

# CHEMICAL REVIEWS

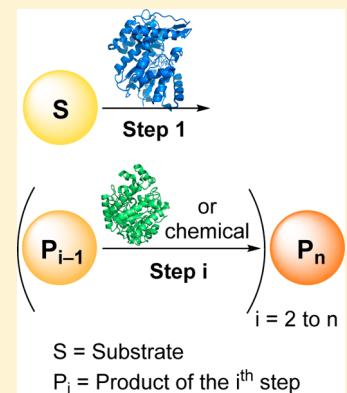
## Artificial Biocatalytic Linear Cascades for Preparation of Organic Molecules

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**ABSTRACT:** The review compiles artificial cascades involving enzymes with a focus on the last 10 years. A cascade is defined as the combination of at least two reaction steps in a single reaction vessel without isolation of the intermediates, whereby at least one step is catalyzed by an enzyme. Additionally, cascades performed *in vivo* and *in vitro* are discussed separately, whereby *in vivo* cascades are defined here as cascades relying on cofactor recycling by the metabolism or on a metabolite from the living organism. The review introduces a systematic classification of the cascades according to the number of enzymes in the linear sequence and differentiates between cascades involving exclusively enzymes and combinations of enzymes with non-natural catalysts or chemical steps. Since the number of examples involving two enzymes is predominant, the two enzyme cascades are further subdivided according to the number, order, and type of redox steps. Furthermore, this classification differentiates between cascades where all reaction steps are performed simultaneously, sequentially, or in flow.



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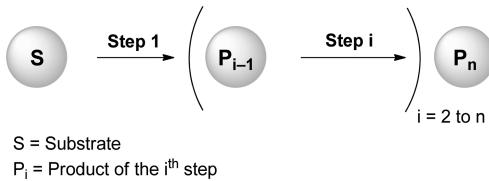
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## 1. INTRODUCTION

### 1.1. Scope of Review and Definitions

A cascade is here defined as the combination of at least two chemical steps in a single reaction vessel without isolation of the intermediate(s).<sup>1</sup> Linear cascade means that the product of one step in the cascade is the substrate of a second subsequent step (**Scheme 1**). In the case of biocatalytic cascades, at least one

**Scheme 1.** Linear Cascade Comprising  $n$  Steps ( $n \geq 2$ )



biocatalyst is required for at least one step in the sequence of transformations. The biocatalyst can be, for instance, an isolated enzyme, a crude cell-free extract, an immobilized enzyme, or enzymes in whole microbial cells, etc. "Artificial cascade" means that the reaction sequence has been designed by combining enzymes from different organisms and the cascade is not part of a metabolism in natural organisms (as far as known) for the specific substrates studied. Actually, artificial biocatalytic cascades can be regarded as an extension of biosynthesis in organisms,<sup>2,3</sup> which can be exploited for the synthesis of (non-natural) organic molecules.

In this review, the term "in vivo" is used for cascades that use (i) the metabolism of a living organism for cofactor regeneration or (ii) a metabolite constantly produced by the organism. If this is not the case, the cascades are classified as *in vitro*. In some publications, *in vivo* is used as a term equal to "using whole cells"; however, this can be confusing, since also, e.g., freeze-dried (whole) cells containing coexpressed enzymes may be used for the desired reaction and cofactor regeneration and are not de facto *in vivo* systems.

The review covers artificial linear cascades with at least one biocatalyzed step; thus, other steps might be catalyzed by chemical catalysts or performed with stoichiometric amounts of reagent or may be spontaneous. The focus is on the development of such cascades over the last 10 years. The review suggests a possible classification of cascades ([section 1.3](#)) and is then structured following the classification proposed: first, according to the number of enzymes involved in the linear sequence; next, whether the subsequent reactions are spontaneous or need a catalyst. If they need a catalyst, it has been differentiated between cascades requiring exclusively enzymes and such cascades combining enzymes and other types of catalysts or chemical steps. Then the cascades have been grouped based on the criteria whether the steps of the sequence can be performed simultaneously or the reaction has to be divided into stages performed sequentially. Importantly, it was also distinguished between cascades performed *in vitro* and *in vivo*. Since cascades with two steps are numerous, these cascades are subdivided whether they encompass two, one, or no redox step; in case a redox step is part of the cascade, the order and type of the redox steps were considered, whereby hydrogen-borrowing cascades and deracemization are treated separately. We name a cascade redox cascade if at least one step is a redox step. If cascades have been described in one publication with a different number of

steps, they will be discussed in the section referring to the longest sequence.

Flow reaction systems may be seen as a special case, since they may not comprise only a single reaction vessel in the classical sense but may consist of several columns connected to each other representing different compartments. Since this can be seen as a vessel system, flow reactions with several columns are also discussed here as cascades.

Various reviews on cascades have been published,<sup>2–17</sup> whereby in some cases the focus was on particular techniques such as deracemization,<sup>18–24</sup> on specific products such as biofuels and biochemicals,<sup>25</sup> on combination of enzymes with metal catalysis,<sup>26–33</sup> or on enzyme classes.<sup>34–37</sup> Some recent reviews have classified *in vitro* cascades as "cell-free metabolic engineering",<sup>38</sup> "cell-free enzymatic reaction cascades",<sup>39</sup> or "systems biocatalysis".<sup>40,41</sup> Other related reviews deal with *de novo* redox pathways in the host *Escherichia coli*,<sup>42</sup> cascade optimization,<sup>43</sup> thermodynamic constraints,<sup>44</sup> aspects of process technology,<sup>45</sup> multienzymatic synthesis in cells,<sup>46</sup> designer microbes,<sup>47–49</sup> and continuous flow processes.<sup>50,51</sup> Studies on glycoconjugates are not the focus of the review and have been reviewed elsewhere.<sup>31,52,53</sup>

### 1.2. History

The first enzymatic cascade studied *in vitro* was most likely the transformation of sugars to ethanol and CO<sub>2</sub>, which led to the discovery of the enzymes and intermediates of glycolysis.<sup>54,55</sup> In the history of biocatalysis,<sup>56</sup> an early publication in which cascades with enzymes were classified especially with respect to glycolysis dates back to 1949.<sup>57</sup> The book composed of four lectures on enzymes already commented that systems of two and more enzymes could be built up synthetically to study the phenomena of these systems. The natural glycolytic system was later reconstituted from individually purified enzymes to study the concentrations of intermediates in this important natural enzyme system.<sup>58</sup> A first artificial *in vitro* cascade with two enzymes in the linear sequence was shown for the transformation of lactic acid to L-alanine via pyruvate in flow as early as 1984.<sup>59,60</sup> The same concept was subsequently applied to phenylalanine.<sup>61,62</sup> Other cascades for amino acid production followed, such as the deracemization of racemic methionine to L-methionine in 1990.<sup>63</sup> An "artificial metabolism" with eight enzymes was designed to produce 1,3-dihydroxyacetone phosphate as intermediate for an aldolase reaction to build a new C–C bond in 1992.<sup>64</sup> The same year, an artificial cascade consisting of an enzymatic and a chemical step was published, which allowed the deracemization of proline; thus, L-proline was prepared from the racemate using a D-amino acid oxidase and sodium borohydride as reducing agent.<sup>65</sup> The combination of an enzyme-catalyzed kinetic resolution with racemization enabled by an ion exchanger was published just 1 year before,<sup>66</sup> extending the concept of the already established dynamic kinetic resolution<sup>67</sup> exploiting spontaneous racemization.

### 1.3. Classification of Cascades

Cascades, especially biocatalytic cascades, may be classified according to various parameters ([Table 1](#)). One obvious descriptor is the number of reaction steps in the linear sequence. The number of catalysts may differ from the number of steps (entry 2). For instance, only a single catalyst is required for (i) domino cascades,<sup>13</sup> which are initiated by one catalyst and all subsequent steps proceed spontaneously, (ii) reaction sequences catalyzed by multifunctional enzymes (e.g., polyketide synthases<sup>68–70</sup>), as well as (iii) cascades in which the substrate bears

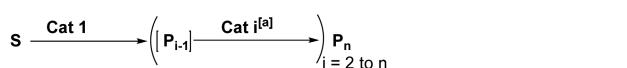
**Table 1. Parameters To Classify an Artificial Cascade Involving (Bio)catalysts**

entry	classification parameter	remark
1	number of steps	range is in theory 2–∞
2	number of catalysts	minimum number of catalysts has to be at least 1; in the special case of one catalyst, (i) an unstable product may react spontaneously to subsequent products (domino cascade); <sup>13</sup> (ii) the catalyst has multiple activities (e.g., polyketide synthase), or (iii) the substrate bears the same functionality several times, which gets transformed by the same catalyst; in general, the range for the number of catalysts can be 1–∞
3	type of combined catalysts	one might distinguish additionally between the number of catalysts in the linear sequence and the number of catalysts required for the overall multistep reaction; two numbers may differ, e.g., if enzymes are required for cofactor recycling, removal of cosubstrates, etc., as in the case, for instance, of combination with orthogonal/parallel/cyclic cascades and convergent/divergent reactions.
4	topology	catalysts may be biocatalysts, organocatalysts, or metal catalysts although the focus in this review is on linear cascades, orthogonal/parallel and cyclic cascades have been described in biocatalysis; for more details see text
5	redox reaction	in case more possibilities exist to define the linear sequence, the sequence with most catalysts is seen as the main sequence enzymatic redox reactions may require additional cofactor recycling systems, such as an additional enzyme and oxidation/reduction reagent; the cascade may be named redox cascade if at least one step in the sequence is a redox step; otherwise, it is a nonredox cascade; in the case of a redox cascade, the cascade may also be classified as redox self-sufficient or redox neutral if no additionally added oxidation or reducing reagent gets consumed; thus, the cascade needs to have an equal number of generated oxidized and reduced cofactor; typical example is termed hydrogen-borrowing cascade, where an oxidation step is balanced by a reduction step and the hydride abstracted in the oxidation is recycled in the reduction step; consequently, the oxidation state of the product is the same as that of the substrate; subclassification may include the number and order of oxidation and reduction steps in the cascade
6	mode of cofactor regeneration	in vivo, in vitro: in vivo refers in general to whole, living organisms; the reason for their use in cascades may be to exploit the cofactor regeneration system of the living metabolism or its produced metabolites; thus, we will use the term <i>in vivo</i> here if the cofactors are recycled by the metabolism or a metabolism is ennobled in a subsequent artificial cascade; everything else can be seen as <i>in vitro</i> ; a special case of <i>in vitro</i> may be <i>flow</i> , whereby the catalysts are immobilized in successive columns and the substrate passes through; this is actually a method for compartmentalization (see also entry 13)
7	chronology	reaction steps of the cascade may be run simultaneously/concurrently or in a sequential mode; in the first case, all reagents are already present in the reaction vessel at the onset of the reaction, while in the latter case reagents are added after a certain sequence is completed; sequential cascade is in the literature sometimes also referred to as stepwise, but this term may cause confusion; therefore, we prefer to use the term sequential mode; the parts of the cascade separated in time are then termed stages; <sup>88</sup> a stage may comprise one or more reaction steps; cascades performed in flow (sequential columns bearing different catalysts) may have all reagents present at the onset of the reaction but have a spatial separation examples may be hydrolysis, reduction (ketone/aldehyde, carboxylic acid, C=C, reductive amination), oxidation (alcohol, aldehyde, C–H···, Baeyer–Villiger), C–C bond formation examples might be deracemization, hydrogen borrowing, alcohol amination, hydroamination, etc.
8	type of chemical reaction steps	EC numbers or enzyme class(es) of involved biocatalysts.
9	descriptor for overall reaction accomplished by the cascade	Possible preparations are purified enzymes, cell-free extracts, <sup>92</sup> whole cells containing recombinant enzyme, freeze-dried catalyst preparations, resting cells, growing cells, immobilized enzymes, cross-linked enzyme aggregates etc.; since in a cascade several biocatalysts are applied, they may differ with respect to the type of preparation; in case all cascade enzymes are expressed in a single host (also for <i>in vitro</i> experiments), the term single-cell catalyst may be used
10	final product formed	spatial organization can be obtained <sup>89–91</sup> by having the catalysts in solution, (co)immobilized, <sup>92,94</sup> compartmentalized (e.g., by a membrane) <sup>95–98</sup> or colocalized, e.g., on a protein or DNA scaffold, <sup>99</sup> artificial protein complexes, <sup>100</sup> multienzyme systems by gene fusion, <sup>101</sup> site-specifically coupled multiple enzymes, <sup>102</sup> scaffolded multienzyme cascade, <sup>103</sup> enzymes in layered nanomaterials <sup>104,105</sup> or on nanoparticles, <sup>106</sup> multicompartimentalized polymersomes, <sup>107</sup> but also separation by having the catalysts in different cartridges as in flow systems and others
11	enzyme classes	
12	type of biocatalyst preparation	
13	spatial organization of biocatalysts	

the same functionality several times and therefore can be transformed by the catalyst more than once; all other cascades require at least two catalysts. In the review, only the number of catalysts of the steps in the linear sequence is considered. In addition to the type of catalysts employed (entry 3), the topology is of interest (entry 4). Although the review focuses on linear cascades, basic reaction concepts which are also referred to as cascades are summarized in **Scheme 2**. While the linear,

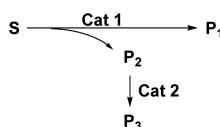
### Scheme 2. Basic Concepts of Multicatalyst/Enzyme Reactions

#### (A) Linear cascade with n linear steps

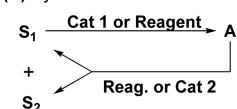


[a]: in case of domino cascades or multiple activities by a single catalyst or several comparable transformable functionalities in S only biocatalyst 1 is required

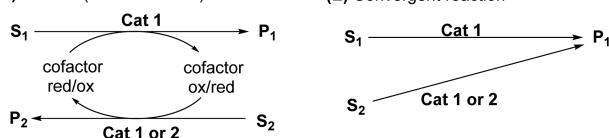
#### (B) Orthogonal cascade



#### (C) Cyclic cascade

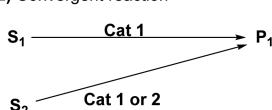


#### (D) Parallel (interconnected) reaction

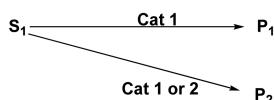


#### (E) Convergent reaction

#### (E) Convergent reaction



#### (F) Divergent reaction



orthogonal, and cyclic cascades<sup>10</sup> are in line with the definition of a cascade, the parallel reaction (**Scheme 2D**) is not compatible with respect to the substrates but with respect to the cofactor. Therefore, this scheme is commonly associated with cofactor recycling systems;<sup>71–75</sup> such schemes are also part of concepts to shift the equilibrium of reactions, such as in the amination of ketones using transaminases.<sup>10,37,76–82</sup> The latter scheme of involving two tandem concurrent processes<sup>83</sup> also applies to the reaction system named “parallel interconnected transformation”.<sup>84</sup> Other reaction systems such as convergent<sup>85</sup> or divergent<sup>86</sup> reactions (**Scheme 2E** and **2F**) do not fulfill the definition of a cascade per se as stated above; in these cases, formally, additional steps in a linear sequence are required to call the overall reaction system a cascade.<sup>87</sup> Thus, very often combinations of the different basic concepts mentioned in **Scheme 2** can be found, especially in redox cascades: for instance, linear cascades are combined with parallel reactions for recycling of the cofactors needed. Consequently, for the classification of cascades, redox steps need special attention. Redox cascades may be classified as redox self-sufficient or redox neutral if no additionally added oxidation or reducing reagents get consumed (**Table 1**, entry 5); thus, the oxidation state of the product is the same as that of the substrate, and the redox equivalents required in the sequence are provided by the substrate(s) or subsequent intermediates thereof. If cofactor regeneration is required, redox equivalents may be provided via parallel reactions by an additional regeneration system (*in vitro*) or from the metabolism (*in vivo*) (**Table 1**, entry 6).

Furthermore, the type and number of redox reactions and their order (i.e., whether an oxidation and a reduction step are combined and in which order or whether oxidation or reduction steps only are involved) might be considered for classifying cascades.

For a clear description of a cascade, the chronological order of events is important, i.e., whether the reactions are run at the same time (simultaneously/concurrently) or sequentially; in the simultaneous case all reagents have to be added at the onset of the reaction, while in the sequential mode some reagents are added to the reaction mixture at a later point of time. The parts of the cascade separated in time are then termed stages;<sup>88</sup> a stage may comprise one or more reaction steps. Cascades performed in flow with sequential columns bearing different, e.g., catalysts may have all reagents present at the onset but have a spatial separation.

Other descriptors for a cascade may refer to the type of reaction of each step or to the overall reaction (entries 8 and 9) and the final product formed (entry 10). The final three parameters (entries 11–13) describe the type of enzymes involved and what kind of enzyme preparation is used as well as possible methods to bring the enzymes involved in a cascade close together,<sup>89,90</sup> which may allow a more efficient cascade due to substrate channeling.<sup>91</sup>

#### 1.4. Advantages of Cascades and Challenges

Cascade reactions offer the advantage of circumventing the isolation of reaction intermediates, which not only saves resources, reagents, and time but are also useful if *in situ* formation of unstable intermediates occurs in the reaction sequence, since they are directly consumed in the next step. Consequently, the cascade approach can lead to higher yields compared to a classical sequence of single-step transformations while at the same time increasing the synthetic efficiency by saving operational workup steps and resources. Additionally, cascades may help to reduce the amount of waste, since less chemicals (e.g., by circumventing work up) are used.

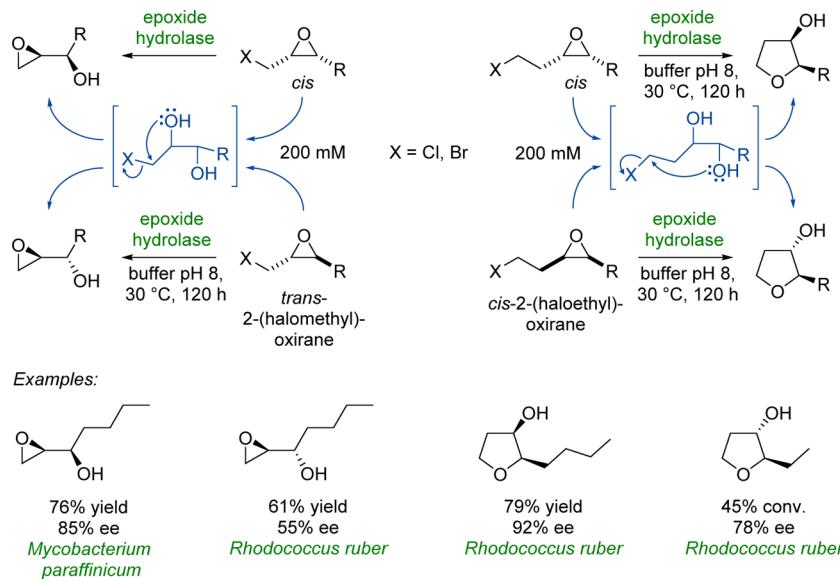
Cascades may be ideal to avoid the need for applying toxic compounds as initial substrate, since these reagents might be generated *in situ* and consumed as soon as formed.

Combining catalysts and reagents in a single pot and performing reactions simultaneously require all reaction conditions to be compatible with each other, which could be challenging, for instance, in case the pH range of the individual catalysts does not overlap; also, the temperature needs to be considered, since multiple enzymes in a cascade may have different optimum operational temperatures, especially if thermophilic enzymes are employed. In case a cosolvent is required to improve the solubility of the substrate in the aqueous phase, all enzymes must tolerate the solvent.

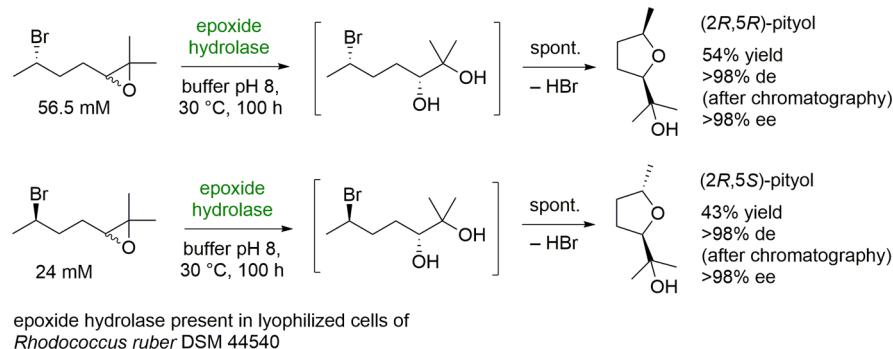
Furthermore, the overall reaction cascade must be energetically favored.<sup>17</sup> If this is not the case, the cascade has to be redesigned to enable high conversion. This can be achieved, for instance, by removing (co)products or in redox reactions by providing suitable redox reagents. Ideally, the last step should be energetically downhill, leading to the thermodynamically preferred final product. Reaction steps within the sequence possessing high activation energy may slow down the overall cascade.

Cascades may provide a solution to enzyme inhibition by reaction intermediates. Inhibition of an enzyme in the cascade by a reagent/compound involved in a later step in the sequence can actually best be solved by performing the cascade in a sequential

**Scheme 3. Epoxide Hydrolase-Triggered Formation of Hydroxy-Epoxides or Tetrahydrofuran Derivatives from Racemic Haloalkyl Oxiranes Using Wild-Type Cells**



**Scheme 4. Synthesis of (2*R*,5*R*)- and (2*R*,5*S*)-Isomers of Pityol through Enzyme-Catalyzed Hydrolysis/Spontaneous Ring-Closure Cascade Using Epoxide Hydrolase Activity of Lyophilized Whole Cells of *R. ruber* DSM 44540**



mode, thus adding the reagents/catalyst for the later step at a later time point. Inhibition of an enzyme by a compound appearing prior in the sequence may be addressed either by controlling the initial concentration (feeding) or again by applying the sequential mode. Performing a cascade in sequential mode may provide the possibility to use noncompatible reaction conditions (e.g., pH, solvents). Furthermore, a sequential cascade may circumvent cross-reactivities of catalysts involved, thus by adding a catalyst which might react with different functionalities present during the reaction sequence at a later time point, a low (chemo)selectivity of the catalyst might be overcome. In the case of *in vivo* cascades or using a single-cell preparation containing all coexpressed enzymes, the inhibition issue can be even more problematic. Additionally, control of the ratio of activities of the individual enzymes present gets more challenging, and fine tuning of the activities of each enzyme present may become more elaborate. The highest flexibility in running a cascade may thus be achieved by using for each enzyme a single preparation, while the highest economy may be obtained by a fine-tuned single-cell catalyst containing all enzymes required at once. It should be stressed that the turnover of an overall cascade is determined by the turnover number of the least stable enzyme.

