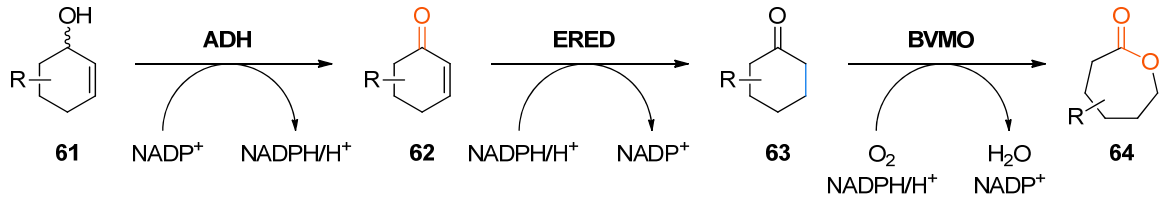
58a (R = *n*-C_5H_{11}) (*R*)-**59a** (87% *ee*) (*R*)-**60a** (83%, 98% *ee*)
58b (R = *n*-C_6H_{13}) (*R*)-**59b** (92% *ee*) (*R*)-**60b** (66%, 97% *ee*)

Scheme 18. Preparation of (*R*)-δ-valerolactones **60** starting from 2-alkylidenecyclopentanones **58**. Enzyme abbreviations: ERED, ene-reductase; BVMO, Baeyer–Villiger monooxygenase; GDH, D-glucose dehydrogenase.

In this study, *Acinetobacter* sp. RS1 was shown to be the best organism for the C=C-reduction step regarding activity, growth and enantioselectivity by screening 200 alkane-, toluene- or benzene-degrading microbial strains. Already after 2 h, the reaction reached >99% conversion, producing (*R*)-**59a** and (*R*)-**59b** with 87% and 92% *ee*, respectively. For the following Baeyer–Villiger oxidation, resting cells of *Escherichia coli* containing a cyclohexanone monooxygenase (CHMO) and a glucose dehydrogenase (GDH) were employed. In initial experiments, an undesired oxidation activity on substrates **58** was observed; consequently, the one-pot cascade was carried out in a sequential fashion, producing (*R*)-**60a** and (*R*)-**60b** with 83% and 66% conversion and 98% and 97% *ee*, respectively.

The improved enantiomeric excess of the products **60** compared to the intermediates **59** was shown to be a consequence of the hydrolysis of the lactone catalysed by the *Acinetobacter* cells. Thereby the (*S*)-enantiomers of lactones **60** were preferentially converted (E = 8–11). Hence, the optical purity of the desired products increased over time due to removal of the minor enantiomer. As a final proof of concept, preparative-scale transformations with **58a** and **58b** (40 mg) were performed and the lactones **60a** and **60b** were isolated in 56% and 41% yield (98% and 97% *ee*), respectively.

A similar combination of ene-reductases and Baeyer–Villiger monooxygenases, extended by an ADH-catalysed step, has been investigated for the preparation of ε-caprolactone derivatives **64** from cyclohexenol derivatives **61** (Scheme 19) [87,88]. This particular cascade was termed an "artificial metabolic mini pathway" by the authors, meaning that all the required enzymes were co-expressed in *E. coli*, while in nature they are not connected in a single organism in a metabolic context.



Scheme 19. "Artificial metabolic mini pathway" for the synthesis of ε-caprolactone derivatives **64**. Enzyme abbreviations: ADH, alcohol dehydrogenase; ERED, ene-reductase; BVMO, Baeyer–Villiger monooxygenase.

Suitable enzyme candidates for each individual step of the cascade were identified whereby the ADH from *Lactobacillus kefir,* the ene-reductase XenB from *Pseudomonas* sp., and the well-known cyclohexanone monooxygenase from *Acinetobacter* sp. performed best for most of the tested substrates. Cofactor regeneration was performed via the metabolism of the *E. coli* host. Table 8 summarises the results of the substrates tested.

Table 8. Overview of results of the "artificial metabolic mini pathway" for the synthesis of ε-caprolactone derivatives **64** (Scheme 19) [a].