## 2. SPONTANEOUS CASCADES TRIGGERED BY A SINGLE ENZYME

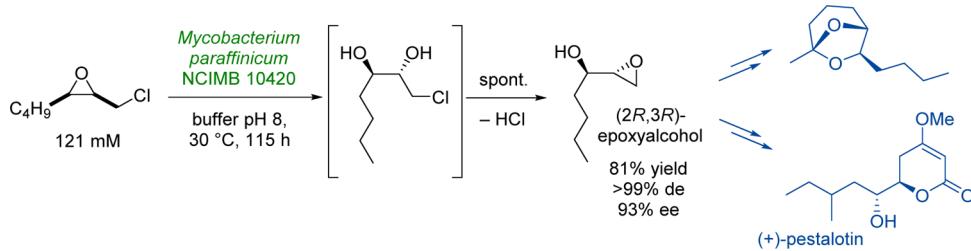
Synthetic chemists aiming at total synthesis of natural products often rely on biomimetic strategies deriving from the analysis of biosynthetic pathways. The generation of complex structures in nature often occurs through astonishingly simple reactions. Cyclic molecules in particular can be obtained from enzyme-triggered cascades, in which a single biocatalyst catalyzes the generation of (unstable) reactive intermediates, which undergo (spontaneous) events, eventually leading to cyclization reactions. Depending on the mechanism, two types of cascade reactions can be distinguished: (i) nucleophilic reactions involving C–O bond formation and (ii) electrophilic reactions involving C–C bond formation.<sup>4,109</sup> Epoxide hydrolases are predominant in the first type of cascades, whereas cyclases are mostly involved in the second type.

Several spontaneous reaction steps can also be found later in the review in various other cascades involving more than one enzyme but will not be considered there as a separate step.

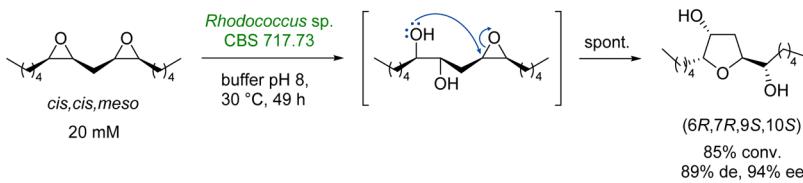
### 2.1. Epoxide Hydrolase-Triggered Cascades Involving C–O Bond Formation

Bacterial epoxide hydrolases were employed in the hydrolysis of 2-(1- or 2-haloalkyl) oxiranes, forming diols as intermediates,

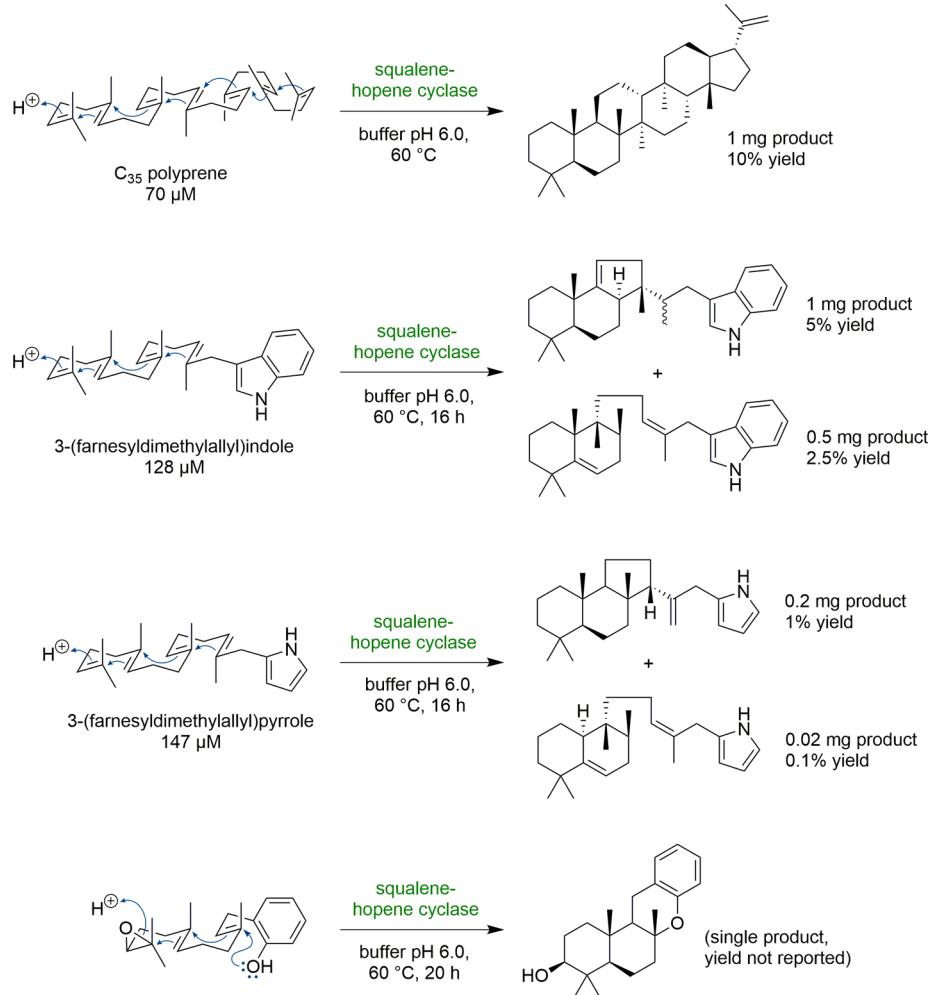
**Scheme 5. Enzyme-Triggered Conversion of Racemic Haloalkyl Oxirane to Epoxyalcohol as Precursor of Natural Products Using Epoxide–Hydrolase Activity of Lyophilized Whole Cells of *Mycobacterium paraffinicum* NCIMB 10420**



**Scheme 6. Access to Dihydroxy–Tetrahydrofuran Derivative from Methylene-Interrupted *meso*-Bis-epoxide through the Action of Epoxide–Hydrolase Activity of *Rhodococcus* sp. CBS 717.73**



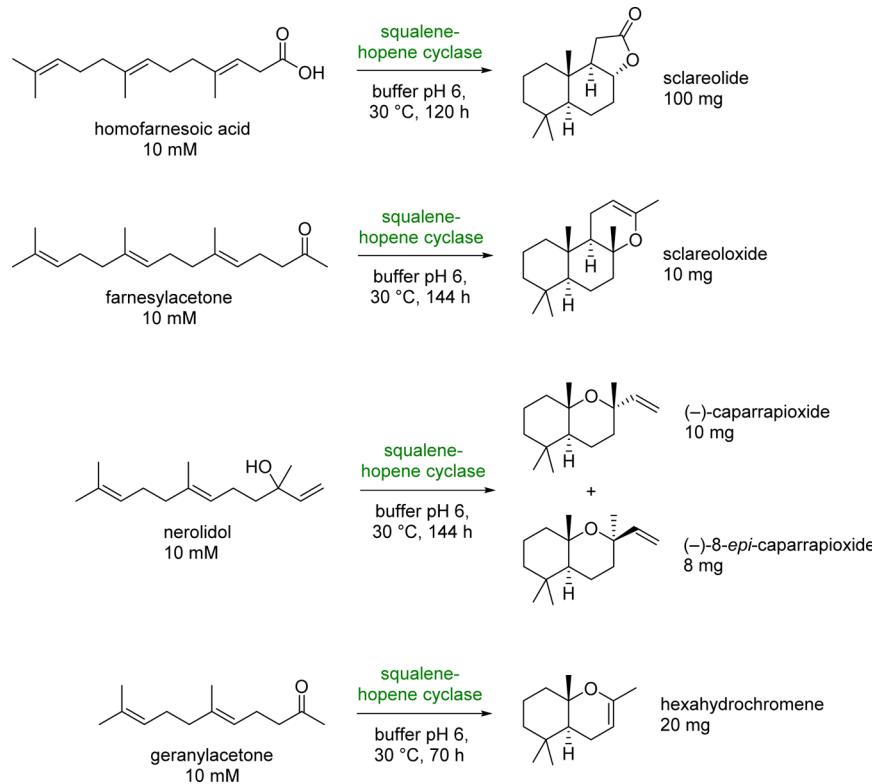
**Scheme 7. Squalene–Hopene Cyclase from *A. acidocaldarius* Catalyzes the Formation of Polycyclic Compounds<sup>119–122</sup>**



which, depending on the substitution pattern, spontaneously cyclized to hydroxy–epoxides or tetrahydrofuran derivatives through intramolecular nucleophilic substitution following Baldwin's rules of ring closure (favored *exo-tet*) (Scheme 3).

Racemic cis- and trans-configured epoxides eventually furnished diastereomerically pure cis- (or syn-) and trans- (or anti-) configured end products, respectively, as single predominant enantiomer in high enantiomeric excess. Reactions were carried

**Scheme 8. Squalene–Hopene Cyclase (*ZmoSHC1*) from *Z. mobilis* Enables the Formation of Polycyclic Compounds<sup>123</sup>**



out in Tris buffer (pH 8) with lyophilized cells from a selection of microorganisms possessing epoxide hydrolase activity.<sup>110</sup>

Similarly, the enzyme-mediated synthesis of two isomers of naturally occurring pheromone pityol was designed based on retrosynthetic analysis, which identified a brominated epoxide as a possible precursor. This compound with fixed absolute configuration for the carbon bearing the halogen was used as a mixture of isomeric epoxides and subjected to diastereococonvergent hydrolysis by epoxide hydrolase contained in whole cells of *Rhodococcus ruber* DSM 44540. The resulting diols underwent spontaneous S<sub>N</sub>2-type ring closure accompanied by liberation of hydrobromic acid (Scheme 4). The minor stereoisomer formed was removed by column chromatography and allowed isolation of both (2*R*,5*R*)- and (2*R*,5*S*)-pityol in high de and ee values.<sup>111</sup>

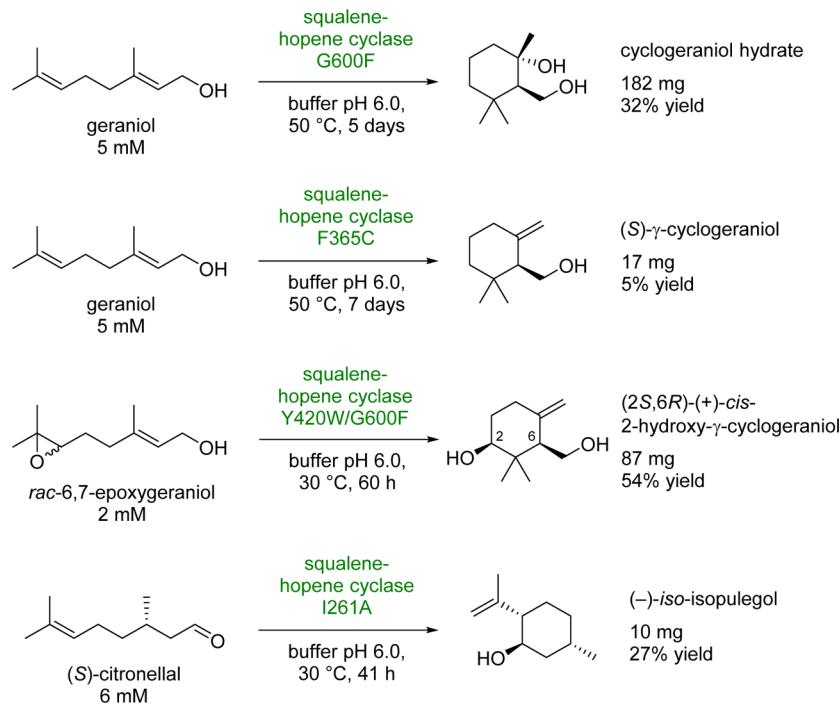
Following similar strategies, the preparation of (+)-pestalotin involved the formation of an epoxyalcohol through epoxide hydrolase-triggered transformation of haloalkyl oxiranes (hydrolysis followed by S<sub>N</sub>2-type ring closure and concomitant release of hydrochloric acid). (+)-Pestalotin is a natural product originally isolated from the culture filtrate of a fungus (*Pestalotia cryptomeriaecola*) and acts as plant growth stimulator. This stereoisomerically pure product of the enzymatic transformation was also used as a building block in the synthesis of a bicyclic acetal isolated from Jamaican rum aroma (Scheme 5). The biotransformation was run on gram scale for extended time (115 h), and after continuous product extraction and flash chromatography, the final product could be isolated in 81% yield as one stereoisomer (>99% de and 93% ee).<sup>112</sup>

Methylene-interrupted bis-epoxides were studied in view of their high reactivity toward epoxide hydrolases and the possibility to strictly control the stereochemistry of up to four centers simultaneously through hydrolysis. A meso-bis-oxirane was transformed in a cascade to the corresponding tetrahydrofuran product via hydrolysis of the first oxirane moiety and

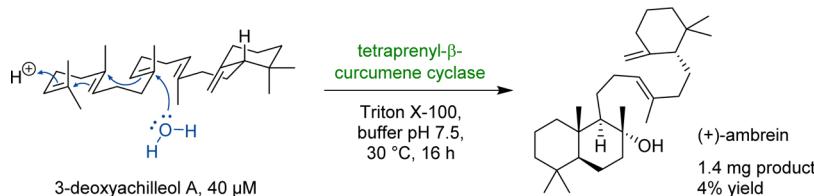
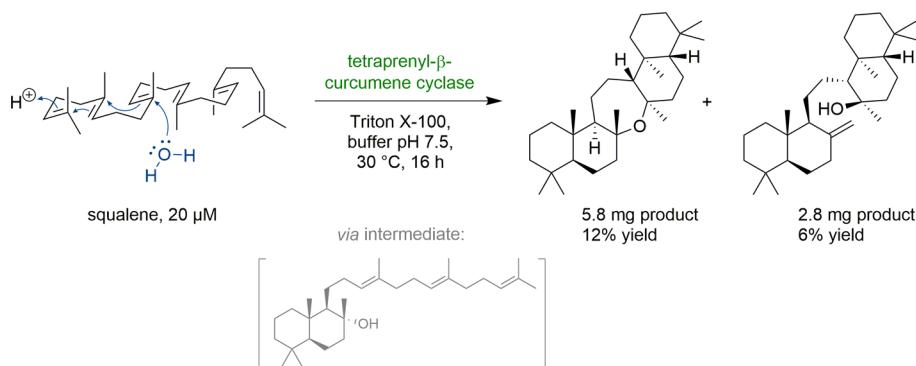
subsequent spontaneous intramolecular cyclization<sup>113</sup> via collapse onto the second oxirane moiety (Scheme 6). The cascade was triggered by epoxide–hydrolase activity of lyophilized whole cells of *Rhodococcus* sp. CBS 717.73 and furnished as sole product the dihydroxy–tetrahydrofuran derivative, which represents the central core of particular natural polyether compounds with biological activities known as annonaceous acetogenins. Despite the possible occurrence of eight stereochemical pathways, a high degree of stereocontrol allowed formation of only one stereoisomer in high purity (89% de and 94% ee).<sup>114</sup>

## 2.2. Cyclase-Triggered Cascades Involving C–C Bond Formation

The formation of polycyclic triterpenes in nature has been fascinating chemists for decades, and subsequent research in the field has led to significant advances in understanding of the enzymatic machinery responsible for a high degree of regio- and stereoselectivity in the formation of C–C bonds in triterpenes.<sup>115</sup> Triterpene cyclases are type II terpenoid cyclases: they rely on a Brønsted acid for the generation of a reactive carbocation, which is responsible for the proliferation of events consisting in the consecutive formation of new C–C bonds to eventually form polycyclic products.<sup>109</sup> Despite improved understanding of two major cyclases—squalene–hopene cyclase (SHC) and 2,3-oxidosqualene cyclase—applications of these enzymes in synthesis are rare, and typically, yields are low and reactions are run on milligram scale. SHC presents exceptional substrate tolerance and can accept various substrate analogues. Most biotransformations of non-natural substrates were performed using homologues from *Alicyclobacillus acidocaldaricus*<sup>116</sup> and lately from *Zymomonas mobilis*<sup>117</sup> and are summarized in Schemes 7 and 8.<sup>118</sup>

**Scheme 9.** Application of Squalene–Hopene Cyclase (SHC) Variants from *A. acidocaldarius* in the Cyclization of Small Substrates<sup>a</sup>

<sup>a</sup>Substrate loading from 100 mg to 1 g.

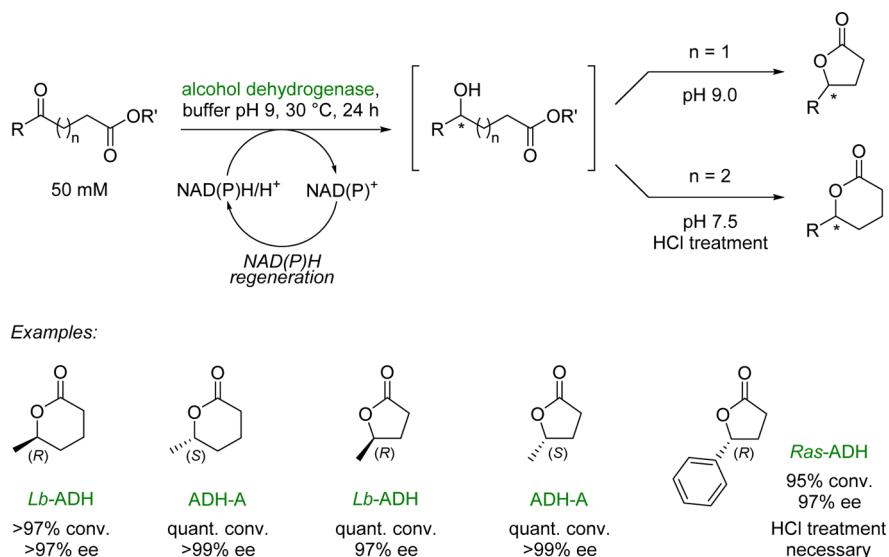
**Scheme 10.** Conversion of 3-Deoxyachilleol to (+)-Ambrein by Tetraprenyl- $\beta$ -curcumene Cyclase from *B. megaterium* (BmeTC)**Scheme 11.** Conversion of Squalene by Tetraprenyl- $\beta$ -curcumene Cyclase from *B. megaterium* (BmeTC) through Formation of Bicyclic Intermediate

Variants of squalene-hopene cyclase from *A. acidocaldarius* were generated by protein engineering and applied to the cyclization of several shorter analogues of squalene. While the wild-type enzyme showed only basal or no level of activity (<1%) for these substrates, single and double mutants could be used in preparative-scale biotransformations, which allowed isolation and characterization of the final products (Scheme 9). This study demonstrates the wide applicability of enzymatic Brønsted acid catalysis and the diversity of functional reactive groups accepted by the enzyme (not only C=C bonds but also epoxides and

carbonyls), including a notable example of Prins/ene-catalyzed conversion of (S)-citronellal, induced from one single-point mutation (I261A).<sup>124</sup>

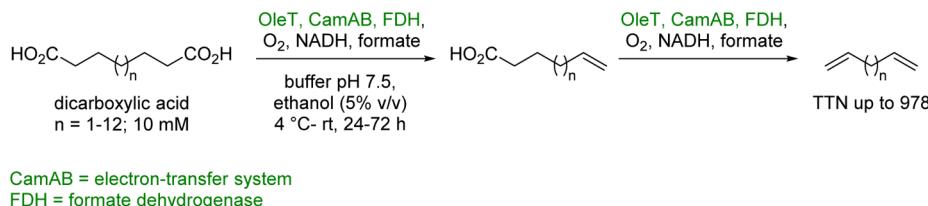
A notable exception to previous examples all based on squalene–hopene cyclase is the use of tetraprenyl- $\beta$ -curcumene cyclase from *Bacillus megaterium* (BmeTC) in the synthesis of (+)-ambrein.<sup>125</sup> BmeTC shares 30% identity with squalene–hopene cyclase and is a bifunctional triterpene/sesquiterpene cyclase. Cell-free extracts containing overexpressed recombinant BmeTC were implemented in the transformation of 3-

**Scheme 12.** Access to Enantiopure Substituted Lactones through Alcohol Dehydrogenase-Catalyzed Reduction and Subsequent Cyclization Reaction<sup>a</sup>



<sup>a</sup>LBADH: ADH from *Lactobacillus brevis*. ADH-A: ADH from *R. ruber*. RasADH: ADH from *Ralstonia* sp.

**Scheme 13.** Bis-decarboxylation of Dicarboxylic Acids with OleT



deoxyachilleol A in Tris-HCl buffer (pH 7.5) (Scheme 10). The substrate was emulsified with Triton X-100, and after multiple extraction and column chromatography steps, pure (+)-ambrein could be isolated as final product (1.4 mg, 4% yield). In this process, two rings (i.e., two C–C bonds) were formed and incorporation of one molecule of water in the final product terminated the intramolecular cascade.

The same enzyme was used in the conversion of squalene in a cascade producing an intermediate which could be isolated and characterized and which underwent further intramolecular cyclization to two major products (Scheme 11). Up to five rings were formed, and one molecule of water was incorporated. In the major product, two new C–O bonds were selectively created in addition to four new C–C bonds.<sup>125</sup>

### 2.3. Laccase-Trigected Cascades

Laccases have been used for dimerization or oligo/polymerization of phenolic compounds. This reaction is triggered by the formation of a reactive radical, which then undergoes follow-up reactions.<sup>126–128</sup> Consequently, in line with the cascades mentioned above, these transformations can also be considered as cascades initiated by a single enzyme. The dimerization is also part of various other cascades, where examples are given (Scheme 35 and 134). In contrast to the cyclase-catalyzed transformation, dimerizations by laccases are not stereoselective leading in general to various products.

### 2.4. Alcohol Dehydrogenase-Triggered Cascade

The alcohol dehydrogenase-catalyzed reduction of  $\gamma$ - and  $\delta$ -keto esters led to the stereoselective formation of substituted lactones

(Scheme 12).<sup>129</sup> Upon asymmetric reduction, the  $\gamma$ -hydroxy acids formed cyclized spontaneously to  $\gamma$ -lactones at pH 9, whereas the cyclization to  $\delta$ -lactones required additional acidic treatment but still proceeded in one pot. The stereoselectivity of the reaction could be controlled by proper choice of the biocatalyst, and enantiopure products were obtained in high ee values. Preparative-scale synthesis on a 200 mg scale allowed isolation of (*R*)-5-phenyldihydrofuran-2(3*H*)-one in quantitative yield and excellent ee value (97%). This compound was further used in follow-up chemistry, including Suzuki, Sonogashira, and Heck coupling reactions.

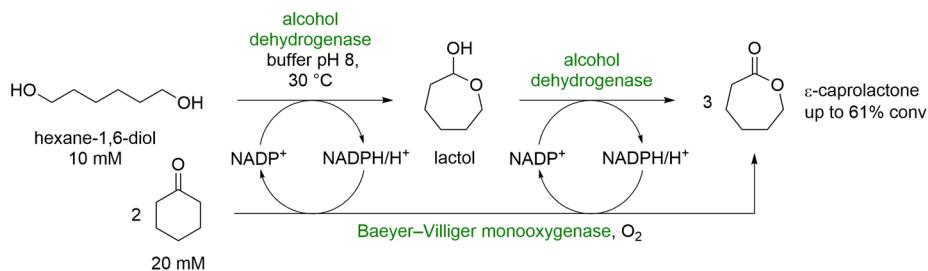
A similar concept was applied to cosubstrates to shift the equilibrium of the reaction, for instance, in ketone reduction leading to cyclized products (e.g., 1,4-butanediol, 1,6-hexanediol, *cis*-1,4-but-2-ene-diol).<sup>87,130–133</sup> In analogy, diamines were employed in transamination reactions.<sup>130,134,135</sup> Spontaneous ring closure was described not only for the coproducts but also for the amination products.<sup>136–142</sup>

## 3. IN VITRO CASCADES REQUIRING FOR EACH STEP A BIOCATALYST

### 3.1. Simultaneous Mode

**3.1.1. Single Catalyst in the Linear Sequence.** The special case that a single enzyme reacts several times on a substrate requires that either the same functionality is found several times in the molecule or two comparable functionalities appear during the cascade (an exception are polyketide synthases,<sup>68–70</sup> which are out of scope of this review). Consequently, examples are rare. For instance, access to terminal

**Scheme 14. Double Oxidation by an Alcohol Dehydrogenase in a Convergent Cascade with Parallel Reactions Leading to  $\epsilon$ -Caprolactone Starting from 1,6-Hexanediol and Cyclohexanone**



dienes was achieved by sequential oxidative decarboxylation of dicarboxylic acids via the ( $\omega$ -1)-alkene carboxylic acid using  $O_2$  and formate and catalyzed by P450 monooxygenase OleT (Scheme 13).<sup>143</sup> The highest productivity of OleT was obtained with tetradecanedioic acid yielding 1,11-dodecadiene ( $n = 8$ , TTN 978, 29% conversion, 0.49 g L<sup>-1</sup>).

Another example is the double oxidation of hexane-1,6-diol to  $\epsilon$ -caprolactone via the intermediate lactol catalyzed by the alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (Scheme 14).<sup>87</sup> The oxidized cofactor required for the oxidation steps was obtained from a parallel reaction, the Baeyer–Villiger oxidation of cyclohexanone, which also leads to  $\epsilon$ -caprolactone. Thus, two substrates were converted to the same product in a convergent cascade. The system produces theoretically three molecules of  $\epsilon$ -caprolactone from two molecules of cyclohexanone and one molecule of hexane-1,6-diol. In practice, the formation of 3 equiv of  $\epsilon$ -caprolactone was not observed due to undesired hydrolysis and oligomerization of  $\epsilon$ -caprolactone. Nevertheless, up to 18.3 mM  $\epsilon$ -caprolactone was formed from 20 mM cyclohexanol and 10 mM diol within a reaction time of 72 h, corresponding to a conversion of 61%.

In a follow-up study, the reaction parameters were optimized using a design-of-experiments approach and an aqueous/organic biphasic reaction system.<sup>144</sup> This led to an increase in turnover numbers for NADPH and the alcohol dehydrogenase by a factor of 50 and 10, respectively.

Formally a single catalyst with multiple activities may also be obtained by fusion of the involved enzymes to each other. This has been done for the two enzymes involved in the biocatalytic conversion of alcohols into esters.<sup>145</sup> However, since the reaction was performed by recycling the cofactor via the metabolism, this reaction is discussed in the *in vivo* section.

### 3.1.2. Two Catalysts in the Linear Sequence.

**3.1.2.1. Redox Cascades—Two Redox Steps.** **3.1.2.1.1. Hydrogen-Borrowing Cascades.** **3.1.2.1.1.1. First Step Oxidation, Second Step Reduction.** Hydrogen-borrowing cascades require intrinsically an oxidation and a reduction step and involve a cofactor which connects the two redox steps; thus, the oxidation and reduction steps are linked to each other in general via the exchange of a hydride, which is abstracted in the oxidation step and consumed in the reduction step. Therefore, the two steps in hydrogen borrowing can only work provided each step is functioning; consequently, this system is sensitive to side reactions and in the case of a redox cofactor [e.g., NAD(P)H] gets oxidized by other enzymes present. Reactions catalyzed by a P450 enzyme are special because they perform an oxidation reaction with respect to the substrate but also with respect to the NAD(P)H cofactor, generating NAD(P)<sup>+</sup>, while, e.g., oxidations with alcohol dehydrogenases generate the reduced cofactor. Hydrogen-borrowing cascades are thus typically made up by two

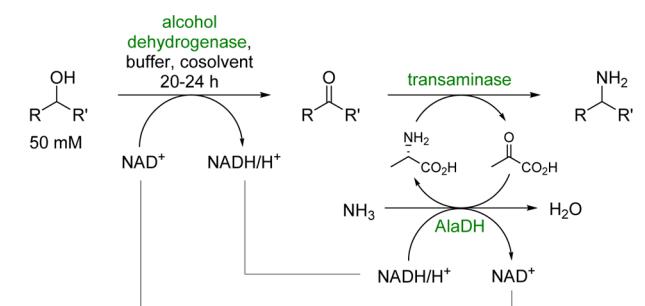
steps and two enzymes. In the review, there is one case of a hydrogen-borrowing cascade which involves three enzymes, whereby a step not consuming the redox cofactors is squeezed between the two linked redox reactions (see section 3.1.3, Scheme 62).

The exploitation of such a concept was suggested as early as 1978<sup>146</sup> and then realized for the transformation of lactic acid to L-alanine via pyruvate in flow over 12 days reaching conversion of close to 90% using immobilized NADH in a membrane reactor in 1984.<sup>59</sup> Finally, an average yield of 134 g of alanine/L d was maintained for 32 days.<sup>60</sup> The same concept was subsequently also applied for phenylalanine using the appropriate enzymes and reaching a space time yield of 456 g/L d.<sup>61,62</sup> In 1995, the coupling of the oxidation of a cyclic sec-alcohol with a Baeyer–Villiger oxidation to form a lactone was reported to be limited by the stability of the employed monooxygenase, since only 15% conversion was achieved after 22 h.<sup>147</sup> In a subsequent study, an alcohol dehydrogenase was coupled but this time with an ene reductase for the transformation of morphine to hydro-morphone.<sup>148</sup>

In more recent studies, the amination of alcohols was realized via a redox-self-sufficient hydrogen-borrowing cascade using a transaminase, an amino acid dehydrogenase, or an amine dehydrogenase for the final amination step (for the amination of alcohols using redox-independent cascade steps see section 3.1.2.1.3, Scheme 30). To ensure reusability of the hydride abstracted in the oxidation step, only alcohol dehydrogenases can be employed, since they can store the hydride at NAD(P)H; oxidases would form hydrogen peroxide or water as side product, which are not suitable reducing agents. In case a transaminase was used, an additional enzyme was required to connect alcohol oxidation and reductive amination steps, namely, an alanine dehydrogenase (Scheme 15).<sup>149</sup> The feasibility of this reaction system was demonstrated preferentially for primary alcohols. Thermostable ADH from *Bacillus stearothermophilus*, transaminase from *Chromobacterium violaceum*, and L-alanine dehydrogenase from *Bacillus subtilis* were used as biocatalysts. At a substrate concentration of 50 mM, the amination of 1-hexanol reached completion within 24 h. Addition of 1,2-dimethoxy ethane (10% v/v) proved beneficial in the double amination of terminal diols, which was demonstrated using 1,8-octanediol and 1,10-decanediol as starting materials. Both compounds were quantitatively aminated within 20 h, and the diamination of 1,10-decanediol was also performed on preparative scale (174 mg of substrate), affording the terminal diamine, a building block for polyamides, in 70% isolated yield.

The responsible enzymes (ADH, TA, AlaDH) were then coexpressed to provide an *E. coli* single-cell catalyst.<sup>150</sup> Biotransformations performed with resting microbial cells did not require the addition of any of the cofactors pyridoxal

**Scheme 15. Hydrogen-Borrowing System for the Amination of Alcohols Employing an Alcohol Dehydrogenase (ADH), Transaminase (TA), and L-Alanine Dehydrogenase (AlaDH)**



Amines obtained from primary alcohols:

<chem>CCCCN</chem>	>99% conv.	<chem>c1ccccc1CCN</chem>	87% conv.
<chem>CCCCCCN</chem>	57% conv.	<chem>c1ccccc1CCCN</chem>	99% conv.
<chem>NCCCCCNC</chem>	99% conv.	<chem>c1ccccc1CCCN</chem>	99% conv.
<chem>NCCCCCCNC</chem>	99% conv.	<chem>c1ccccc1CCCN</chem>	70% conv.
<chem>COCCOCN</chem>	up to 60% conv. from 10 mM substrate	<chem>c1ccccc1CCCN</chem>	

Selected amines obtained from secondary alcohols:

<chem>NCC1CCCC1</chem>	64% conv. 24 h	<chem>NCC1(C)CCCC1</chem>	25% conv. 98% ee 24 h	<chem>O[C@H]1[C@H](N)O[C@H]1O</chem>	up to 7% conv. from isosorbide
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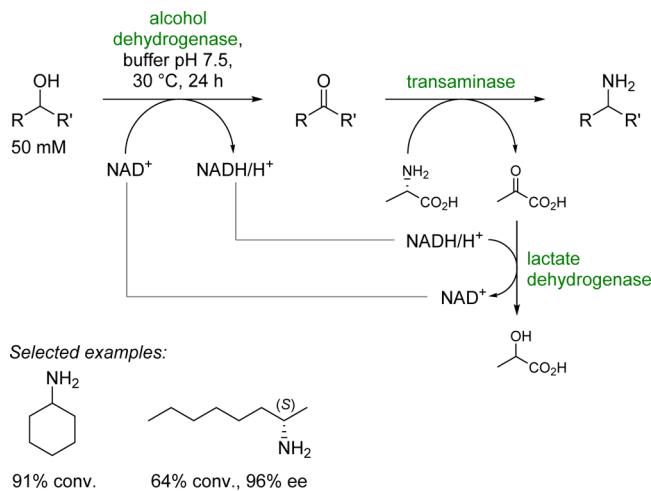
phosphate (PLP) and NAD<sup>+</sup>, while both ammonium chloride ( $\geq 50$  mM) and L-alanine ( $\geq 100$  mM) had to be supplied to enable complete conversion of 10 mM 1,10-decanediol. Later, detailed investigations on the role of L-alanine revealed that it was partially consumed by cell catabolism. Nevertheless, it was demonstrated that addition of 20 mM L-alanine along with 100 mM NH<sub>4</sub>Cl is sufficient for the complete amination of 10 mM 1,10-decanediol and that substantial conversion can also be achieved using stoichiometric levels of L-alanine.<sup>151</sup> In the case of selected ether alcohols, thermodynamic limitations became apparent due to possible intramolecular H-bond formation; under optimized conditions using stepwise addition of 280 mM ammonia, conversion up to 60% was reached.<sup>152</sup>

Studies aiming at extending the substrate scope to secondary alcohols revealed additional challenges. Racemic alcohol substrates, for instance, may require the use of two stereo-complementary alcohol dehydrogenases for their complete oxidation.<sup>153</sup> Furthermore, in contrast to aliphatic primary alcohols, secondary alcohols were converted with maximum 64% conversion (cyclopentanol), indicating thermodynamic limitations. In a related study, the biocatalytic amination of isosorbide, a bicyclic secondary diol obtainable from D-glucose, was investigated.<sup>154</sup> Choosing levodione reductase from *Leifsonia aquatica*, an engineered transaminase from *Paracoccus denitrificans* and alanine dehydrogenase from *B. subtilis*, isosorbide was transformed to the monoaminated (2S,5S)-amino alcohol with 7% conversion (20 mM from 300 mM isosorbide).

When using *E. coli* whole-cell biocatalysts in hydrogen-borrowing cascades with poorly reactive substrates, significant amounts of the intermediate ketones accumulated, which indicated an insufficient coupling of the oxidation and reductive amination reactions; this was attributed to the presence of NADH oxidase activity in *E. coli*, which consumed hydride equivalents.<sup>153</sup> Additionally, a decrease of the ee of the amine was observed over time for selected substrates.<sup>153</sup>

To overcome some of these limitations and to introduce a better thermodynamic driving force, a modified reaction system was designed using alanine as the only nitrogen source, which was transformed to pyruvate and further on to lactate as final coproduct; thus, the alanine dehydrogenase of the previous scheme got substituted by a lactate dehydrogenase (Scheme 16).

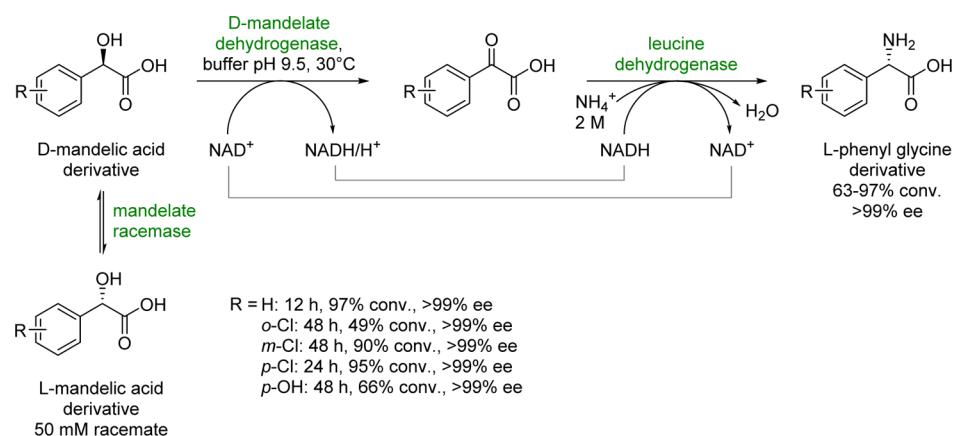
**Scheme 16. Redox-Neutral Multienzyme System for Alcohol-to-Amine Interconversion Using Alanine as Nitrogen Source Leading to Lactate as Coproduct**



This modified redox-neutral cascade led to higher levels of amine formation and significantly improved optical purities when implemented with purified enzymes (e.g., 91% conversion for cyclohexylamine from cyclohexanol; 64% of (S)-octan-2-amine in 96% ee from (S)-2-octanol).

Instead of a transaminase, using an amino acid dehydrogenase or amine dehydrogenase for the amination step simplified the reaction system, since the NAD(P)H gained in the alcohol oxidation step could be used directly by the aminating enzyme as already shown in the very beginning of the history of artificial cascades.<sup>59–62</sup> A cascade transforming mandelic acid derivatives to L-phenylglycine derivatives involved an alcohol dehydrogenase and an amino acid dehydrogenase (Scheme 17). The transformation of D-mandelates would require in theory only two enzymes; however, to be able to use racemic substrates, a mandelic acid racemase was added as well. Thus, for the L-mandelate substrate enantiomer, the cascade involves three enzymes but is discussed here (see consideration on hydrogen-borrowing cascades above). While in a first study only mandelic acid was transformed,<sup>155</sup> further mandelic acid derivatives were investigated in a subsequent publication.<sup>156</sup> Thereby, an engineered mandelate racemase from *Pseudomonas putida*, a D-mandelate dehydrogenase from *L. brevis*, and an L-leucine dehydrogenase from *Exiguobacterium sibiricum* were combined to give L-phenyl glycine derivatives in a one-pot simultaneous fashion. Racemic mandelic acid was transformed into phenylglycine with 97% conversion and >99% ee. Increasing the

**Scheme 17.** Alcohol–Amine Cascade for Transforming Mandelic Acid Derivatives to Phenylglycine Derivatives via a Hydrogen-Borrowing Cascade<sup>a</sup>



<sup>a</sup>To allow consumption of both enantiomers of the racemic substrate racemization by a mandelate racemase was introduced.

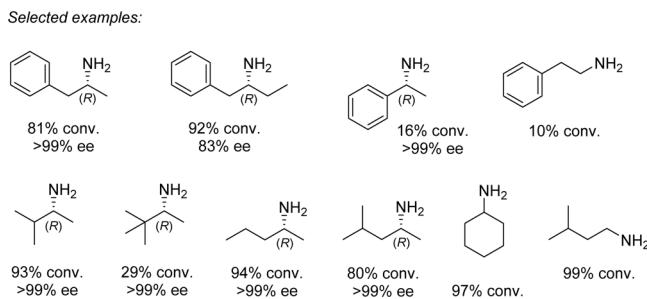
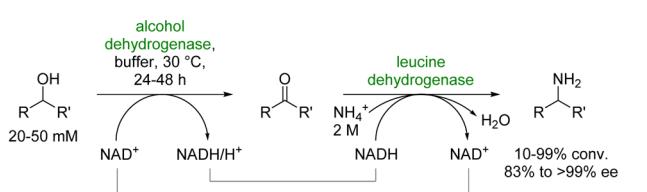
substrate loading to 200 mM led to very good conversions of >95%, but at higher concentrations (500 mM) only 63% of the substrate was transformed into the amino acid under the conditions employed. To prove the applicability of this system, a 1 L scale reaction with 30.4 g (200 mM) of *rac*-mandelic acid was performed, resulting in >96% conversion and an isolated product yield of 86.5%. Several other mandelic acid derivatives were successfully converted to the corresponding phenylglycine products with moderate to good conversion levels. Of particular interest is the *ortho*-chloro product, which is a building block for Clopidogrel, an antiplatelet drug.

Alternatively, the combination of alcohol dehydrogenases and amine dehydrogenases<sup>157–159</sup> enabled also the amination of alcohols via a direct coupling of the alcohol oxidation and reductive amination steps (Scheme 18). The amination of a broad range of *sec*- and *prim*-alcohols following the hydrogen-borrowing concept has been reported,<sup>160</sup> whereby an engineered amine dehydrogenase (from *Bacillus badius* phenylalanine dehydrogenase, Ph-AmDH)<sup>161</sup> as well as a chimeric amine dehydrogenase (Ch1-AmDH)<sup>162</sup> were chosen for the reductive

amination step. Alcohol dehydrogenases from *L. brevis* (*Lb*-ADH) and *Aromatoleum aromaticum* (*Aa*-ADH) were employed for alcohol oxidation. Preliminary experiments indicated that crude cell-free extracts resulted in an interference from NADH oxidases present in the *E. coli* expression host, leading to low overall conversions and the accumulation of the intermediate ketone. Furthermore, a His-tag on Ph-AmDH was found to destabilize the *Lb*-ADH, which also prevented an efficient biotransformation. Hence, purified enzymes from which the His-tag had been cleaved were required. Employing pure enzyme preparations, a wide range of (*R*)-configured, (*S*)-configured, and racemic secondary alcohols were transformed into the corresponding (*R*)-amines with moderate to high conversions (16–93%) and good to excellent optical purities (from 83% to >99%). The amination of racemic alcohols required the presence of two enantiocomplementary ADHs; therefore, in this case three enzymes in the linear sequence were required, instead of two. The amination of primary alcohols was successful for aliphatic substrates with a chain length smaller than or equal to C<sub>6</sub>. In an independent study published almost at the same time, the same concept—but with different enzymes—was applied leading to similar results.<sup>163</sup> The amine dehydrogenase used in this work was created by protein engineering of a leucine dehydrogenase from *E. sibiricum* while the ADH originated from *Streptomyces coelicolor*.<sup>164</sup> The latter enzyme offers the advantage of oxidizing a variety of *sec*-alcohols with low enantioselectivity, hence allowing the complete conversion of racemic substrates without the need for a second ADH. In contrast to the first report,<sup>160</sup> purification of the enzymes was not required, since no accumulation of the intermediate ketones was reported for reactions performed with crude cell-free extracts. A selection of aliphatic secondary alcohols (acyclic and cyclic) was aminated, providing the corresponding amines in up to 97% conversion and, for chiral amines with >99% ee for the (*R*)-enantiomer. Thermodynamic limitations, which were observed in the transaminase-based amination of *sec*-alcohols, did not become apparent in either of the two studies, as evidenced by the high conversions achieved. Most likely, the surplus of ammonia (2 M for reactions containing 20 mM substrate in one case and 50 mM in the other) pushed the equilibrium to the product side, offering an alternative to other concepts.<sup>165</sup>

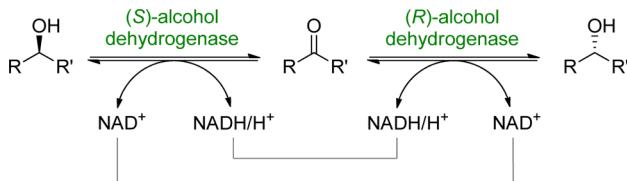
Combinations of two stereocomplementary alcohol dehydrogenases have been used for the racemization of chiral secondary

**Scheme 18.** Functional Group Interconversion of Alcohols to Amines via a Hydrogen-Borrowing Cascade Using Alcohol and Amine Dehydrogenases



alcohols: the two ADHs are employed together with catalytic amounts of NADH and NAD<sup>+</sup>, allowing one to establish an equilibrium between the two alcohol enantiomers via a coupled oxidation–reduction process (Scheme 19). Thus, the two

**Scheme 19.** Racemization of Secondary Alcohols Using Two Stereocomplementary Alcohol Dehydrogenases

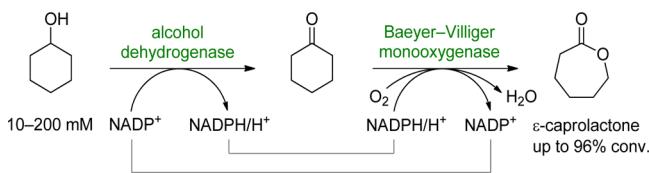


enzymes “emulate” the activity of a racemase.<sup>166</sup> Alternatively, also a single alcohol dehydrogenase with low stereoselectivity may be applied. The same racemization concept has also been applied for the racemization of  $\alpha$ -hydroxyacids using two stereocomplementary  $\alpha$ -hydroxy-isocaproate dehydrogenases (HicDHs).<sup>167</sup>

The racemization of  $\alpha$ -chiral primary amines has been achieved in an analogous approach using two stereocomplementary transaminases relying on an amine shuttle molecule, although the racemization process in this case was far less efficient than the racemization of  $\alpha$ -hydroxycarboxylic acids or alcohols using dehydrogenases.<sup>168</sup>

A cascade designed to transform cyclohexanol to  $\epsilon$ -caprolactone was realized in a hydrogen-borrowing fashion.<sup>169,170</sup>  $\epsilon$ -Caprolactone is a valuable chemical for polymer synthesis that is being used for the production of biodegradable polymers, such as polycaprolactone,<sup>171</sup> and as a precursor to  $\epsilon$ -caprolactam. An alcohol dehydrogenase and a Baeyer–Villiger monooxygenase were coupled in a redox-neutral fashion, similar to a previously described system,<sup>147</sup> with respect to the required nicotinamide cofactor (consumption of NADP<sup>+</sup> by ADH and generation of NADPH), which is required for activity of the monooxygenase (Scheme 20). Molecular oxygen is the only

**Scheme 20.** Conversion of Cyclohexanol into  $\epsilon$ -Caprolactone Using an Alcohol Dehydrogenase and a Baeyer–Villiger Monooxygenase

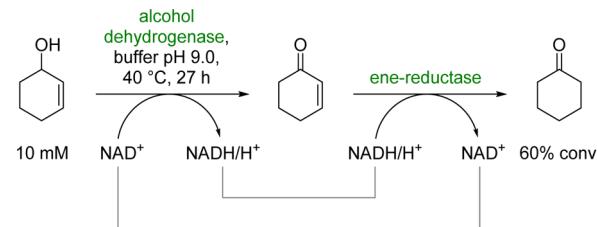


stoichiometric cosubstrate in the overall process. Two reports in 2013 demonstrated that an efficient transformation of cyclohexanol into  $\epsilon$ -caprolactone is possible (94% conversion of 60 mM substrate in one case, 80% conversion of 10 mM in the other), but limited conversions at elevated substrate concentrations were observed due to inhibition and partial deactivation of the Baeyer–Villiger monooxygenase. This limitation was addressed in a follow-up study,<sup>172</sup> whereby the  $\epsilon$ -caprolactone formed in the cascade was subjected to *in situ* ring-opening oligomerization catalyzed by lipase A from *Candida antarctica* (CAL-A) (see cascades with three enzymes, Scheme 63). The sequence from cyclohexanol to  $\epsilon$ -caprolactone was also part of a longer cascade (Scheme 72) and successfully performed at a 200

mM substrate concentration employing a variant of the Baeyer–Villiger monooxygenase leading to 96% lactone within 20 h.<sup>173</sup>