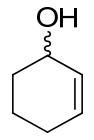
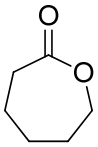
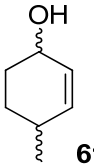
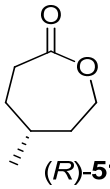
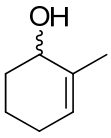
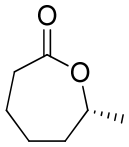
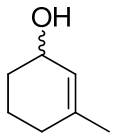
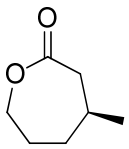
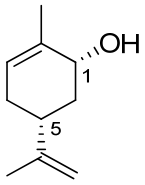
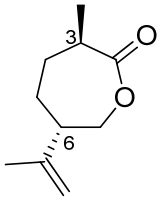
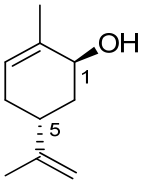
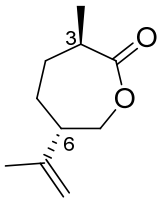
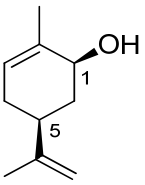
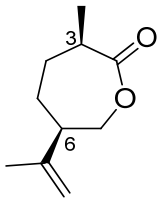
Substrate	Product	Time (h)	Conv. (%)	*ee*/*de* (%)
 **61a**	 **49**	21	>99	*n.a.* [b]
 **61b**	 (*R*)-**51**	20	>99	>99
 *rac*-**61c**	 (*R*)-**64c**	20	64	>99

Table 8. *Cont.*

Substrate	Product	Time (h)	Conv. (%)	*ee*/*de* (%)
 *rac*-**61d**	 (*S*)-**64d**	20	86	>99
 (1*R*,5*R*)-**61e**	 (3*R*,6*S*)-**64e**	20	63	>99
 (1*S*,5*R*)-**61e**	 (3*R*,6*S*)-**64e**	20	93	>99
 (1*S*,5*S*)-**61e**	 (3*R*,6*R*)-**64e**	20	>99	>99

[a] Reaction conditions: Substrate (2.5–4.0 mM), resting *E. coli* cells (concentration not reported), 24 °C, 48 h. [b] *n.a.*: not applicable.

Interestingly, substrate *rac*-**61d** was oxidized by ADH from *Lactobacillus kefir* with 100% conversion, but the ene-reductase from *Pseudomonas* sp. did not accept intermediate **62d**, although it was active on the regioisomeric **62c**. Therefore, an additional ERED was investigated, namely old yellow enzyme 1 (OYE1) from *Saccharomyces carlsbergensis*, which yielded optically pure (*S*)-**63d**, which was then further oxidized to lactone (*S*)-**64d**.

Preparative-scale biotransformations with substrates *rac*-**61d** and (1*S*,5*S*)-**61e** (100 mg) afforded the products (*S*)-**64d** and (3*R*,6*R*)-**64e** in 55% and 60% isolated yield, respectively, and in >99% *ee*/*de*.

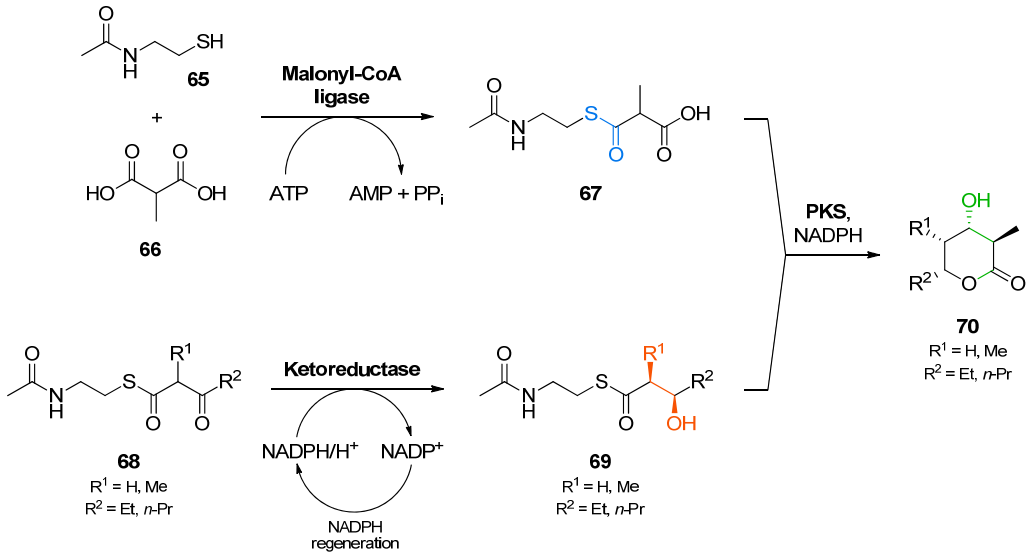
In a subsequent study, three ene-reductases from *Pseudomonas putida* were used in a very similar cascade, in this case implemented using isolated enzymes (purified enzymes and crude cell-free extracts) [89]. Cyclohexenol, cyclohexanone, and cyclopentenone were investigated, leading to δ-valerolactone or ε-caprolactone as products. Conversions between 19% and 99% were obtained at a substrate concentration of 3 mM within 1 h.