The isomerization of allylic alcohols to the saturated ketones has already been described for morphine to hydromorphone<sup>148,174</sup> and has recently been reinvestigated using cyclohex-2-en-1-ol as substrate (Scheme 21).<sup>175</sup> Two thermostable

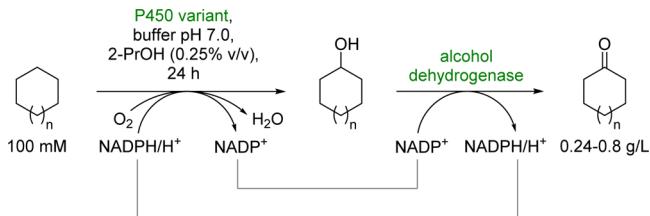
**Scheme 21.** Biocatalytic Redox-Self-Sufficient Isomerization of Cyclohexenol to Cyclohexanone



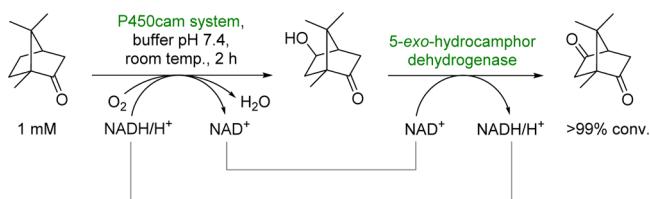
biocatalysts, an alcohol dehydrogenase from *Thermus* sp. ATN1 and an ene–reductase from *Thermus scotoductus*, were used in this study. Thorough optimization of pH and NAD<sup>+</sup> concentration as well as a careful balancing of the enzyme ratio was required to achieve a high conversion of the starting material and suppress the undesired formation of cyclohexanol as an “over-reduction” side product. Under optimized conditions, 6 mM cyclohexanone was obtained from 10 mM cyclohex-2-en-1-ol within 27 h. In a related study, primary allylic alcohols were isomerized to the saturated aldehyde and reduced to the corresponding saturated alcohols by addition of stoichiometric amounts of NADH.<sup>176</sup>

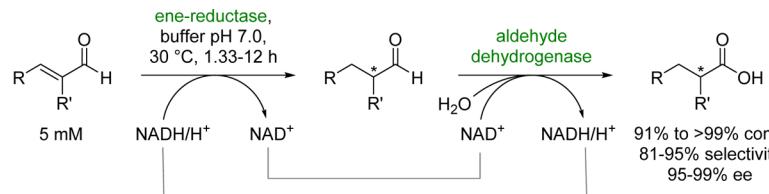
P450 enzymes perform an oxidation reaction with respect to the substrate but also oxidize the NAD(P)H cofactor generating NAD(P)<sup>+</sup>. For this reason, these enzymes can be coupled with another oxidation reaction consuming NAD(P)<sup>+</sup>. For instance, alcohol formation via hydroxylation by P450 was coupled to a subsequent alcohol oxidation in a hydrogen-borrowing fashion (Schemes 22 and 23).<sup>177</sup>

**Scheme 22.** Hydroxylation/C–H Oxidation Followed by Alcohol Oxidation for the Synthesis of Cycloalkanones Starting from Cycloalkanes

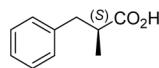
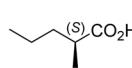
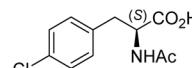
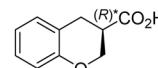


**Scheme 23.** Hydroxylation/C–H Oxidation Followed by Alcohol Oxidation Producing 2,5-Diketobornane from Camphor



**Scheme 24. Biocatalytic Redox Isomerization of  $\alpha,\beta$ -Unsaturated Aldehydes to Carboxylic Acids**

Selected examples:

quant. conv.  
95% selectivity  
99% eequant. conv.  
92% selectivity  
>98% ee99% conv.  
81% selectivity  
95% eequant. conv.  
81% selectivity  
95% ee\* in the publication  
erroneously assigned as (S)

The biocatalytic transformation of carbocycles such as cyclohexane, cyclooctane, and cyclodecane was achieved using a P450-mediated hydroxylation followed by oxidation by an ADH leading to the corresponding cycloalkanone.<sup>177</sup> A variant of the P450 BM-3 monooxygenase originating from *B. megaterium* was coupled with an ADH from *Lactobacillus kefir* for the second reaction step. All substrates tested were converted to the desired products. A productivity of 0.24–0.8 g/L for the final product cycloalkanone was obtained from 100 mM of cycloalkane.

The keto functionalization of a natural compound, namely, camphor, was described based on the same cascade concept (Scheme 23).<sup>178</sup> In this case, the regioselective hydroxylation in the 5 position of camphor was performed by applying the P450cam system from *P. putida* followed by oxidation of the hydroxy group by the 5-exo-hydrocamphor dehydrogenase, producing the desired product 2,5-diketobornane. Reaction parameters were optimized in terms of pH, temperature, amount of cofactor and enzyme loading; under these conditions, 1 mM camphor was converted within 2 h at room temperature.

### 3.1.2.1.1.2. First Step Reduction, Second Step Oxidation.

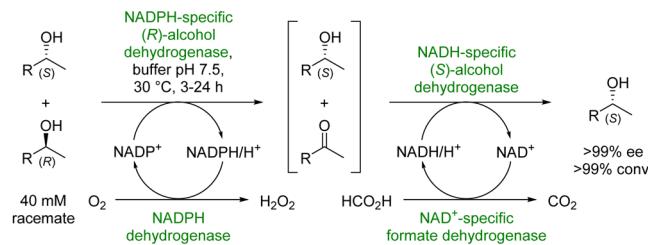
The previous cascade, starting with an oxidation (Scheme 23), was the first cascade consuming the reduced nicotinamide cofactor in the first step and regenerating it in the second step, which is also happening in the next cascade. In the subsequent example, the first step is a reduction with respect to the substrate followed by aldehyde oxidation. This hydrogen-borrowing cascade employs ene-reductase and aldehyde dehydrogenase for the conversion of  $\alpha$ -substituted  $\alpha,\beta$ -unsaturated aldehydes to corresponding saturated carboxylic acids (Scheme 24).<sup>179</sup> The combination of ene-reductase OYE2 from Baker's yeast or XenB from *Pseudomonas aeruginosa* with the aldehyde dehydrogenase from *E. coli* or from bovine lens led to the formation of a range of five structurally diverse  $\alpha$ -substituted carboxylic acids with high conversions (from 91% to >99%) and chemo-selectivities (81–95%) and in excellent optical purities (ee = 95–99%).

A case of cascade following the hydrogen-borrowing concept and involving three enzymes can be found in section 3.1.3 (Scheme 62).

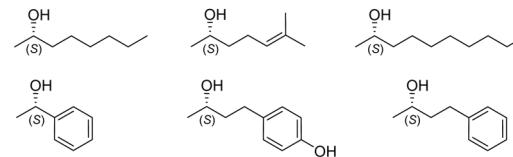
**3.1.2.1.2. Deracemization Cascades.** Deracemization via redox reactions requires an oxidation as well as a reduction step, as in the case of hydrogen-borrowing cascades. However, in contrast to hydrogen-borrowing cascades, deracemization cascades are not interlinked via their cofactor and the product

is identical to the substrate, except for its optical purity. Deracemization cascades have been described for *sec*-alcohols and  $\alpha$ -chiral amines.

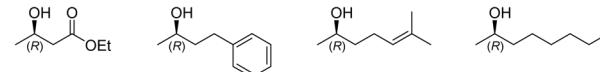
For the deracemization<sup>18–24</sup> of racemic *sec*-alcohols to optically pure compounds, a simultaneous oxidation–reduction cascade was designed based on two stereocomplementary alcohol dehydrogenases (ADHs) from *R. ruber* and *L. brevis* with complementary cofactor preference (NADH<sup>+</sup> vs NADPH<sup>+</sup>) (Scheme 25).<sup>180</sup> Thereby, the (R)-enantiomer of

**Scheme 25. Deracemization of Secondary Alcohols by the Simultaneous Action of a Pair of Stereocomplementary Alcohol Dehydrogenases (ADHs)**

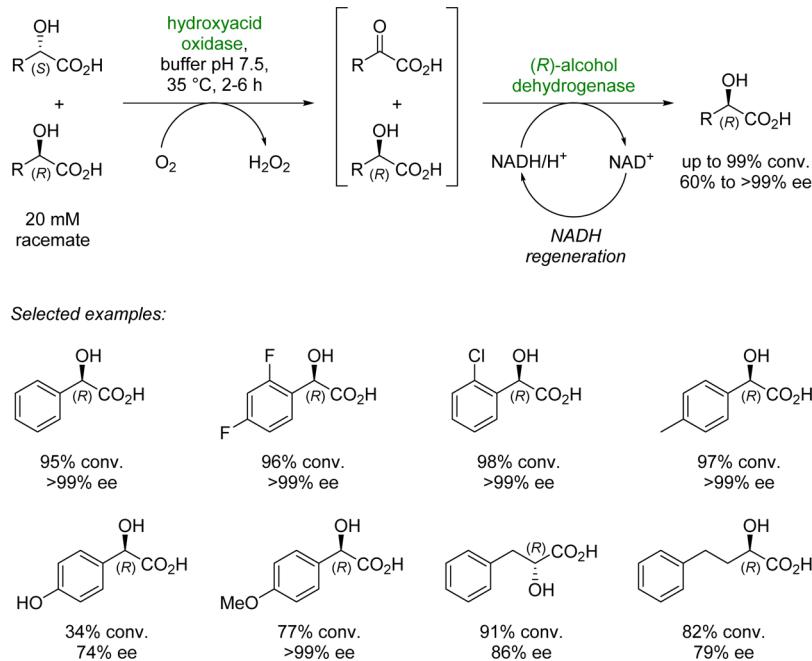
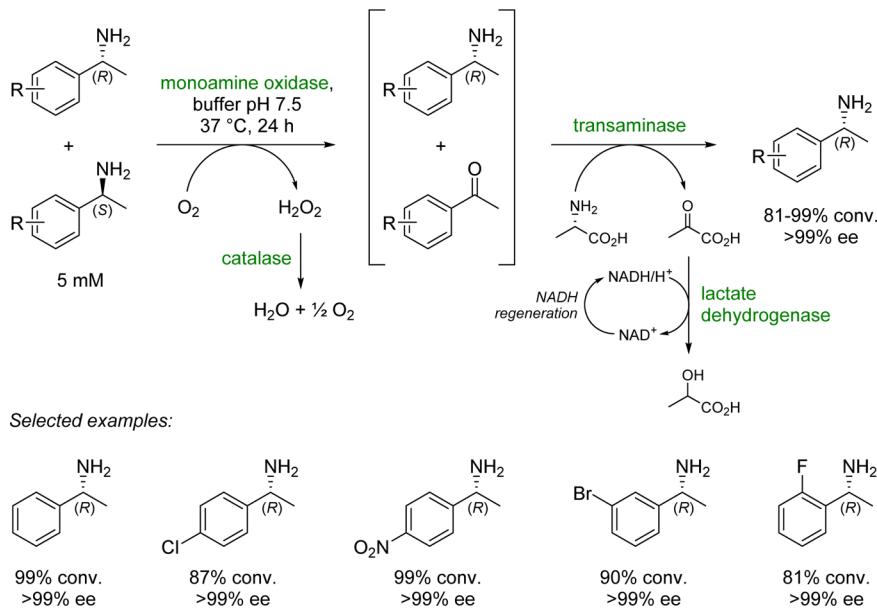
Selected examples (all &gt;99% ee and &gt;99% conversion):



Examples using an alternative set of ADHs:



the racemic alcohol was oxidized to the ketone, leaving the desired (S)-stereoisomer untouched, or vice versa. By using a second stereocomplementary ADH, the intermediate ketone was reduced to the (S)-alcohol. In this system, oxidation and reduction work concurrently with different cofactor pairs, and consequently, two cofactor recycling systems have to run simultaneously without interference: while NADP<sup>+</sup> is recycled by an oxidase, NADH is recycled by a formate dehydrogenase. By

**Scheme 26.** Deracemization of  $\alpha$ -Hydroxycarboxylic Acids via Enantioselective Aerobic Oxidation and Stereoselective Reduction**Scheme 27.** Deracemization of  $\alpha$ -Chiral Primary Amines Using an Amine Oxidase and a Transaminase

choosing enzymes with opposite stereopreference, also the (*R*)-enantiomer was obtained in optically pure form.

In a preceding study,<sup>181</sup> the enantioselective ketone oxidation was achieved by using resting cells of the microorganism *Alcaligenes*. This design enabled the compartmentalization of oxidation and reduction, which was required since in this case the two ADHs were both NAD dependent. Using this system, model substrate *rac*-1-phenylethanol was converted into its optically pure (*R*)-form within 30 min. Preparative-scale reaction (50 mg) afforded enantiopure (*R*)-phenylethanol with an isolated yield of 82% after purification.

Following a previously established concept,<sup>182,183</sup> the deracemization of  $\alpha$ -hydroxy acids via the combination of enantioselective aerobic oxidation to the corresponding  $\alpha$ -keto acids followed by stereoselective reduction was recently

extended (Scheme 26).<sup>184</sup> The oxidation biocatalyst used was a FMN-dependent (*S*)-2-hydroxy acid dehydrogenase (actually an oxidase) from *P. aeruginosa*, while the reduction of the keto acid was achieved employing an (*R*)-keto acid reductase from *Leuconostoc mesenteroides* in combination with glucose dehydrogenase for cofactor regeneration. The deracemization of 19 hydroxy acids—mostly mandelic acid derivatives—was successfully performed using either a mixture of two *E. coli* whole-cell biocatalysts expressing the enzymes for the oxidation step and the reduction step separately or a single *E. coli* strain coexpressing all three required enzymes. In both cases, analytical yields above 90% and excellent optical purities (ee > 99%) were achieved in the majority of the investigated reactions.

In a related process, but including a nonenantioselective oxidation, the synthesis of enantiopure 2-phenyl-1-propanol was

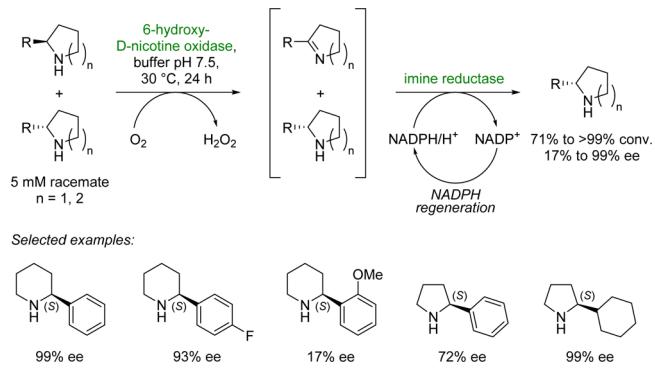
investigated in simultaneous as well as sequential fashion.<sup>185</sup> For this purpose, the racemic substrate was oxidized nonstereoselectively via a laccase/TEMPO system to produce the aldehyde intermediate, which was then selectively reduced by an ADH to generate the enantiopure product. (*R*)-Selective reduction was achieved by the use of a horse liver ADH, whereas the (*S*)-enantiomer was obtained by employing the commercial evo-1.1.200 ADH. Under optimized conditions, the simultaneous biotransformation gave slightly better results for the (*R*)-enantiomer in terms of conversion (85% vs 75%) than for the sequential cascade. The ee's were in both modes in a similar range (86–87%).

Using similar concepts, racemic amines have been deracemized. For instance, a simultaneous cascade was realized using an amine oxidase in the oxidation step and a commercially available transaminase (Scheme 27).<sup>186</sup> A range of racemic 1-arylethylamines was deracemized to the optically pure (*R*)-enantiomers (ee > 99%) using an engineered variant of monoamine oxidase from *Aspergillus niger*. However, the feasibility of a simultaneous cascade was only demonstrated in one case (*R* = *p*-NO<sub>2</sub>), while the other reactions were performed in a stepwise fashion. The selective cleavage of aliphatic substituents from secondary benzylamines was also shown to be possible using this reaction system.

The deracemization of amines using stereocomplementary transaminases has also been successfully performed in simultaneous mode,<sup>187</sup> as well as in a sequential cascade (Scheme 85).

The (*S*)-enantiomers of a panel of racemic 2-substituted piperidines and pyrrolidines (Scheme 28) were obtained using a

**Scheme 28. Deracemization via Amine Oxidation and Imine Reduction**



deracemization cascade that combined a variant of 6-hydroxy-D-nicotine oxidase (6-HDNO) from *Arthrobacter nicotinovorans* and an imine reductase (IRED) from *Streptomyces* sp. GF3587.<sup>188</sup> An interesting cooperation of the two enzymes with respect to the overall stereoselectivity was described for this system, as in several cases the deracemization afforded the product amine in significantly higher enantiomeric excess (95–99%) than the asymmetric reduction of the corresponding imine using the IRED alone would imply (37–89%).

Phenylalanine derivatives were deracemized by a simultaneous combination of *E. coli* whole cells expressing an L-amino acid deaminase and an engineered D-amino acid dehydrogenase to prepare enantiomerically pure D-phenylalanines (Scheme 29).<sup>189</sup> Instead of starting from the racemic mixture, optically pure L-enantiomers were used, allowing inversion of the absolute configuration. Several substituted derivatives of the D-amino

acids were obtained in good isolated yields (69–83%) and excellent ee's (from 95% to >99%).

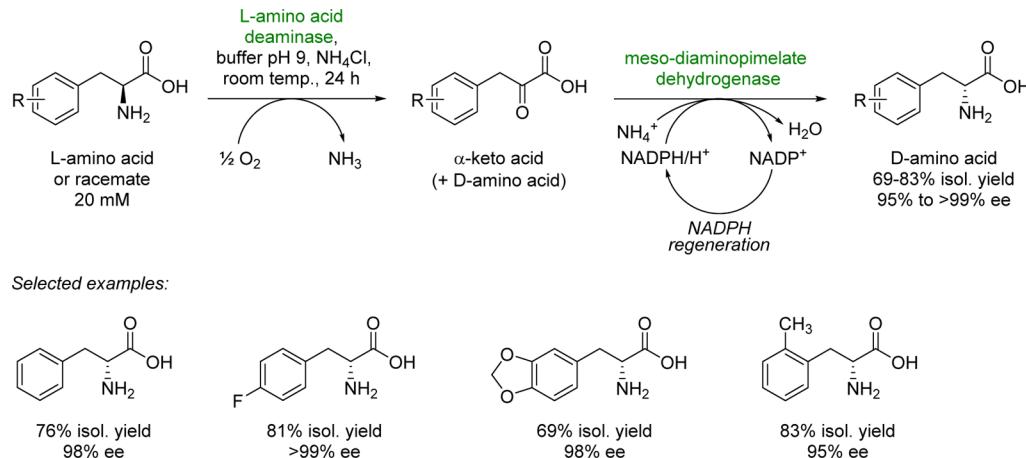
Deracemization may also be achieved via the combination of a racemization and a kinetic resolution. Since a chemical/metal catalyst is in general used for the racemization, this dynamic kinetic resolution is discussed in section 4.1.2.

**3.1.2.1.3. Other Redox Cascades Involving Two Redox Steps.** **3.1.2.1.3.1. Oxidation Followed by Reduction.** The amination of alcohols was described above via various hydrogen-borrowing cascades. The same transformation may also be achieved by employing two independent redox steps; thus, the two redox steps are not interlinked to each other as in the case of hydrogen borrowing. For instance, an alcohol oxidase may be applied for oxidation of the alcohol consuming molecular oxygen leading to hydrogen peroxide as coproduct (Scheme 30). Alternatively, an alcohol dehydrogenase may be coupled to an NAD(P)H oxidase (see also *Deracemization Cascades*). For the amination, transaminases have been employed.

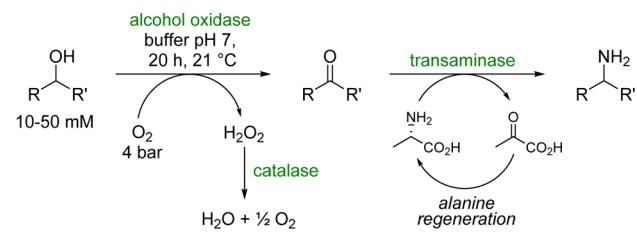
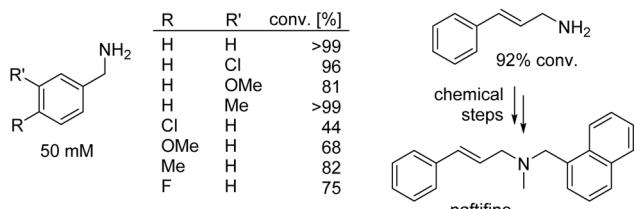
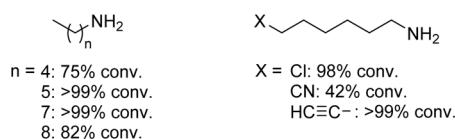
For instance, a variant of galactose oxidase (GalOx) from *Fusarium* sp. NRRL 2903<sup>190</sup> was used for alcohol oxidation and transaminases from *Vibrio fluvialis* or *P. denitrificans* for reductive amination.<sup>191</sup> The formed hydrogen peroxide was removed in this case by horseradish peroxidase in combination with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Alanine, required for the amination step, was recycled employing an L-alanine dehydrogenase (AlaDH) and glucose dehydrogenase (GDH). For sufficient supply of molecular oxygen in the first step, the cascade was carried out in a pressurized chamber at 4 bar of oxygen. This set up was applicable to benzylic alcohols and cinnamyl alcohol; benzyl alcohol and *p*-methylbenzyl alcohol were quantitatively aminated within 20 h at room temperature at 50 mM substrate concentration. Cinnamyl alcohol was also efficiently converted into the corresponding amine (*E*)-3-phenyl-2-propen-1-amine, which was transformed further by chemical methods to the antifungal agent naftifine (51% overall yield via four steps from cinnamyl alcohol). For the amination of aliphatic primary alcohols of medium chain length (C<sub>4</sub>–C<sub>11</sub>), the long chain alcohol oxidase from *Aspergillus fumigatus* was required.<sup>192</sup> Quantitative conversion to the amine was observed for several substrates (e.g., 1-hexanol, 1-octanol, 7-octyne-1-ol); however, the reactions were run at a substantially lower substrate concentration (10 mM) than in the cascade using GalOx (50 mM).