4.2. Formation of Lactones from Acyclic Precursors

The formation of lactones form acyclic precursors is not only a common strategy in organic synthesis, but also takes place in the biosynthesis of many naturally occurring lactones, for instance macrolides. These molecules are produced by polyketide synthases (PKSs)—large multi-enzyme

complexes—from acyclic precursors that are assembled by consecutive Claisen condensations while bound to an acyl-carrier protein and are finally cyclised by a thioesterase domain of the PKS [90,91].

The exploitation of this biocatalytic machinery for polyketide synthesis in vitro has proven challenging, but recently researchers have succeeded in producing triketide lactones via multi-enzyme reactions that combine functional domains from several polyketide synthases [92]. The authors used the malonyl-CoA ligase from *Streptomyces coelicolor* for producing the extender unit **67** from methylmalonic acid (**66**) and *N*-acetylcysteamine (**65**) at the expense of stoichiometric amounts of ATP (Scheme 20). In parallel, an asymmetric reduction of β-keto-thioesters **68** by isolated ketoreductase domains of bacterial PKSs was performed to furnish the starter units for the PKS-catalysed Claisen condensation. The crude reaction mixtures obtained from the two biotransformations were then combined and supplemented with the terminal PKS module (consisting of acyl carrier protein, acyl transferase, ketoreductase, and ketosynthase domains) fused to the thioesterase of the erythromycin PKS from *Saccharopolyspora erythraea*. This "minimal PKS" catalysed the Claisen condensation of **67** and **69** as well as the reduction of the resulting ketone and the final cyclization to the product lactone **70**. Although the isolated yields of the overall sequence did not exceed 13%, this method allowed the preparation of substantial amounts of the triketide lactones **70** (3.4–77 mg) as single stereoisomers. The corresponding 3-ketolactones could also be accessed by isolating intermediate **69** and performing the final biotransformation in the absence of an NADPH regeneration system.

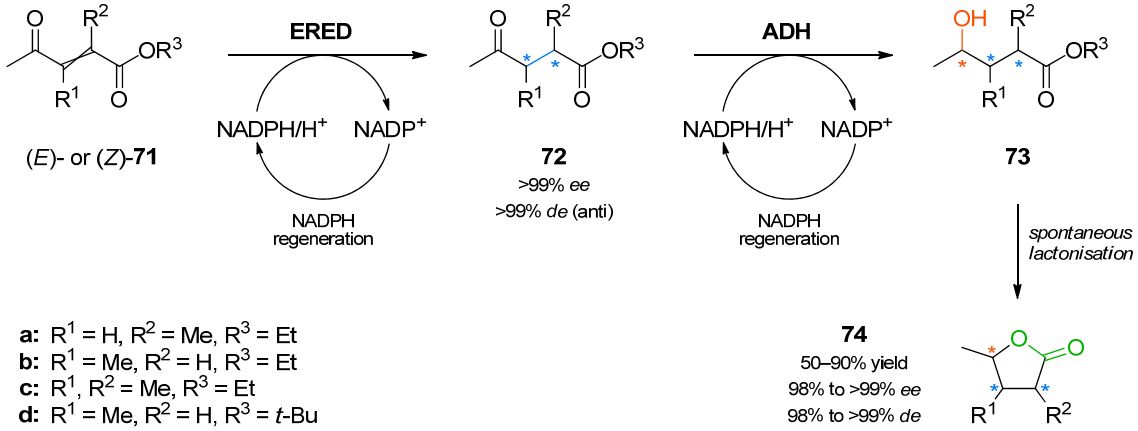


Scheme 20. Synthesis of triketide lactones **70** via coupling of enzymatically prepared precursors **67** and **69** by a polyketide synthase (PKS).

Another study aimed at the synthesis of chiral lactones from acyclic precursors relied on spontaneous ring closure and used ene-reductase YqjM from *Bacillus subtilis* and alcohol dehydrogenases from different microbial sources for controlling the formation of up to three contiguous stereogenic centres (Scheme 21) [93].

In the first step of the synthetic sequence, (*E*)- or (*Z*)-oxo-esters **71** were reduced by YqjM—a reaction that proceeded with varying stereochemical outcomes: While both double bond isomers of substrate **71a** (R^1 = H, R^2 = Me, R^3 = Et) afforded the same product (*R*)-**72a** and only the (*E*)-isomer of substrate **71c** (R^1, R^2 = Me, R^3 = Et) was converted, substrate **71b** (R^1 = Me, R^2 = H, R^3 = Et) displayed an *E*/*Z*-dependent switch in selectivity. The (*E*)-isomer was reduced to (*S*)-**72b**, while the (*Z*)-isomer gave the (*R*)-enantiomer, and in both cases optically pure products (*ee* > 99%) were obtained. The subsequent reduction of intermediates **72** catalysed by alcohol dehydrogenases from *Lactobacillus kefir* (*Lk*-ADH), *Lactobacillus brevis* (*Lb*-ADH), *Thermoanaerobacter* sp. (ADH-T), or from

a commercial supplier (evocatal, evo-1.1.030) was more predictable, proceeding with the expected selectivity in all cases. The ethyl esters **73a–c** underwent spontaneous cyclisation to the desired lactones **74a–c**, while the reaction employing a *tert*-butyl ester stopped at the hydroxyacid stage (**73d**). All products were easily isolated by extraction and purified by column chromatography. The isolated yields and optical purities are summarised in Table 9.



Scheme 21. Two-step cascade leading to substituted γ-butyrolactones (**74**) using an ene-reductase (ERED) and an alcohol dehydrogenase (ADH) for controlling up to three adjacent stereogenic centres.

Table 9. Results of the two-step biocatalytic synthesis of substituted γ-butyrolactones (**74**; Scheme 21) [a].

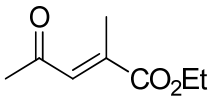
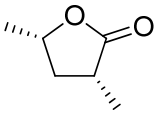
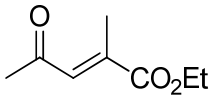
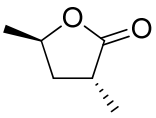
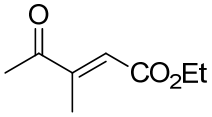
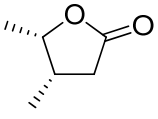
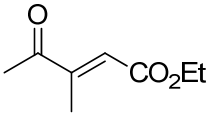
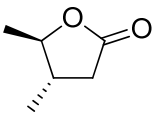
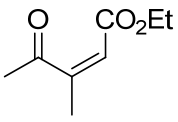
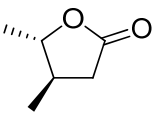
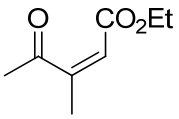
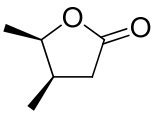
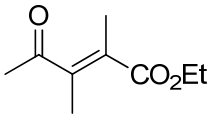
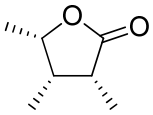
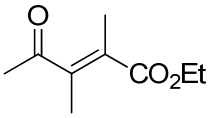
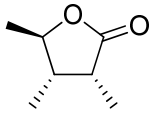
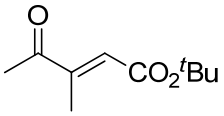
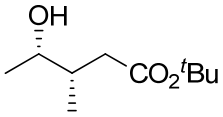
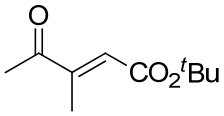
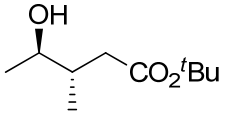
Substrate	ADH [b]	Product	Yield [c] (%)	*ee* (%)	*de* (%)
 (*E*)-**71a**	ADH-T	 (2*R*,4*S*)-**74a**	90	>99	>99
 (*E*)-**71a**	*Lk*-ADH	 (2*R*,4*R*)-**74a**	80	>99	>99
 (*E*)-**71b**	evo-1.1.030	 (3*S*,4*S*)-**74b**	82	>99	>99
 (*E*)-**71b**	*Lk*-ADH	 (3*S*,4*R*)-**74b**	90	>99	>99
 (*Z*)-**71b**	evo-1.1.030	 (3*R*,4*S*)-**74b**	63	98	>99