The reverse transformation from amines to alcohols has also been realized in a two-step cascade (Scheme 31).<sup>193</sup> However, since the overall reaction goes energetically uphill, the hydrogen-borrowing concept as described for the transformation of alcohols to amines (a thermodynamically downhill reaction) cannot be applied to reach preparative useful conversions. Starting from renewable resources, L-amino acids were transformed to corresponding (*R*)- or (*S*)- $\alpha$ -hydroxy acids applying an L-amino acid deaminase (L-AAD) from *Proteus myxofaciens*,<sup>194–196</sup> followed by asymmetric reduction controlled by either a D- or a L-hydroxyisocaproate dehydrogenase (HicDH), which originated from *Lactobacillus paracasei* DSM 20008<sup>197</sup> and *Lactobacillus confusus* DSM 20196,<sup>198</sup> respectively. The transformation of all amino acids tested reached conversions of 97–99% and allowed one to obtain optically pure products (>99% ee) with 73–85% isolated yield within 7 h. The substrate concentrations were up to 200 mM. For instance, L-tyrosine was transformed at 200 mM substrate concentration on a 100 mg scale affording the pharmacologically relevant corresponding (*S*)- $\alpha$ -hydroxy acid in 80% isolated yield and >99% ee. This

Scheme 29. In Vitro Oxidation-Reduction Deracemization Cascade for the Preparation of D-Amino Acids



Scheme 30. Alcohol Oxidation Followed by Amination via Two Independent Redox Steps

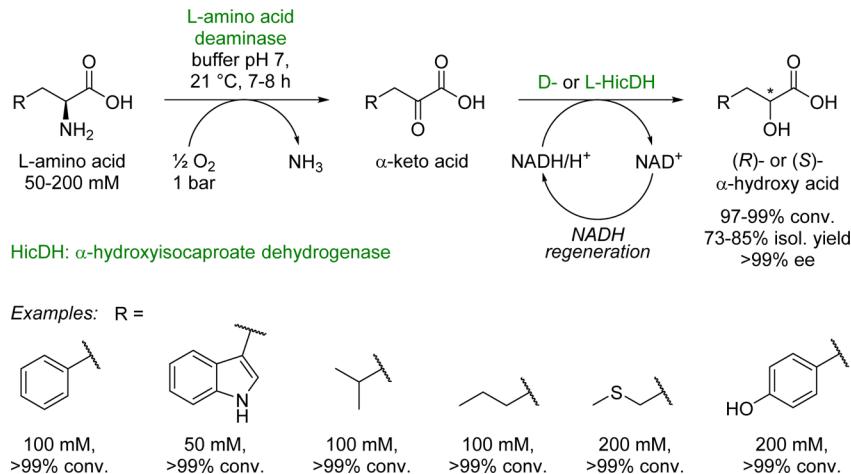
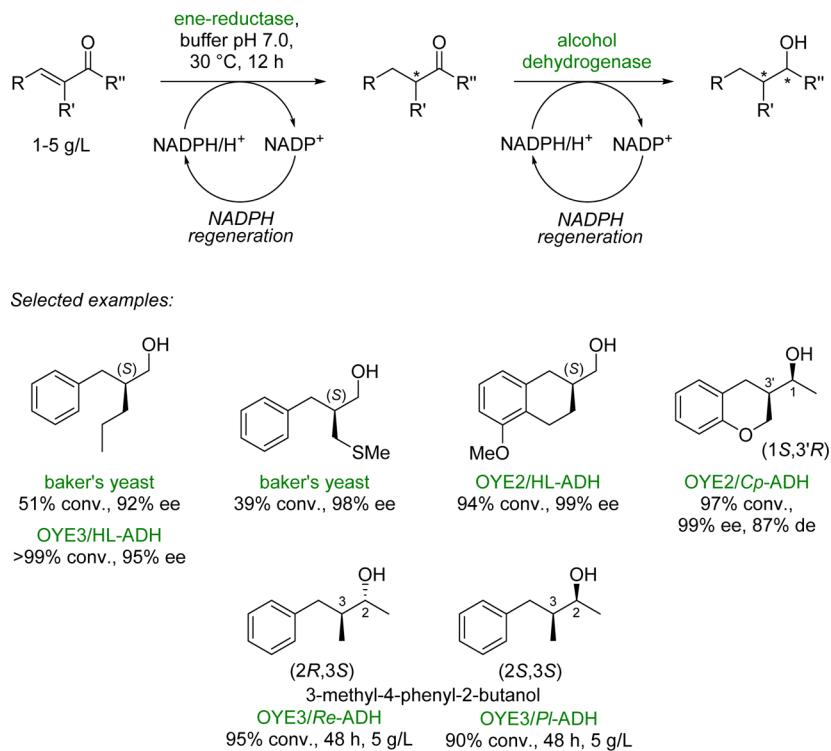
Employing galactose oxidase from *Fusarium* sp. NRRL 2903 as oxidase:Employing long-chain alcohol oxidase from *Aspergillus fumigatus*:

product may serve as a building block for various biologically active compounds, for example, Saroglitazar, which is used as a treatment for diabetes Type II.<sup>199</sup> In a subsequent study, the three enzymes required for the cascade (including cofactor recycling) were encoded on a single plasmid and coexpressed in *E. coli*, leading to an improved system.<sup>200</sup> The *E. coli* cells containing the three enzymes were then employed in the reaction as freeze-dried cell preparation and showed higher activity as the combination of the individual enzymes. After only 6 h of reaction time, hydroxy acids were formed with >99% conversion and isolated with 71%–86% yield and perfect ee's (>99%).

**3.1.2.1.3.2. Two Reduction Steps.** The combination of C=C reduction of  $\alpha,\beta$ -unsaturated ketones catalyzed by ene-reductases with C=O reduction of the resulting saturated carbonyl compounds provides access to chiral  $\beta$ -branched alcohols (Scheme 32). This cascade sequence was the focus of

several studies.<sup>201–203</sup> In a first approach, Baker's yeast was used to generate  $\alpha$ -substituted primary chiral alcohols starting from cinnamic aldehydes.<sup>201</sup> Although the ee's of the primary alcohol products were reasonable, ranging between 93% and 96%, conversions were moderate, reaching a maximum of 51%. Results were improved using purified ene-reductases OYE2 and OYE3 in combination with horse liver alcohol dehydrogenase (HL-ADH). Additionally, substrates were adsorbed on polymeric adsorbent beads to allow for in situ substrate feeding and product removal. With this modified system, conversions reached completion in selected cases (36% → 99%); optical purities were comparable to those obtained in the whole-cell reactions. The transformation of bicyclic substrates leads to synthetic precursors of the pharmaceuticals Robalzotan, Ebalzotan, and Rotigotine.<sup>202</sup> Corresponding substrates were again transformed with purified ene-reductase OYE2 and ADHs originating either from horse liver (HL-ADH) or *Candida parapsilosis* (Cp-ADH) depending on the substrate. The cyclic products were prepared on a preparative scale (1.25 mmol, 200–238 mg) with high isolated yields (83–96%) and optical purities (91–99% ee). Alcohols such as 3-methyl-4-phenyl-2-butanol (tradename Muguesia) are used as flavors and fragrances. Out of the four possible stereoisomers, only two—the (S)-isomers in position C3—display the floral notes desired in fragrance industry. To access this stereoisomer, OYE3 (*cerevisiae*) was chosen,<sup>203</sup> which produced the intermediate (S)- $\alpha$ -branched ketone with 98% ee. The desired product alcohols were obtained by using two different alcohol dehydrogenases (ADHs), namely, Re-ADH from *Rhodococcus erythropolis* to produce the (2S,3S)-product and Pl-ADH from *Parvibaculum lavamentivorans* for the synthesis of the (2R,3S)-product. A preparative synthesis with 5 g/L substrate loading showed 90–95% conversion after 48 h.

The combination of the same types of enzymes in the cascade with  $\alpha,\beta$ -unsaturated  $\gamma$ -keto esters led to the formation of chiral  $\gamma$ -butyrolactones (Scheme 33).<sup>204</sup> Ene-reductase YqjM from *B. subtilis* and alcohol dehydrogenases from different microbial sources performed the two reduction steps leading to up to three adjacent stereogenic centers. In the first step of the synthetic sequence, (E)- or (Z)-unsaturated oxo-esters were reduced by YqjM. Subsequent reduction of the saturated  $\gamma$ -keto ester was catalyzed by alcohol dehydrogenases from *L. kefir* (Lk-ADH), *L. brevis* (Lb-ADH), *Thermoanaerobacter* sp. (ADH-T), or a commercial supplier (Evocatal, evo-1.1.030). The ethyl esters underwent spontaneous cyclization to the lactones, while the reaction employing a *tert*-butyl ester stopped at the hydroxyacid

**Scheme 31.** In Vitro Oxidation-Reduction Cascade for the Conversion of L-Amino Acids into Corresponding  $\alpha$ -Hydroxy Acids**Scheme 32.** Access to  $\alpha$ -Substituted Alcohols from Unsaturated Carbonyls via Two Reduction Steps Using an Ene-Reductase (ERED) and an Alcohol Dehydrogenase (ADH)

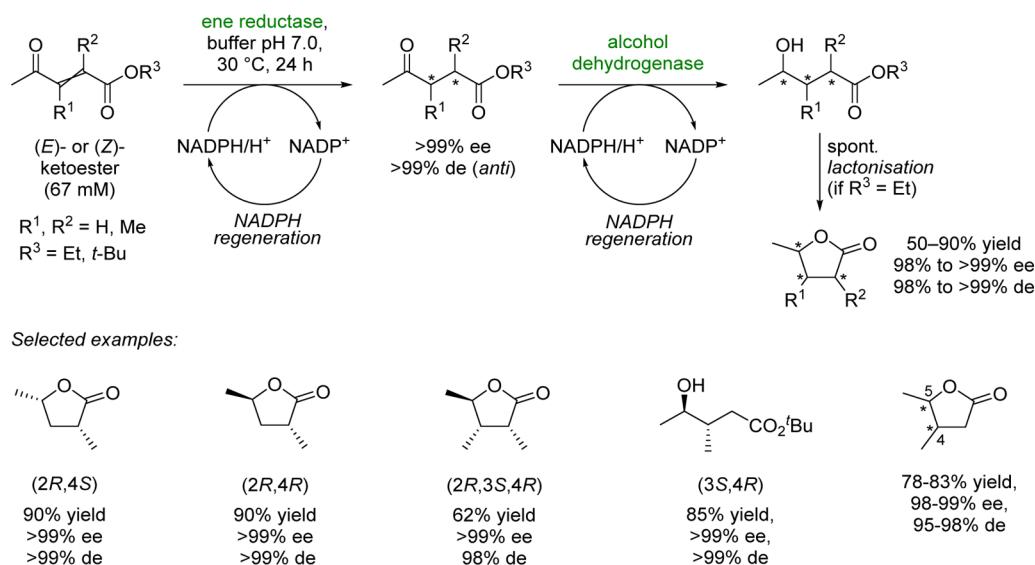
stage. All products were isolated by extraction and purified by column chromatography. Isolated yields varied between 50% and 90%, and optical purities were in most cases perfect (>99% ee, >99% de).

In a related publication, the synthesis of odoriferous 4,5-dimethyl- $\gamma$ -butyrolactones found in dried *Nicotiana tabacum* leaves was described.<sup>205</sup> In this case, OYE2 from *Saccharomyces cerevisiae* was used for C=C reduction, followed by carbonyl reduction catalyzed, e.g., by an alcohol dehydrogenase from *R. erythropolis*. With a substrate loading of 2 g/L, desired products were obtained with 78–83% isolated yield and excellent ee (98–99%) and de (95–98%).

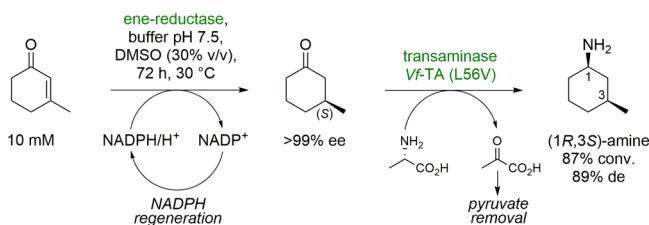
In a similar fashion, ene-reductases (EREDs) were combined with transaminases for the transformation of enones to amines containing two stereogenic centers (Scheme 34). Although

enone reduction using OYE1 from *Saccharomyces carlbergensis* proceeded with excellent stereoselectivity (ee > 99%), the transaminase from *V. fluvialis* furnished a mixture of diastereomers with only a slight excess (14% de) of the (1R,3S)-product.<sup>206</sup> The variant L56 V of the transaminase provided the (1R,3S)-amine with increased diastereomeric purity of 65% de, while the stereoselectivity was inverted for variant L56I, which gave the (1S,3S)-amine in 70% de. The optical purity of the (1R,3S)-isomer was further improved to 89% de by medium engineering, performing the biotransformation in the presence of 30% (v/v) DMSO. In a second report, the authors used an analogous cascade process for accessing the (1R,3R)-isomer in 97% de using ene-reductase variant YqjM C26D/I69T and *Vf*-TA variant L56I.<sup>207</sup> It is worth noting that a simultaneous combination of both biocatalytic steps was possible without

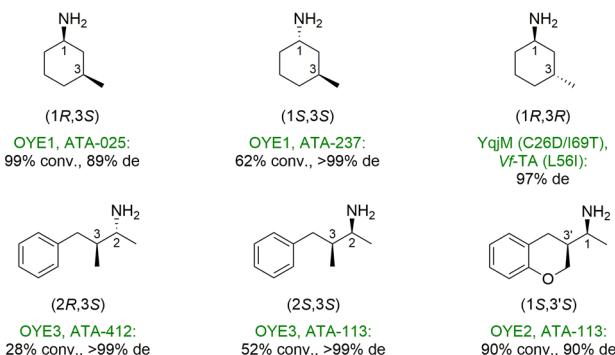
**Scheme 33.** Two-Step Biocatalytic Synthesis of Substituted  $\gamma$ -Butyrolactones Using an Ene-Reductase (ERED) and an Alcohol Dehydrogenase (ADH) by Control of up to Three Contiguous Stereogenic Centers



**Scheme 34.** Two Reduction Steps Catalyzed by an Ene-Reductase and a Transaminase for Asymmetric Synthesis of Chiral Amines Bearing Two Stereogenic Centers



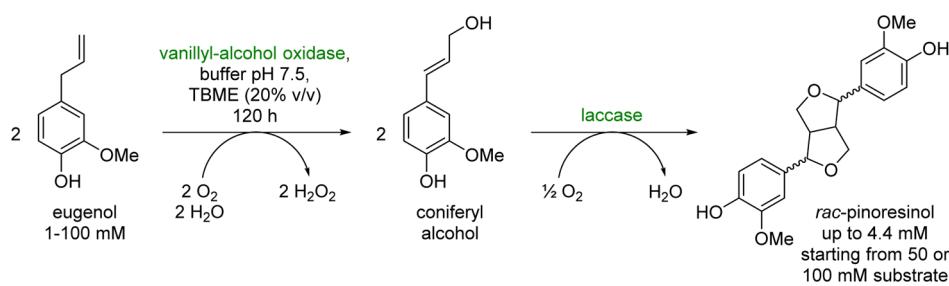
*Selected examples using different combinations of ene-reductases and transaminases:*



undesired side reactions, although the transaminase could in theory also react with the enone; however, the investigated transaminases were unreactive toward the starting enone, while they readily converted the saturated ketone. This chemoselectivity was exploited in a related study where three enones were reduced with old yellow enzymes from *S. cerevisiae* or *S. pastorianus*, and a set of 24 commercially available transaminases from Codexis was screened for reduction of the corresponding saturated ketones.<sup>208</sup> Using this enzyme “toolbox”, two of the four possible stereoisomers of each target amine were obtained in high diastereomeric excess, independent of whether the biotransformation was carried out as a sequential one-pot process or simultaneous cascade.

**3.1.2.1.3.3. Two Oxidation Steps.** Two combined oxidation steps allowed the biocatalytic synthesis of pinoresinol, a phytoestrogen and one of the simplest lignans. Vanillyl-alcohol oxidase from *Penicillium simplicissimum* and a bacterial laccase derived from *Corynebacterium glutamicum* were combined in this cascade starting form eugenol, whereby the first enzyme performed the oxidation of eugenol to coniferyl alcohol and the laccase allowed subsequent dimerization (**Scheme 35**).<sup>209</sup> The reaction was performed on a semipreparative scale (50 or 100 mM) and led to the formation of 4.4 mM racemic pinoresinol within 120 h under optimized conditions. When the synthesis was run in a sequential mode, no further improvement in productivity could be observed. It is worth noting that during the dimerization step various other products are formed,<sup>210,211</sup> which led to reduced yield. In a follow-up study the cascade was

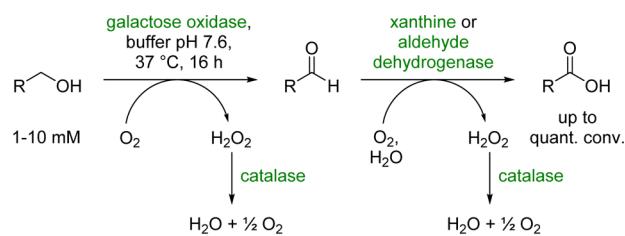
**Scheme 35.** Synthesis of Phytoestrogen *rac*-Pinoresinol Using a Vanillyl-Alcohol Oxidase and a Laccase



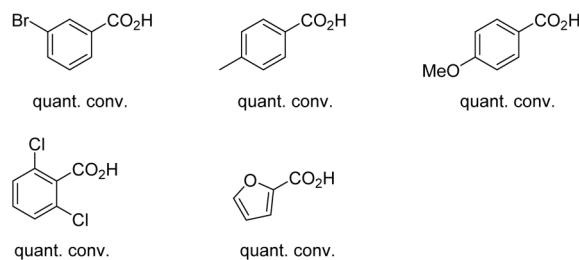
extended by a reductive opening of the tetrahydrofuran ring (see three enzymes *in vivo* in the linear sequence, Scheme 134). Enantiopure synthesis of either (+)- or (-)-pinoresinol was investigated in a whole-cell biocatalyst.<sup>212</sup> Therefore, a kinetic resolution approach was established making use of two additional enzymes: pinoresinol reductase from *Arabidopsis thaliana* for the synthesis of (+)-pinoresinol and pinoresinol-lariciresinol reductase from *Forsythia intermedia* to access the other enantiomer. By performing the cascade in a sequential mode, 876  $\mu\text{M}$  (+)-pinoresinol with an ee of 98% and 610  $\mu\text{M}$  (-)-pinoresinol with an ee > 99% were produced within 28 h starting from 10 mM eugenol.

The double oxidation of alcohols at the expense of molecular oxygen allowed the transformation of primary alcohols to carboxylic acids. In very early work horse liver alcohol dehydrogenase (HLADH) was employed for this type of transformation; thus, a single enzyme was doing two subsequent oxidation steps using FMN for NAD<sup>+</sup> recycling.<sup>213,214</sup> In a related fashion an  $\alpha$ -keto acid ( $\alpha$ -keto adipate) was used as oxidant.<sup>215</sup> More recently, engineered variants of galactose oxidase (GalOx) from *Fusarium graminearum* have been employed in combination with aldehyde oxidases. In a first study, GalOx variant M<sub>3-5</sub> was used for the oxidation of various benzylic or heteroaryl alcohols to the corresponding aldehydes, which were converted further into carboxylic acids using xanthine dehydrogenase from *E. coli* (Scheme 36).<sup>216</sup> At 10 mM substrate concentration, 16 tested alcohols were converted

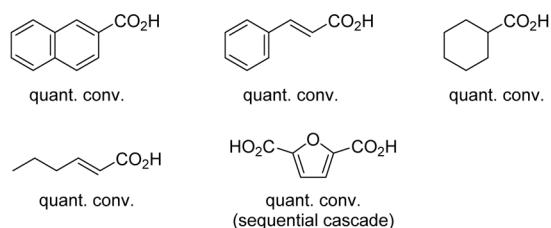
**Scheme 36. Conversion of Primary Alcohols into Carboxylic Acids Using Oxidases in a Two-Step Biocatalytic Aerobic Oxidation Cascade**



Selected examples using *xanthine dehydrogenase*:



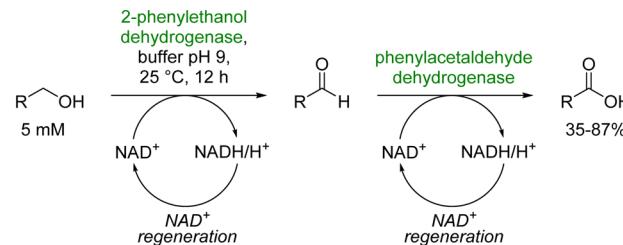
Selected examples using *periplasmic aldehyde dehydrogenase*:



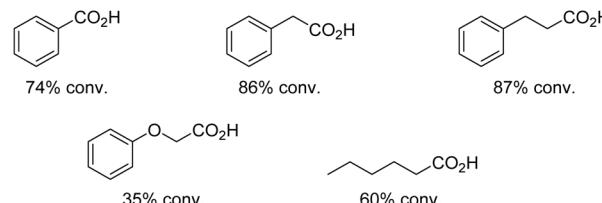
quantitatively into carboxylic acids within 16 h. The presence of catalase, which disproportionates the hydrogen peroxide formed as coproduct, was found to be essential for achieving high conversion. In a second study, a more structurally diverse set of substrates was investigated and xanthine dehydrogenase was replaced by a periplasmic aldehyde dehydrogenase from *E. coli*, which offered a broader substrate scope.<sup>217</sup> The oxidation of hydroxymethyl furfural to furandicarboxylic acid as well as the oxidation of allylic, benzylic, and aliphatic alcohols to corresponding carboxylic acids was achieved, and quantitative conversion was observed in most cases.

The biocatalytic oxidation of primary alcohols to carboxylic acids at the expense of molecular oxygen has also been achieved using NAD<sup>+</sup>-dependent dehydrogenases for the two oxidation steps and NADH oxidase for regeneration of the cofactor (Scheme 37).<sup>218</sup> Employing 2-phenylethanol dehydrogenase and

**Scheme 37. Conversion of Primary Alcohols into Carboxylic Acids Employing NAD<sup>+</sup>-Dependent Dehydrogenases**



Selected examples:

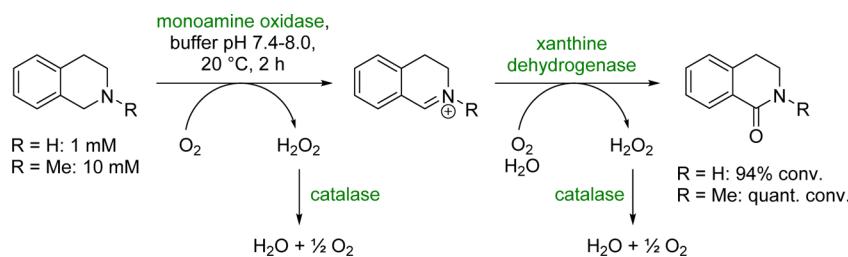


phenylacetaldehyde dehydrogenase from *Brevibacterium* sp. KU 1309 together with H<sub>2</sub>O-producing NADH oxidase from *L. brevis*, several arylaliphatic, benzylic, and aliphatic alcohols were oxidized to the corresponding acids with 35–87% analytical yield (GC).

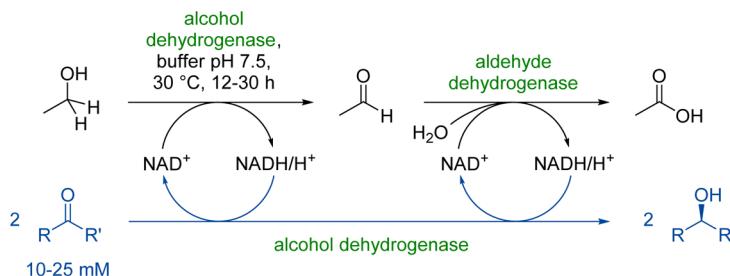
Another double oxidation allowed the transformation of a benzylic cyclic amine into the corresponding lactam (Scheme 38).<sup>216</sup> The conversion was achieved by variant D9 of monoamine oxidase from *A. niger* catalyzing the oxidation of the starting material to the imine or iminium ion, which was further oxidized to the lactam by xanthine dehydrogenase from *E. coli*. The addition of catalase ensures rapid decomposition of the reaction coproduct hydrogen peroxide and thereby prevents oxidative inactivation of the enzymes. 1,2,3,4-Tetrahydroisoquinoline was converted with 94% to the lactam at 1 mM substrate concentration, while the *N*-methyl derivative was quantitatively converted at 10 mM concentration.