Table 9. *Cont.*

Substrate	ADH [b]	Product	Yield [c] (%)	*ee* (%)	*de* (%)
 (*Z*)-**71b**	*Lk*-ADH	 (3*R*,4*R*)-**74b**	50	98	>99
 (*E*)-**71c**	ADH-T	 (2*R*,3*S*,4*S*)-**74c**	70	>99	>99
 (*E*)-**71c**	*Lb*-ADH	 (2*R*,3*S*,4*R*)-**74c**	62	>99	98
 (*E*)-**71d**	evo-1.1.030	 (3*S*,4*S*)-**73d**	75	>99	>99
 (*E*)-**71d**	*Lk*-ADH	 (3*S*,4*R*)-**73d**	85	>99	>99

[a] Reaction conditions: Substrate **71** (67 mM), D-glucose (250 mM), $NADP^+$ (0.67 mM), GDH (0.067 U/mL), ERED (0.167 U/mL; purified enzyme), ADH (0.73–1.67 U/mL; cell free extract), phosphate buffer (100 mM, pH 7.0), 30 °C, 24 h. [b] ADH-T, alcohol dehydrogenase from *Thermoanaerobacter* sp.; *Lb*-ADH, alcohol dehydrogenase from *Lactobacillus brevis*.; *Lk*-ADH, alcohol dehydrogenase from *Lactobacillus kefir*; evo-1.1.030, commercial alcohol dehydrogenase from evocatal. [c] Isolated yields after column chromatography.

In a later study, the cascade was extended, starting from allylic cyclic alcohols using fusion proteins; however, conversions did not exceed 34% [94].

5. Conclusions

Cascade reactions possess the advantage of circumventing the need of isolation of reaction intermediates, which saves resources, reagents, and time. They may also minimize the risk of undesired side reactions of unstable intermediates. Furthermore, the cascade approach is expected to lead to higher yields compared to a classical sequence of single-step transformations and at the same time can increase the synthetic efficiency by reducing operational work-up steps and resources. Consequently, cascades enable to reduce the amount of waste, due to minimization of the amount of chemicals used (e.g., by circumventing work-up).

The cascades discussed in this review represent a fraction of possible artificial biocatalytic cascades [1ac], of which the number will significantly increase during the next years. This will be a result of (novel) enzymes becoming more easily available, improved expression tools, as well as improved enzyme engineering methods.

This review shows that artificial cascades may can use different feedstocks as substrates: either from renewables such as amino acids but also from fossil resources, such as phenol or styrenes, etc.

The target compounds discussed in the review, namely hydroxy acids, amino acids, and lactones represent important building blocks and intermediates. The cascades shown enabled the successful preparation of these compounds within 2–10 artificially combined steps.

A number of cascades have already been described to be performed at elevated substrate concentration, e.g., reaching in the best case e.g., 600 mM as reported in this review and are therefore ideally suited for preparative transformations and applications; however, in other cases the substrate concentration is as low as 1 mM, which is due to the limitation of the most sensitive enzyme in the cascade. Thus, a cascade can only be as good in terms of efficiency (space time yield) as is given by the "weakest" enzyme. Limiting reactions are still hydroxylation transformations of C–H bonds but also cross-inhibition by products/substrates of various steps may force to keep concentrations low. Therefore, there is still a need for further improved enzymes. Another point which would be desirable is that co-expression constructs for various cascades may be deposited and made broadly available for subsequent studies. This may allow to generate the possibility to design rather fast novel complex networks including the now designed and optimized cascades.

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