Although nicotinamide cofactors are mostly regenerated by a single enzyme, selected studies have investigated linear cascade systems for cofactor regeneration with the aim of performing two or more successive oxidations of a sacrificial substrate and thereby regenerating more than 1 equiv of NAD(P)H. For instance, alcohol dehydrogenase and aldehyde dehydrogenase from Baker's yeast have been used for regenerating NADH via

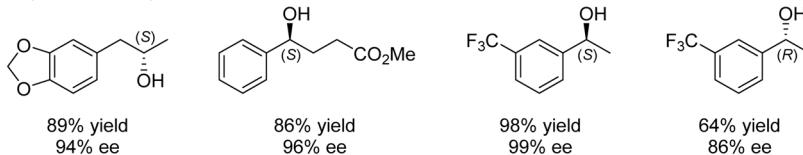
**Scheme 38. Conversion of Tetrahydroisoquinolines into the Corresponding Lactams via Two Biocatalytic Aerobic Oxidation Steps**



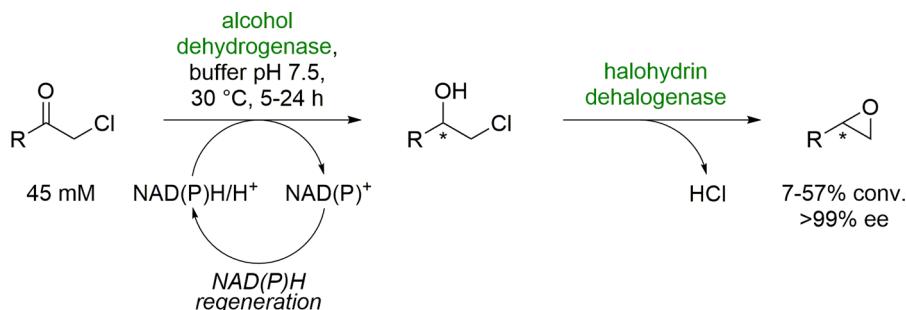
**Scheme 39. Two Oxidation Steps for the Regeneration of 2 Equiv of NADH from a Single Molecule of Ethanol**



Preparative examples:

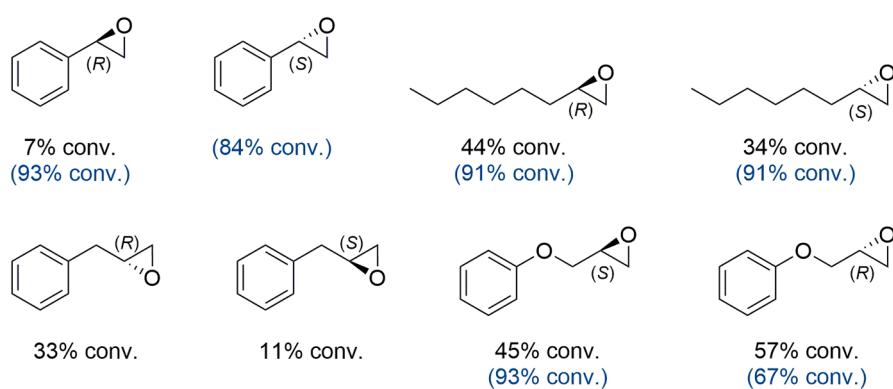


**Scheme 40. Combining Asymmetric Reduction of  $\alpha$ -Chloroketones and Epoxide Formation**



Examples:

ee >99% in all cases; conversion values in parentheses are for reactions in the presence of anion exchangers:



the four-electron oxidation of ethanol to acetic acid (**Scheme 39**).<sup>219</sup> When the yeast ADH is replaced with ADH from *L. kefir*, the regeneration of NADPH is possible as well. For systems generating three redox equivalents from the transformation of methanol to CO<sub>2</sub>, please refer to section 3.1.3, **Schemes 64** and **65**.

**3.1.2.2. Redox Cascades—One Redox Step.** **3.1.2.2.1. Reduction as First Step.** Chiral epoxides have been obtained via a cascade that combines an asymmetric reduction of prochiral  $\alpha$ -chloroketones by an alcohol dehydrogenase followed by ring closure of the intermediate chlorohydrins catalyzed by a halohydrin dehalogenase (**Scheme 40**).<sup>220</sup> Employing stereocomplementary alcohol dehydrogenases from *R. ruber* DSM 44541 and *L. brevis*, both enantiomers of the chlorohydrins were formed in excellent enantiomeric excess (ee > 99%). The use of a halohydrin dehalogenase with low enantioselectivity (HheB from *Mycobacterium* sp. GP1, *E* = 1.1–2.3 for the investigated substrates) ensured efficient cyclization of either of the two chlorohydrin enantiomers to the target epoxides. However, the halohydrin dehalogenase reaction is reversible, and hence, only limited conversions to the epoxides were attained (7–57%), even if the reduction of the chloroketone went to completion. The limitation of the equilibrium was later overcome by carrying out the biotransformation in the presence of anion exchange resins, which were thought to bind the chloride ion liberated in the ring-closure step and thereby shifted the equilibrium to the product side.<sup>221</sup> This strategy raised the conversion to >90% in several cases. In some reactions, however, inhibition of the halohydrin dehalogenase by the  $\alpha$ -chloroketone substrates was identified as another yield-limiting issue, which was addressed by adding the halohydrin dehalogenase once the ADH-catalyzed reduction had run to completion, thus performing in selected cases the reaction in sequential mode.

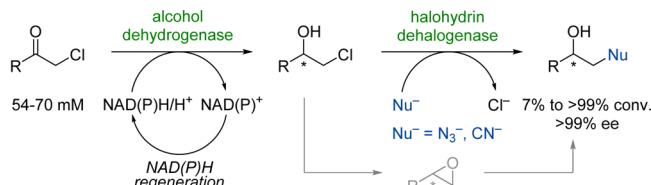
Halohydrin dehalogenases not only form epoxides from halohydrins but can also open epoxides, accepting a broad range of nucleophiles (e.g., halides, cyanide, azide, nitrite, cyanate, thiocyanate) in the ring-opening step.<sup>222,223</sup> Consequently, the above-mentioned cascade was extended by a third step using the halohydrin dehalogenase for ring closing as well as epoxide opening (**Scheme 41**). The cascade was first demonstrated with

azide as nucleophile using alcohol dehydrogenases from *R. ruber* and *L. brevis* and halohydrin dehalogenases from *Mycobacterium* sp. GP1 (HheB) and *Agrobacterium radiobacter* AD1 (HheC) as biocatalysts.<sup>224</sup> The ring-opening step by azide allowed also one to shift the equilibrium of the reaction to the product side affording  $\beta$ -azidoalcohols in good conversions (>90% in four cases) and optically pure form (ee > 99%). Since the cascade using cyanide as nucleophile led to inhibition of the zinc(II)-dependent ADH from *R. ruber* as well as to undesired side reactions, the cascade was performed in sequential fashion (see section sequential cascades 3.2, **Scheme 79**). Despite this, the cascade with cyanide was reported in another publication to be run in simultaneous fashion using a single-cell catalyst coexpressing one of a pair of stereocomplementary alcohol dehydrogenases from *L. kefir* DSM 20587, halohydrin dehalogenase HheC, and—if required—an additional enzyme for cofactor regeneration.<sup>225</sup> In this case, neither enzyme inhibition by cyanide nor any side reaction (formation of cyanoketone) was observed. The whole-cell approach allowed significantly higher substrate loadings and productivities compared to the cascade employing individually expressed enzymes. For instance, 100 mM 2'-chloroacetophenone was quantitatively transformed into the corresponding (R)-hydroxynitrile (ee > 99%) within 1 h with 20 g/L (dry cell weight) of biocatalyst. A similar sequence of chloroketone reduction, epoxide formation, and epoxide ring opening by cyanide has been implemented on large scale for the synthesis of a statin side chain building block; however, in this case the steps were not performed in a cascade.<sup>226,227</sup>

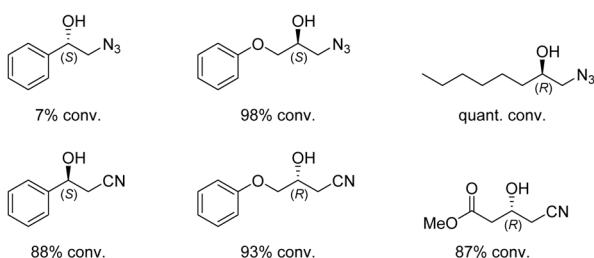
A two-point variant of halohydrin dehalogenase HheC (P175S/W249P) has been used in combination with a variant of epoxide hydrolase from *Agromyces mediolanus* ZJB120203 for the synthesis of (S)-epichlorohydrin from 1,3-dichloropropanol.<sup>228</sup> In this system, the HHDH variant catalyzes ring closure of the prochiral starting material with good but not exclusive stereoselectivity (ee = 92%). The epoxide hydrolase is employed to upgrade the enantiomeric excess of the product by selectively hydrolyzing the minor (R)-enantiomer to the corresponding vicinal diol. Under optimized conditions, (S)-epichlorohydrin was obtained with 91% conversion and an ee of >99%.

A reductive dynamic kinetic resolution of cyclic  $\beta$ -ketonitriles has been combined with biocatalytic nitrile hydrolysis to furnish cyclic 2-hydroxycarboxylic acids (**Scheme 42**).<sup>229</sup> The  $\beta$ -ketonitriles investigated, based on a 5-membered to 7-membered carbocyclic scaffold, are prone to spontaneous racemization at

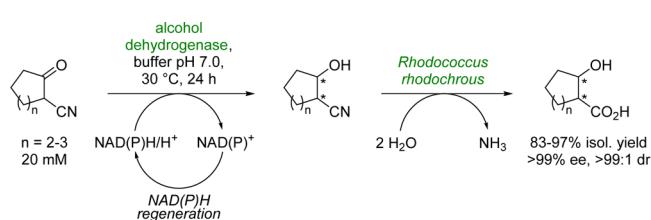
**Scheme 41. Asymmetric Synthesis of  $\beta$ -Azidoalcohols and  $\beta$ -Hydroxynitriles from  $\alpha$ -Chloroketones Using Alcohol Dehydrogenases and Halohydrin Dehalogenases**



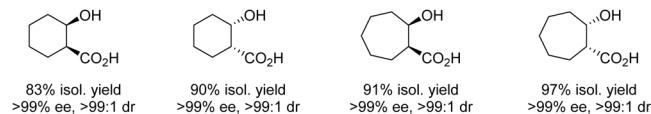
Selected examples (ee >99% in all cases):

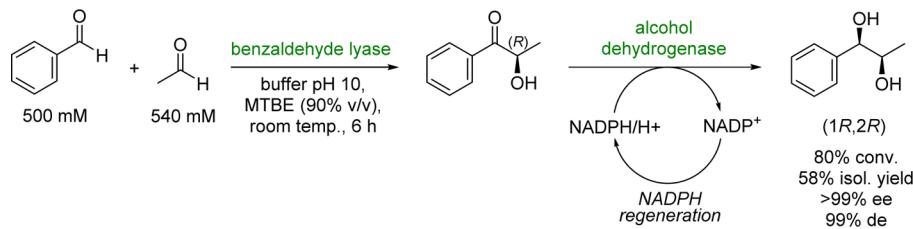


**Scheme 42. Synthesis of Cyclic 2-Hydroxycarboxylic Acids via Concurrent Biocatalytic Reductive Dynamic Kinetic Resolution and Nitrile Hydrolysis**



Examples:



Scheme 43. Biocatalytic Synthesis of a Vicinal (1*R*,2*R*)-Diol via C–C Bond Formation and Ketone Reduction

neutral pH enabling dynamic kinetic resolution of the labile stereogenic center. From reactions with a set of commercial alcohol dehydrogenases, the trans isomer was in general the predominant product and in some cases the product was stereoisomerically pure (*dr* > 99:1, ee > 99%). Due to the low activity of commercial nitrilases in hydrolysis of the resulting hydroxynitriles, whole cells of *Rhodococcus rhodochrous* were employed, a bacterium known to display nitrile hydratase and amidase activity.<sup>230</sup> The cells efficiently hydrolyzed the investigated hydroxynitriles independent of their absolute configuration but were inactive toward the ketonitrile starting materials. These characteristics enabled a simultaneous cascade of reduction and hydrolysis, which afforded the *cis*-hydroxycarboxylic acids in 83–97% isolated yield and in stereoisomerically pure form. When *R. rhodochrous* cells were grown in the presence of an amidase inhibitor, chemoselective hydrolysis of the nitrile group to the amide was achieved.

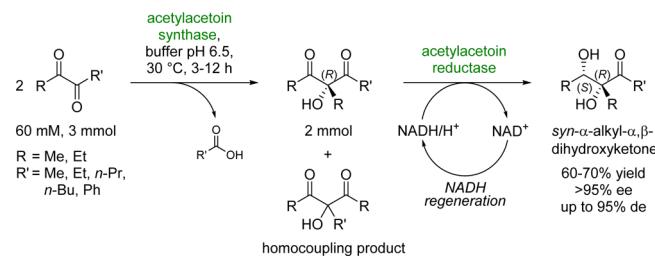
**3.1.2.2. Reduction as Second Step.** The combination of a ThDP-dependent benzylkadehyde lyase with an alcohol dehydrogenase allowed the preparation of chiral vicinal diols via C–C bond formation and ketone reduction. For instance, a vicinal (1*R*,2*R*)-diol was prepared using a two-enzyme two-step simultaneous cascade involving a benzaldehyde lyase (BAL) from *Pseudomonas fluorescens* and an ADH from a *Ralstonia* sp. (Scheme 43).<sup>231</sup>

The reaction was performed in a microaqueous system containing 10% (v/v) water or buffer and 90% (v/v) of methyl *tert*-butyl ether (MTBE) at a concentration of 500 mM benzaldehyde. The system enables elevated concentrations of benzaldehyde to levels beyond its solubility limit in aqueous media. Comparing the simultaneous cascade with the cascade performed in sequential mode (adding the ADH after completion of the BAL reaction), the latter gave better results concerning conversion (93% vs 75%) while ee and de were the same in both cases (>99% ee; 99.6% and 99.8% de, respectively). This was attributed mainly to the competing reduction of benzaldehyde by *Ralstonia* ADH that was observed in the simultaneous cascade, which led to the accumulation of benzyl alcohol (39 mM). Performing the two-step reaction on a preparative scale (1.06 g of benzaldehyde), the (1*R*,2*R*)-product was obtained with 58% isolated yield and excellent optical purity (>99% ee, 99% de). In a follow-up study, the same cascade was investigated using whole cells placed in a polyvinylidene fluoride (PVDF) teabag, aiming at a better recyclability of the biocatalysts by simple removal of the bag from the reaction vessel.<sup>232</sup> This new reaction setup was tested in (i) sequential mode, adding the ADH after completion of the BAL reaction, (ii) simultaneous mode, adding all enzymes and reagents at the start of the reaction, and (iii) mixed cascade mode, adding the ADH after 3 h, when benzaldehyde had been largely but not completely consumed by BAL. The highest levels of product formation (339 mM from 500 mM benzaldehyde) were attained in the mixed mode, while the simultaneous cascade led to the highest space-

time yield ( $3.25 \text{ g L}^{-1} \text{ h}^{-1}$ ) due to the shorter time required for the reaction to reach completion.

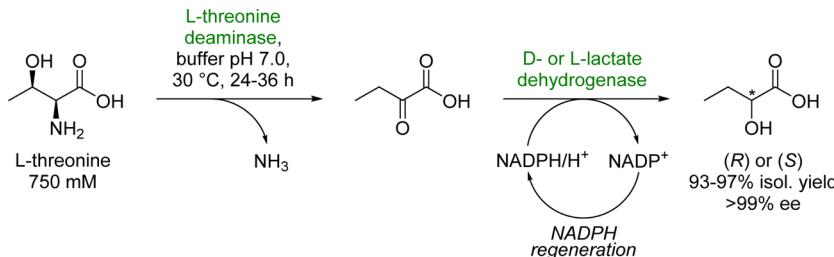
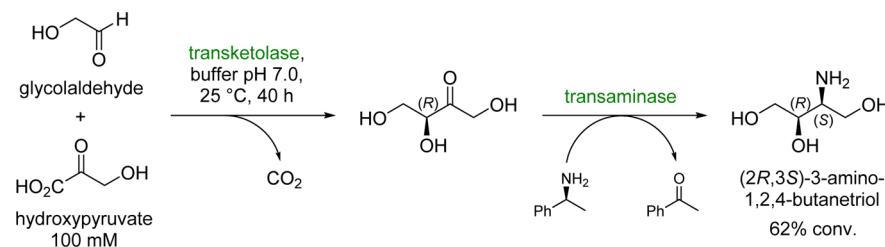
All four stereoisomers of diol have been prepared in a follow-up study in a microaqueous solution, although not in a cascade mode, since the solution of a first step was separated from the catalyst and transferred to a fresh flask.<sup>233</sup> Nevertheless, this processes enabled up to  $63 \text{ g L}^{-1}$  product concentration (98% yield), space–time yields up to  $144 \text{ g L}^{-1} \text{ d}^{-1}$  and a target isomer content of at least 95%.

Coupling a ThDP-dependent acetylacetoin synthase (AAS) from *Bacillus licheniformis* to the NADH-dependent acetylacetoin reductase (AAR) from the same organism allowed to access *syn*- $\alpha$ -alkyl- $\alpha$ , $\beta$ -dihydroxyketones with high enantioselectivities (>95% ee; Scheme 44) and good isolated yields (60–

Scheme 44. Cascade for the Synthesis of Enantioenriched *syn*- $\alpha$ -Alkyl- $\alpha$ , $\beta$ -dihydroxyketones via C–C Bond Formation and Ketone Reduction Using an Acetylacetoin Synthase and a Reductase

70%).<sup>234,235</sup> It is important to note that symmetrical diketones obtained from homocoupling were not reduced by the (*S*)-stereospecific reductase (AAR) and could be separated from the final products by flash chromatography. The reaction sequence was also employed for the synthesis of the chiral green tea flavor compound 3-hydroxy-3-methylnonane-2,4-dione.<sup>236</sup>

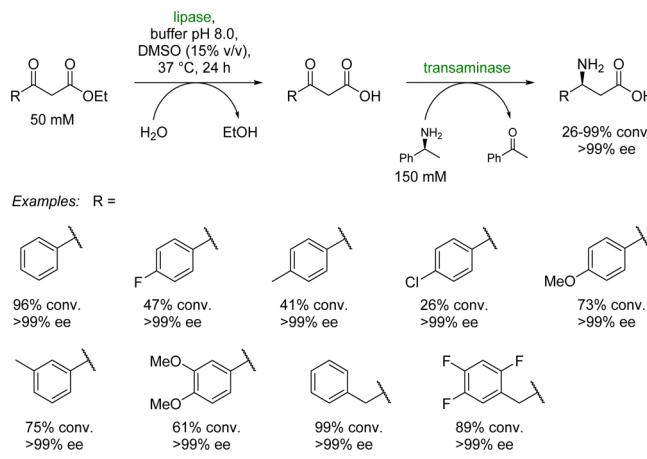
In Scheme 31, a cascade was depicted in which  $\alpha$ -amino acids were transformed to corresponding  $\alpha$ -hydroxy acids via an oxidation reduction cascade. In the following example an  $\alpha$ -amino acid, namely, threonine, was transformed to the corresponding  $\alpha$ -hydroxy acid; however, the first step relied on water elimination and thus it is not a redox step (Scheme 45).<sup>237</sup> The first step was catalyzed by L-threonine deaminase from *E. coli* followed by reduction by either an L- or an D-lactate dehydrogenase (D-/L-LDH) derived from *Oryctolagus cuniculus* or *Staphylococcus epidermidis*, respectively, leading to (*R*)- or (*S*)-2-hydroxybutyric acid (2-HB). *E. coli* whole-cell preparations were used for the reaction whereby the desired enzymes had been expressed separately. L-Threonine (750 mM) was converted within 24 h for (*R*)-2-HB or 36 h for (*S*)-2-HB) with 95% and 97% isolated yield, respectively, and perfect ee (>99%). To further improve the handling of the catalysts, deaminase, D-/L-LDH and NADPH-recycling enzyme (FDH)

**Scheme 45.** Cascade Synthesis of (R)/(S)-2-Hydroxybutyric Acid from L-Threonine**Scheme 46.** Formation of (2R,3S)-3-Amino-1,2,4-butanetriol via C–C Bond Formation and Amination Catalyzed by a Transketolase and a Transaminase

were coexpressed in *E. coli*, allowing complete conversion after 8 and 24 h, respectively, with 93% and 97% isolated yield and perfect ee (>99%).

ThDP-dependent lyases have also been combined with transaminases for the formation of 1,2-amino alcohols.<sup>238</sup> The feasibility of such a cascade has first been demonstrated by combining a transketolase from *E. coli* and  $\beta$ -alanine:pyruvate aminotransferase from *P. aeruginosa* PAO2 coexpressed in a single *E. coli* host, furnishing 3-amino-1,2,4-butanetriol from achiral starting materials hydroxypyruvate and glycolaldehyde, using (S)-1-phenylethylamine as amino donor (Scheme 46).<sup>239</sup> Both enzymes displayed excellent stereoselectivity, forming the (2R,3S)-amino alcohol as a single stereoisomer,<sup>88</sup> but their activities were misbalanced: the transketolase was more active than the transaminase by 3 orders of magnitude, and thus, the first step was complete within 30 min, while more than 4 days were required for the second step to reach 21% conversion. Moreover, the product was found to be slowly degraded under the reaction conditions employed. These points were addressed in follow-up studies by switching to a different transaminase originating from *C. violaceum* and by optimizing the fermentation conditions to achieve more balanced expression levels of the two enzymes.<sup>240–242</sup> These adjustments, in combination with mathematical reaction modeling and model-based process optimization, formed the basis for an improved cascade run at 100 mM initial substrate concentration with continuous (S)-1-phenylethylamine feeding, which afforded (2S,3R)-amino alcohol in 62% conversion after 2.5 days. A process for the synthesis of (2S,3S)-2-amino-1,3-pentanetriol has been developed and optimized in a similar manner, in this case using isopropylamine as amino donor.<sup>242,243</sup>

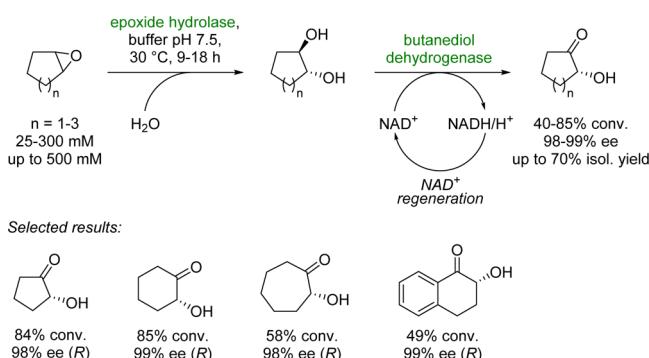
Various aromatic  $\beta$ -amino acids were obtained via a simultaneous two-step cascade starting from  $\beta$ -keto esters. Step one involved hydrolysis of the ethyl ester by a lipase originating from *Candida rugosa*, followed by reductive amination of the keto acid by an transaminase (TA) from *Polaromonas* sp. JS666 (Scheme 47).<sup>244</sup> Several other  $\beta$ -keto esters were transformed into their corresponding amino acids with conversions of 26–99% and excellent ee (>99%).  $\beta$ -Amino acids have attracted

**Scheme 47.** Lipase/Transaminase Cascade To Transform  $\beta$ -Keto Esters to  $\beta$ -Amino Acids

attention due to their application in the synthesis of bioactive compounds as well as in the design of hybrid peptides.<sup>245–248</sup>

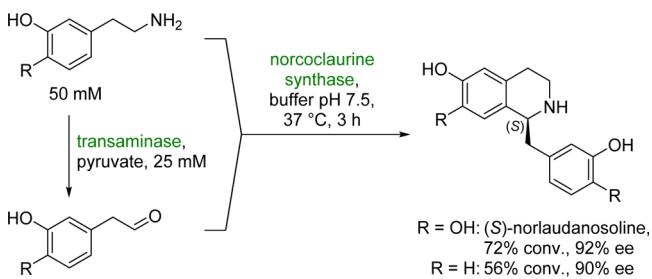
Various cyclic  $\alpha$ -hydroxy ketones were accessed in a simultaneous two-step cascade via epoxide hydrolysis and alcohol oxidation (Scheme 48).<sup>249</sup> The epoxides were hydrolyzed by an epoxide hydrolase from *Sphingomonas* sp. (SpEH) producing the vicinal diols, which were then enantioselectively oxidized to the corresponding  $\alpha$ -hydroxy ketones by butanediol dehydrogenase (BDHA) from *B. subtilis* BGSC1A1. Different options for applying catalyst preparations were tested such as using cell-free extracts of each enzyme or using single cells containing all cascade biocatalysts. The best results in terms of conversion were obtained applying *E. coli* whole cells coexpressing SpEH, the alcohol dehydrogenase, and an NADH oxidase for cofactor recycling. With this system, up to 300 mM cyclohexene oxide was converted to 70%, leading to an excellent ee of 98% for the (R)-enantiomer product. The applicability of this system was further examined on a preparative scale with 500 mg of cyclohexene oxide producing 350 mg (70% isolated yield) of the product with a perfect ee of 98% for the (R)-enantiomer.

**Scheme 48. Epoxide Hydrolysis Followed by Alcohol Oxidation Leading to Cyclic  $\alpha$ -Hydroxyketones**



**3.1.2.2.3. Oxidation as First Step.** Formal oxidative deamination of a terminal primary amine catalyzed by a transaminase from *C. violaceum* was coupled with C–C bond formation catalyzed by the lyase norcoclaurine synthase (NCS) from *Thalictrum flavum* to produce 1,2,3,4-tetrahydrobenzylisoquinolines (Scheme 49).<sup>250</sup> One equivalent of the 2 equiv of

**Scheme 49. Deamination Coupled with C–C Bond Formation Leading to 1,2,3,4-Benzyltetrahydroisoquinolines**



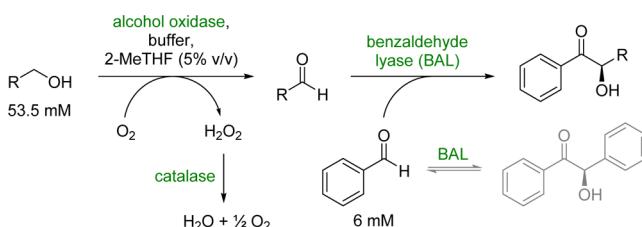
substrate amine was transformed via a two-enzyme sequence, while the other equivalent was touched only by one enzyme. Thus, the transaminase catalyzed the conversion of the amine into a 1:1 mixture of primary amine and aldehyde at the expense of pyruvate as amino group acceptor. Norcoclaurine synthase coupled this mixture in an asymmetric biocatalytic Pictet–Spengler reaction, affording the benzylisoquinoline alkaloid (S)-norlaudanosoline in reasonable conversion (72% from 50 mM substrate; up to 87% from 20 mM substrate) and very good

enantioselectivity (90–99% ee). Due to the reaction scheme, the cascade was named a “triangular cascade”.

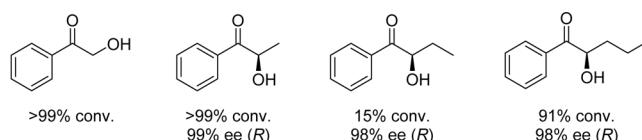
Another two-step cascade with one redox step as the first reaction was designed to access L-tyrosine derivatives from substituted benzene, whereby the benzene ring was hydroxylated in the first step in the ortho position to the substituent (Scheme 50).<sup>251</sup> The hydroxylation was catalyzed by the P450 monooxygenase BM3 from *B. megaterium* followed by C–C bond formation using a tyrosine phenyl lyase from *Citrobacter freundii*. As side reactions, small amounts (<5%) of *p*-hydroxylated product as well as *p*-hydroquinone were detected. Various substituents were tolerated in the cascade. The best result in terms of productivity was obtained with the methoxy substituent leading to 68% conversion. Side product *p*-hydroquinone formation was particularly high with halogenated substrates.

Alcohol oxidation preceded C–C bond formation for the preparation of chiral hydroxyketones. ThDP-dependent C–C bond-forming benzaldehyde lyase (BAL) was used in combination with an alcohol oxidase from *Hansenula* sp. (Scheme 51).<sup>252</sup>

**Scheme 51. Alcohol Oxidation Followed by C–C Bond Formation Leading to Chiral  $\alpha$ -Hydroxyketones from Benzaldehyde and Aliphatic Alcohols**

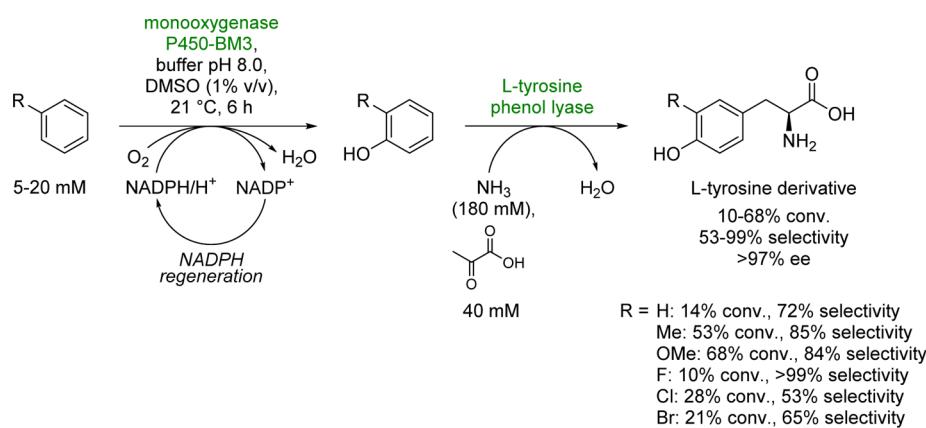


Selected results:

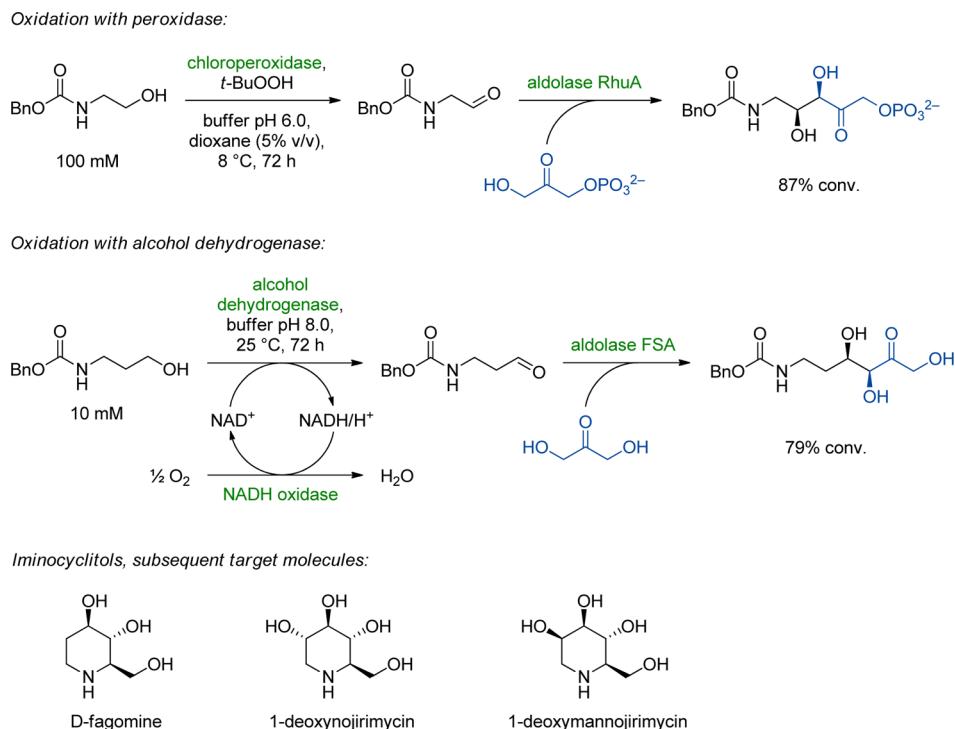


The latter *in situ* oxidized aliphatic alcohols to corresponding aldehydes, which were then coupled with benzaldehyde. Methanol, ethanol, and 1-butanol were found to be good substrates for the oxidase, resulting in from 91% to >99%

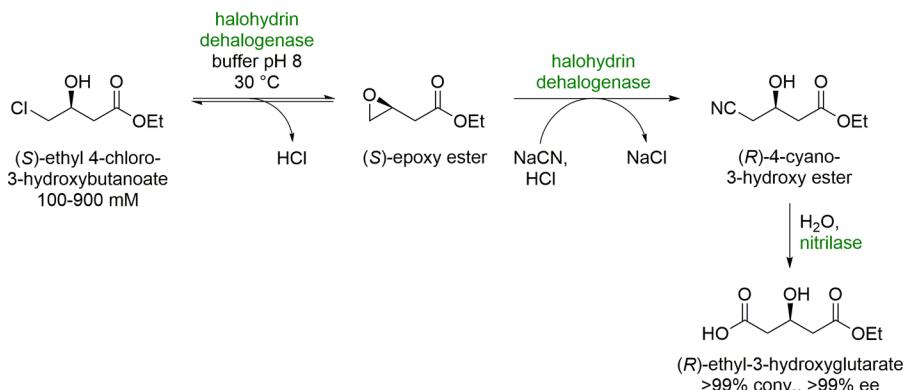
**Scheme 50. Hydroxylation of Monosubstituted Benzene Followed by C–C Bond Formation to L-Tyrosine Derivatives**



**Scheme 52.** Oxidation of Primary Alcohols Followed by Aldolase-Catalyzed C–C Bond Formation To Prepare Synthetic Precursors of Iminocyclitols



**Scheme 53.** Two Enzymes Catalyzing Three Steps To Transform a Halohydrin Moiety to a  $\beta$ -Hydroxy Carboxylic Acid Moiety Employing a Halohydrin Dehalogenase and a Nitrilase

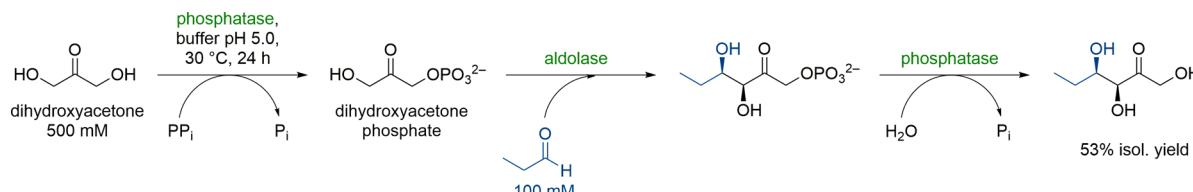


conversion to the product with perfect ee's (98–99%) for the (*R*)-enantiomer. Interestingly, the coupling product of 1-propanol and benzaldehyde led only to low amounts of the hydroxyketone although with good ee (98%).

Iminocyclitols (e.g., D-fagomine, 1-deoxynojirimycin, 1-deoxymannojirimycin; Scheme 52, bottom) are cyclic sugar analogues in which the ring oxygen atom is replaced by nitrogen. Many of these molecules are plant natural products and have been associated with a broad range of biological activities, including antidiabetic, anticancer, and anti-infective properties.<sup>253–257</sup> Their chemoenzymatic synthesis employing aldolases has been investigated since the late 1980s,<sup>258–261</sup> and carboxybenzyl-protected aminoaldehydes (Scheme 52) have emerged as the most common aldol acceptors in these synthetic routes. The inherent sensitivity of these aminoaldehydes has prompted several studies toward their *in situ* formation via biocatalytic oxidation reactions. For instance, the oxidation of *N*-Cbz-ethanolamine using a laccase/TEMPO system<sup>262</sup> has been

reported.<sup>263</sup> Aldehyde formation in this system was accompanied by overoxidation to the corresponding carboxylic acid—a problem that could be alleviated to some extent by performing the reaction in a biphasic system of toluene and buffer (2:1 v/v, containing a cationic surfactant). Furthermore, the divergent pH optima of laccases and aldolases allowed only a stepwise coupling of the oxidation and carboligation steps. A simultaneous cascade of *N*-Cbz-aminoalcohol oxidation and an aldol reaction was demonstrated using chloroperoxidase from *Caldariomyces fumago* and L-rhamnulose-1-phosphate aldolase from *E. coli* as biocatalysts (Scheme 52).<sup>264</sup> Enzyme deactivation was identified as a major yield-limiting factor in this study, causing reactions employing free enzymes to stop at 33% substrate conversion (initial concentration 20 mM) and 24% aldol product formation. The operational stability and productivity of the two-enzyme process were increased significantly by using immobilized enzymes and 5% (v/v) of dioxane as organic cosolvent, which allowed a higher substrate loading (100 mM) and resulted in the

**Scheme 54. Two Enzymes, Three Steps Cascade: In Situ Formation of Dihydroxyacetone Phosphate (DHAP) from Dihydroxyacetone and Its Aldol Coupling with Propionaldehyde**



formation of 87% of the aldol product within 72 h. Recently, an alternative oxidation–aldolization cascade system was developed for the synthesis of (3*S*,4*R*)-6-(Cbz-amino)-5,6-dideoxy-2-hexulose, a precursor of D-fagomine.<sup>265</sup> In this cascade, oxidation of Cbz-protected 3-aminopropanol was achieved by combining alcohol dehydrogenase from horse liver (HL-ADH) with an NADH oxidase from *Lactococcus lactis*. The aldehyde thus formed then underwent an aldol reaction with dihydroxyacetone, catalyzed by an engineered variant (A129S) of D-fructose-6-phosphate aldolase from *E. coli*.<sup>266</sup> Overoxidation of the aldehyde to the corresponding carboxylic acid was observed as a major side reaction, while dihydroxyacetone and the aldol product were not oxidized by HL-ADH. Systematic variation of the reaction conditions using a design-of-experiments approach led to an optimized cascade system that afforded 79% of the aldol product from 10 mM *N*-Cbz-aminoalcohol. The cascade coupling of alcohol oxidation using variants of galactose oxidase and aldol reactions catalyzed by rabbit muscle aldolase was also reported recently; however, a quantitative analysis of the coupled reactions was not performed.<sup>267</sup>

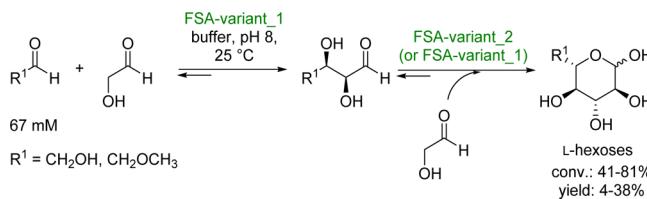
**3.1.2.3. Nonredox Cascades.** Although the following cascade requires just two enzymes, it involves three steps, since two steps are catalyzed by the same enzyme (Scheme 53).<sup>268</sup> Thereby, ethyl (*S*)-4-chloro-3-hydroxybutyrate was converted to the corresponding epoxide by a halohydrin dehalogenase from *A. radiobacter* AD1, which also catalyzed ring opening with cyanide. Subsequent hydrolysis to the monocarboxylic acid product ethyl (*R*)-3-hydroxyglutarate was achieved by a nitrilase from *A. thaliana*. The biocatalytic synthesis led to the key intermediate in the preparation of rosuvastatin, a drug used for the treatment of high cholesterol levels. Using *E. coli* cells coexpressing the two enzymes, up to 300 mM substrate was converted within 1.5 h. At a substrate loading of 600 mM, only about 20% of the target hydroxy acid was formed after 5 h reaction time due to inhibition of the nitrilase. In order to access high product titers, the cascade was performed first in a fed-batch mode, adding 300 mM substrate twice over a period of 2 h, whereby the reaction went to completion after 6 h. Actually, poorly balanced activity levels of the two coexpressed enzymes were finally identified as the reason limiting high titers, as the nitrilase was expressed at a significantly smaller activity level compared to the halohydrin dehalogenase. Consequently, two whole-cell preparations expressing each enzyme individually were used and their cell loadings adjusted to obtain comparable activity levels of the two enzymes. Using this setup, 900 mM substrate was converted into the final product within 6 h, and the (*R*)-product was isolated in 84% yield.

Another example for a cascade in which two enzymes catalyze three steps includes an alternative method for the in situ preparation of dihydroxyacetone phosphate (DHAP) using a phosphatase instead of a kinase (Scheme 54) (for kinase see Schemes 67 and 68, bottom).<sup>269</sup> The phosphate donor in this case is cheap inorganic pyrophosphate, and the process does not require ATP. On the other hand, the phosphatase also catalyzes

the hydrolysis of phosphate esters, and hence upon long reaction times, DHAP is converted back to dihydroxyacetone. Simultaneous coupling of DHAP-generating reaction with carboligation catalyzed by a DHAP-dependent aldolase prevented this undesired hydrolysis, as DHAP was constantly consumed as it was formed. Hydrolysis of the phosphorylated aldol product—which usually is the desired reaction—also took place, and it was demonstrated that the phosphatase could catalyze a phosphate transfer from the aldol product to dihydroxyacetone, leading to “recycling” of the phosphate group. In a first proof-of-principle study, this approach was used for the aldol coupling of dihydroxyacetone and propionaldehyde employing rabbit muscle aldolase and phosphatase from *Shigella flexneri* (PhoNSf) as biocatalysts. The reaction of 500 mM dihydroxyacetone, 100 mM propionaldehyde, and a total of 720 mM pyrophosphate (added in three equal portions) afforded the desired aldol product in 53% isolated yield (based on propionaldehyde). In later studies, the productivity of the cascade was improved by using an engineered phosphatase,<sup>270</sup> and the process was transferred to a flow setup (see section 3.3).

Avoiding the need for a phosphorylated substrate, a series of aldose carbohydrates with up to four chiral centers was constructed by de novo connection of simple achiral aldehyde precursors in one pot. The reaction of 2 equiv of glycolaldehyde and 1 equiv of an acceptor aldehyde ( $\text{R}^1\text{CHO}$ ) resulted in the desired aldose derivatives (Scheme 55).<sup>271</sup> Stereochemical

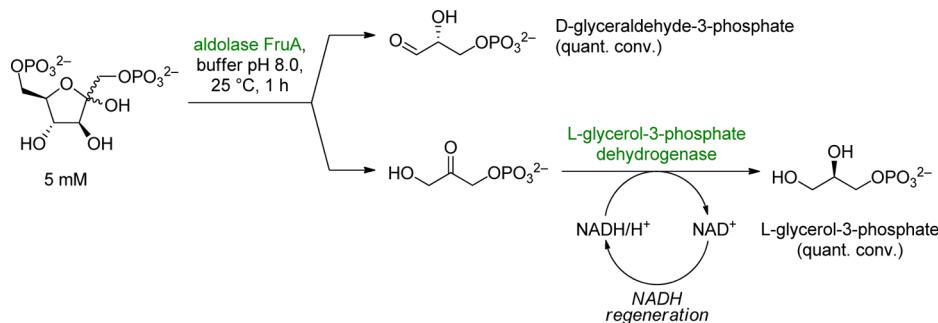
**Scheme 55. Biocatalytic Synthesis of L-Hexose Derivatives from Simple Achiral Precursors by Engineered *E. coli*-FSA Variants**



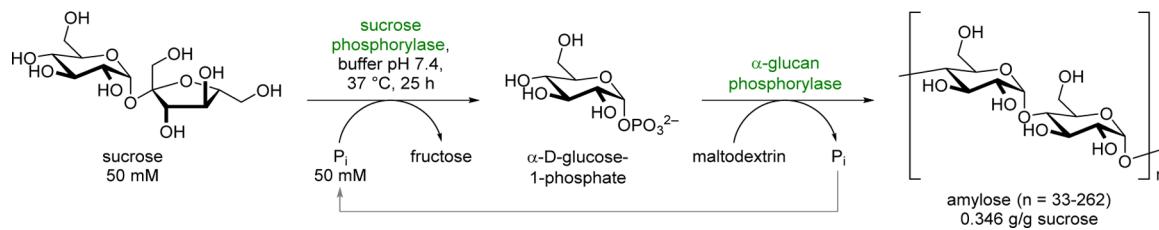
control was assured by engineered variants of D-fructose-6-phosphate aldolase (FSA), which afforded D-aldoses with excellent diastereoselectivities and high conversions (50–98%) and isolated yields. Two double variants (FSA-A129T/S166G and FSA-A129T/A165G) and one triple variant (FSA-A129T/A165G/S166G) emerged as best candidates for the preparation of several deoxy- and O-substituted-D-hexoses. To prepare the corresponding L-aldoses, two FSA variants were employed, whereby for installing the inverted CS center of the L-aldose, e.g., the variant FSA-A129G was employed in the second step (conversions 41–81%).

The D-fructose-1,6-bisphosphate aldolase (FruA) is a key enzyme of a cascade which produces D-glyceraldehyde-3-phosphate and L-glycerol-3-phosphate from D-fructose-1,6-

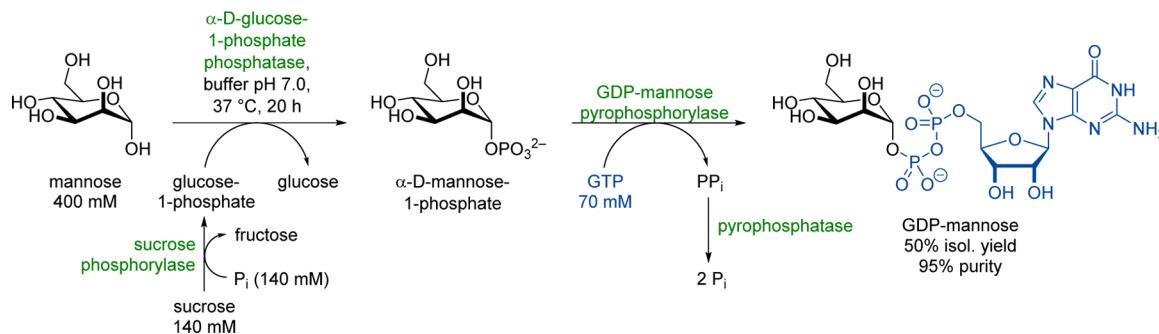
**Scheme 56.** Enzymatic Formation of D-Glyceraldehyde-3-phosphate and L-Glycerol-3-phosphate from D-Fructose-1,6-bisphosphate



**Scheme 57.** Sugar Activation and Transfer Leading to Amylose



**Scheme 58.** Two Enzymes in the Linear Sequence to Transform Mannose to GDP-Mannose Circumventing Nucleotide Triphosphate (NTP)-Dependent Phosphorylation Steps by Using Sucrose as Activated Reagent

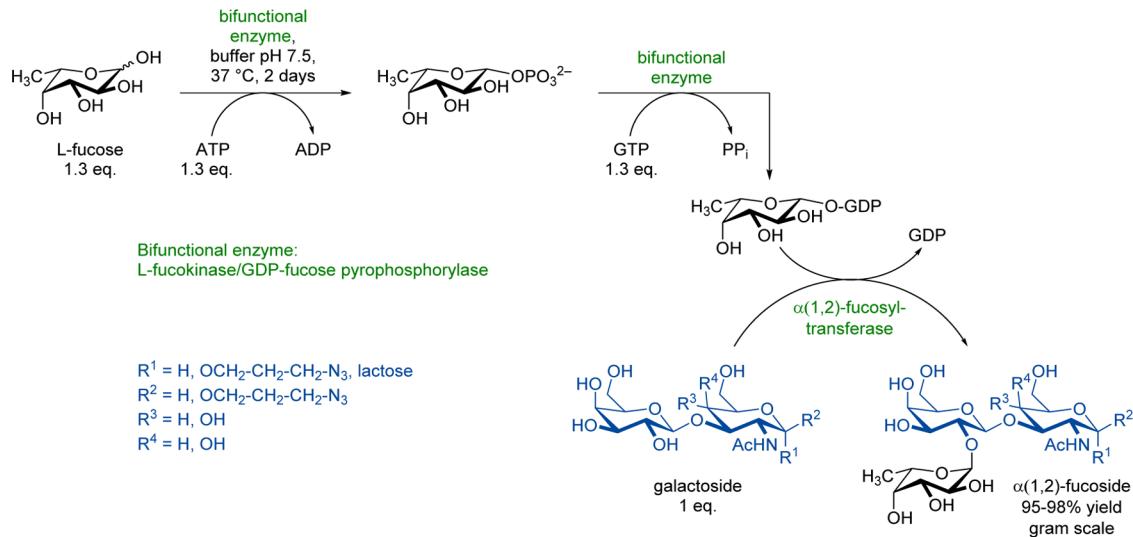


bisphosphate (Scheme 56).<sup>272</sup> In this system, the DHAP formed first in the aldol cleavage reaction is reduced by L-glycerol-3-phosphate dehydrogenase from rabbit muscle, while the NADH cofactor required by this enzyme is regenerated using formate dehydrogenase from *Candida boidinii*. The removal of DHAP from the aldol equilibrium allows complete conversion of D-fructose-1,6-bisphosphate into the two C<sub>3</sub> fragments.

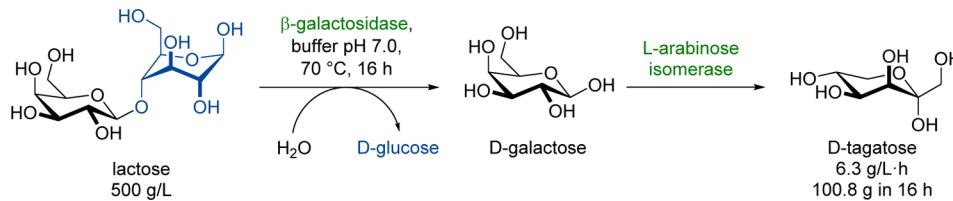
D-Fructose-1,6-bisphosphate aldolase was applied in the reverse direction, i.e., for aldol coupling of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate, in a three-enzyme system that also comprised triosephosphate isomerase for the conversion of D-glyceraldehyde-3-phosphate into DHAP and D-fructose-1,6-bisphosphatase for the irreversible dephosphorylation of D-fructose-1,6-bisphosphate at C1. This cascade was used as a model system for studying substrate-channeling effects resulting from protein self-assembly via dockerin–scaffoldin interactions.<sup>273,274</sup> Follow-up studies dealt with the immobilization of the resulting “synthetic metabolon” on regenerated amorphous cellulose<sup>275</sup> or on cellulose-containing magnetic nanoparticles,<sup>276</sup> the effect of aldolase activity on the overall system,<sup>277</sup> and with the mutual stabilization of the free enzymes—in this study completed by a fourth enzyme, phosphoglucose isomerase—at high protein concentrations.<sup>278</sup>

The polysaccharide amylose has been synthesized in a two-step simultaneous cascade starting from sucrose (Scheme 57).<sup>279</sup> For this purpose, a sucrose phosphorylase from the thermophilic bacterium *Thermoanaerobacterium thermosaccharolyticum* was combined with an α-glucan phosphorylase from potato. In the first step of the cascade, glucose-1-phosphate is produced as an intermediate which is then utilized in the next step as a glycosylating agent in combination with maltodextrin, which in turn acts as a primer for the synthesis of the amylose chain. Under optimized conditions, 0.346 g of amylose was produced per gram of sucrose. Although fructose is described as an inhibitor of the first enzyme, its removal via an orthogonal cascade employing glucose isomerase and glucose oxidase (together with a catalase) did not lead to significant improvements. The number of glucose units in the obtained amylose was controlled from 33 to 262 by adjusting the concentration of the added maltodextrin as well as the reaction time.

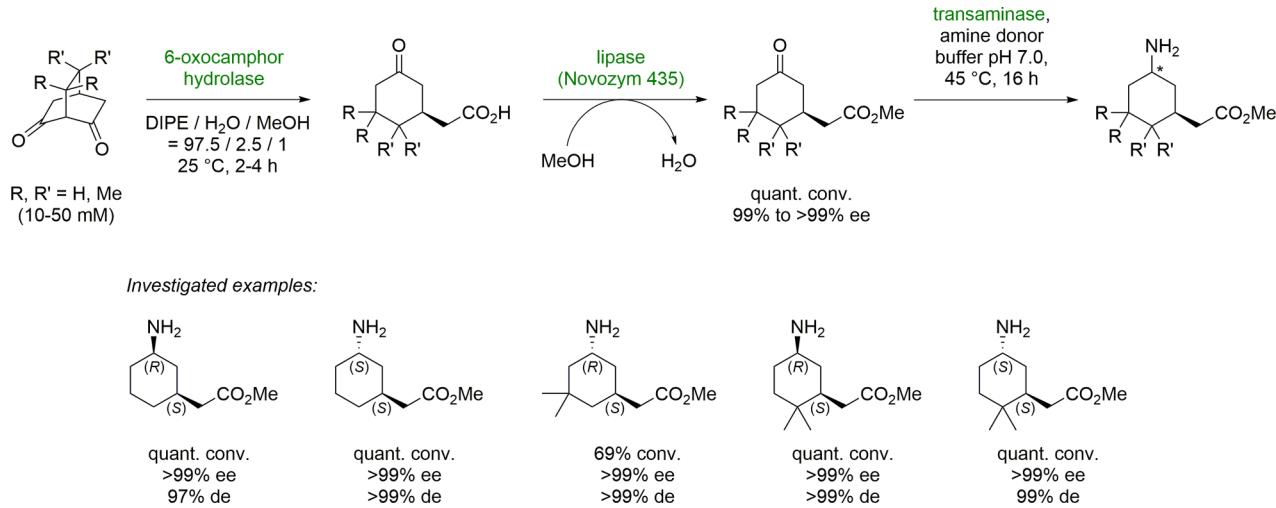
To circumvent energy intensive substrate activation for the synthesis of guanosine 5'-diphospho-D-mannose (GDP-Man), the following cascade was designed using sucrose for activation (Scheme 58).<sup>280</sup> The α-mannose-1-P building block was obtained from mannose via an orthogonal activation cascade, thereby first substituting fructose in sucrose by phosphate and

Scheme 59. Bifunctional Enzyme in Combination with a Transferase for the Synthesis of  $\alpha$ 1-2-Fucosides

Scheme 60. Hydrolysis of Lactose Followed by Isomerization To Give D-Tagatose



Scheme 61. Asymmetric Synthesis of Amino Esters from Oxocamphor Derivatives via a Cascade (Retro-Claisen Condensation and Transesterification) and Thereafter Transamination

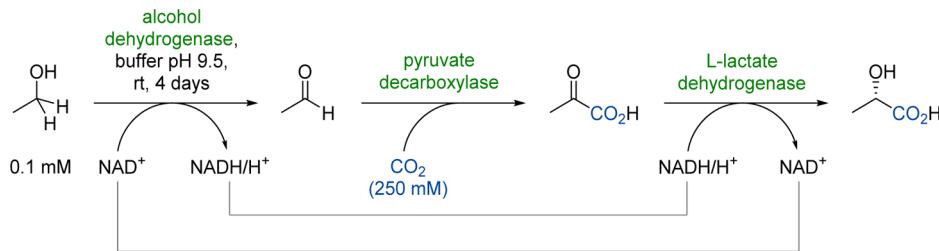
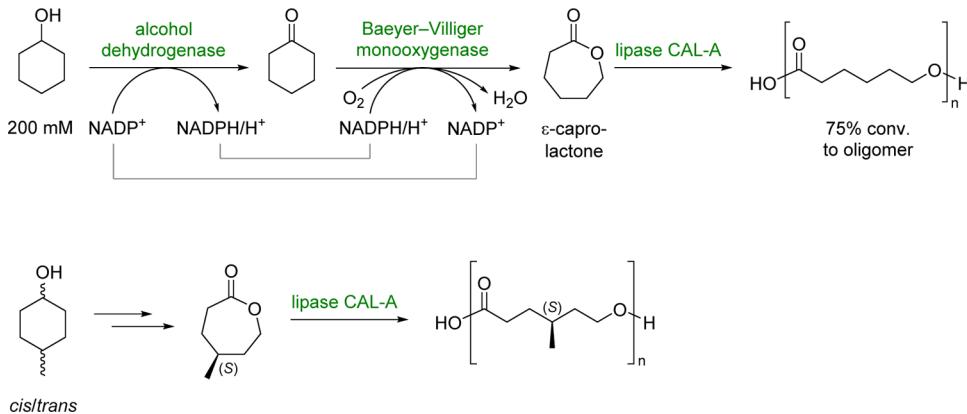


then transferring the phosphate group to mannose. Finally, the phosphate group was substituted by GDP, whereby pyrophosphate was liberated. To shift the equilibrium, pyrophosphatase was hydrolyzed by a pyrophosphatase. Under optimized conditions, 100 mM (67 g/L) GDP-Man was prepared from 140 mM sucrose and phosphate using 400 mM Man as the phosphoryl acceptor. The product was recovered by anion-exchange and size-exclusion chromatography in >95% purity in about 50% yield (100 mg).

Although the next cascade has three reaction steps, two steps are catalyzed by the same protein possessing two different functionalities. The bifunctional L-fucokinase/GDP-fucose py-

rophosphorylase from *Bacteroides fragilis* generates fucose-GDP from fucose, stoichiometric amounts of ATP, and guanine 5'-triphosphate (GTP) to provide the donor substrate for the third step (Scheme 59).<sup>281</sup> In this step,  $\alpha$ 1-2-fucosyltransferase catalyzes the addition of fucose-GDP to galactosides to form  $\alpha$ 1-2-fucosylated galactosides (fucosides), which are important components of human blood group H antigens and human milk oligosaccharides. The generated GDP-fucose can then be transferred to various disaccharides to form fucosylated derivatives.  $\beta$ 1-3-Linked galactosides were excellent acceptors for this reaction, leading to 95–98% yield under optimized conditions. Furthermore, human milk tetrasaccharide lacto-N-

Scheme 62. Biocatalytic Synthesis of L-Lactic Acid from Carbon Dioxide and Ethanol

Scheme 63. Lactone Formation from Cyclohexanone and Oligomerization in Buffer as Well as Transformation of 4-Methylcyclohexanol into Oligo-(S)-4-methyl- $\epsilon$ -caprolactone

tetraose was proven to be an excellent substrate and could be fucosylated on a 1.15 g scale showing 95% yield.

D-Tagatose, an industrially valuable carbohydrate, was produced in *E. coli* cells coexpressing a hyperthermophilic  $\beta$ -galactosidase from *Thermus thermophilus* and a thermophilic L-arabinose isomerase from *Lactobacillus fermentum* using a suitable polycistronic plasmid encoding the two target genes (Scheme 60).<sup>282</sup> Optimization of the reaction parameters allowed the production of 6.3 g/L h d-tagatose from 500 g/L lactose within 16 h.

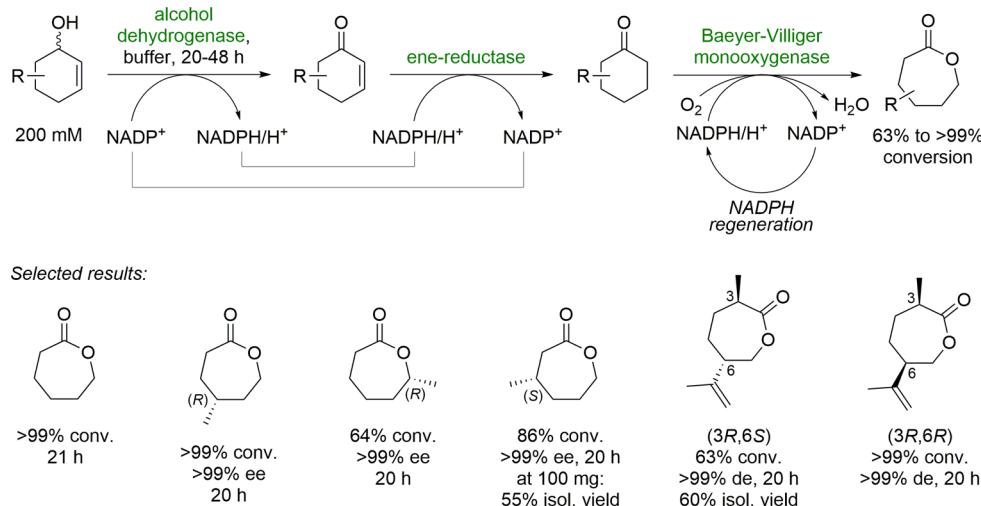
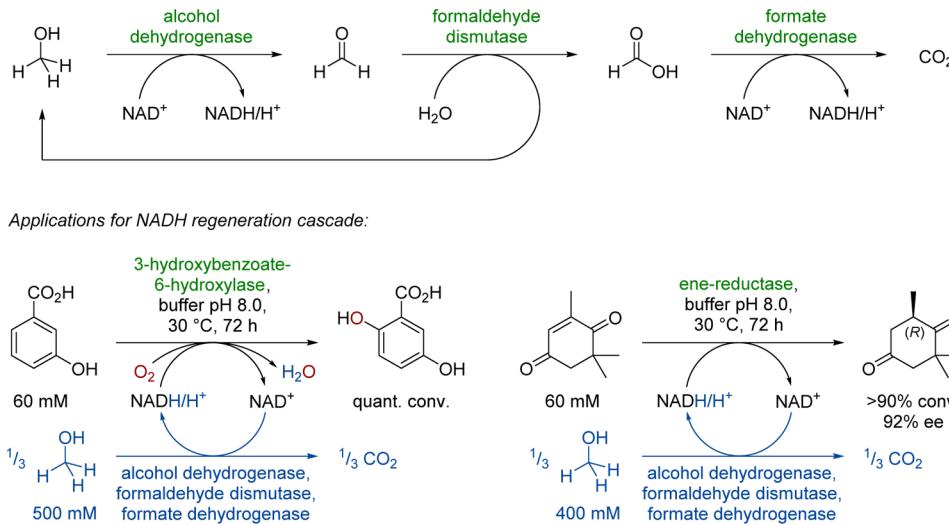
A C–C hydrolase and a lipase have been used in a cascade including a retro-Claisen/esterification process in organic solvent (Scheme 61):<sup>283</sup> 6-Oxocamphor hydrolase (OCH) from *Rhodococcus* sp. NCIMB 9784 cleaved prochiral bicyclic diketones to chiral monocyclic keto acids, which were immediately converted into their methyl esters by immobilized lipase B from *C. antarctica* (Novozym 435). Despite the presence of significant amounts of water (reaction medium diisopropyl ether/water/methanol = 97.5/2.5/1), competing ester hydrolysis was not an issue and complete conversion into the desired methyl esters was observed. The crude products were then subjected to reductive amination catalyzed by various transaminases—either in organic solvent or in buffer—to give the (1*S*,3*S*)- or (1*S*,3*R*)-diastereomer of the corresponding amino esters, depending on the stereopreference of the transaminase used. In all cases, the stereoisomeric purity of the products was excellent (ee > 99%, de = from 97% to >99%).

Cascades involving C–C bond formation using an aldolase and a hydrolytic enzyme for phosphate hydrolysis have been used to access nitrocyclitols. These cascades have been described using these two enzymes in the linear sequence<sup>266,284–286</sup> or using a third enzyme for the in situ synthesis of the phosphorylated substrate for the aldolase.<sup>287</sup> Consequently,

these cascades are described in the section involving three enzymes in the linear sequence.

**3.1.3. Three Catalysts in the Linear Sequence.** The formation of L-lactic acid from ethanol and CO<sub>2</sub> via a biocatalytic hydrogen-borrowing three-enzyme cascade has recently been demonstrated.<sup>288</sup> The reaction system comprises three commercial enzymes (source organisms not specified), an alcohol dehydrogenase, a pyruvate decarboxylase, and a L-lactate dehydrogenase, and establishes internal NADH cycling between the two dehydrogenases (Scheme 62). From a conceptual point of view, the overall transformation is highly interesting since CO<sub>2</sub> is captured and linked to ethanol. In practice, a huge excess of carbonate and continuous addition of ethanol had to be used to drive the transformation into the desired direction, and product yields (41%) as well as NADH turnover numbers (<9) were low.

To circumvent inhibition in the cascade transforming cyclohexanol to  $\epsilon$ -caprolactone (Scheme 20), the  $\epsilon$ -caprolactone formed was removed by a follow-up reaction catalyzed by a third enzyme. For this purpose,  $\epsilon$ -caprolactone was oligomerized in situ via ring opening catalyzed by lipase A from *C. antarctica* (CAL-A) (Scheme 63),<sup>172</sup> which avoided product inhibition. The produced oligo- $\epsilon$ -caprolactone was obtained with an average molecular weight of 375 g/mol. Interestingly, no hydrolysis of  $\epsilon$ -caprolactone to the open chain hydroxycarboxylic acid was observed. For further optimization, a stabilized variant of cyclohexanone monooxygenase (CHMO variant C376L/M400I)<sup>289</sup> was employed as well as two individual *E. coli* whole-cell preparations of the ADH and the monooxygenase. Moreover, despite the redox-neutral nature of the process, acetone and D-glucose (1 equiv each) were added to facilitate cofactor regeneration. These adjustments enabled the complete conversion of 200 mM cyclohexanol, affording a mixture of 75% oligo- $\epsilon$ -caprolactone and 25% of monomeric  $\epsilon$ -caprolactone within 48 h. The transformation of prochiral 4-methylcyclohex-

Scheme 64. Transformation of Cyclohexenols to  $\epsilon$ -Caprolactone DerivativesScheme 65. Three Redox Steps Transforming Methanol to CO<sub>2</sub> for Regeneration of 3 Equiv of NADH

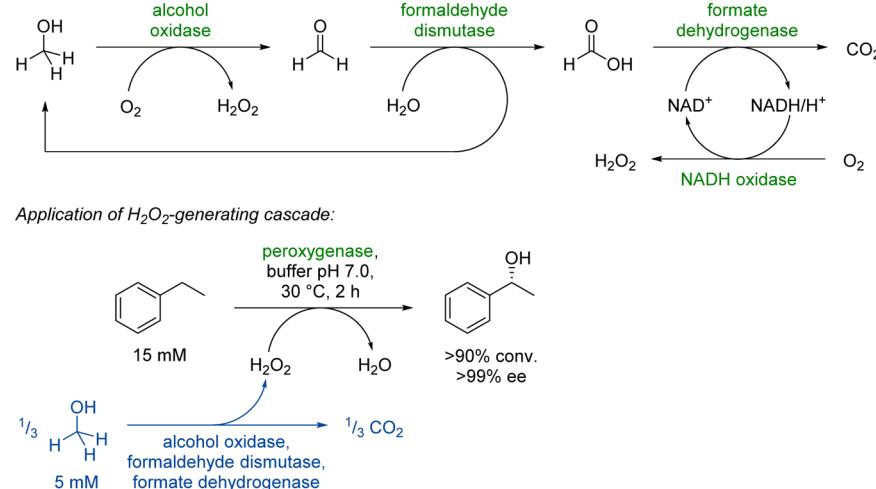
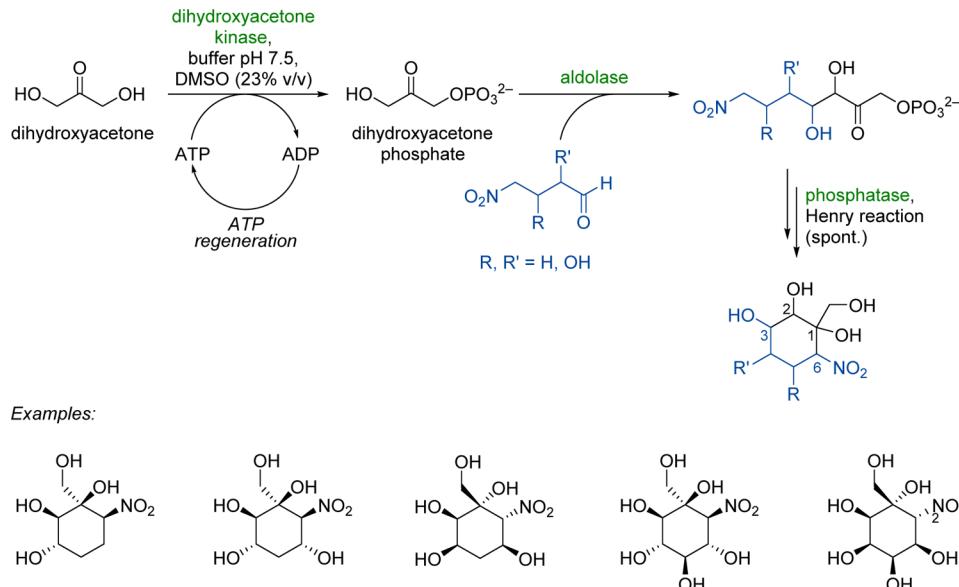
anol (employed as a mixture of cis and trans isomers) gave access to a chiral lactone, (*S*)-4-methyl- $\epsilon$ -caprolactone, which provided after oligomerization a chiral polymer.<sup>290</sup>

A related combination of ene-reductases and Baeyer–Villiger monooxygenases and an alcohol dehydrogenase-catalyzed step allowed the transformation of cyclohexenol derivatives to  $\epsilon$ -caprolactone derivatives (Scheme 64).<sup>291,292</sup> All required enzymes were coexpressed in a single *E. coli* host. Suitable enzyme candidates for each step of the cascade were identified whereby ADH from *L. kefir*, ene-reductase XenB from *Pseudomonas* sp., and cyclohexanone monooxygenase from *Acinetobacter* sp. gave the best results for most of the tested substrates. Alternatively, old yellow enzyme 1 (OYE1) from *Saccharomyces carlsbergensis* was used as ene-reductase in selected cases. Preparative biotransformations (100 mg) afforded the corresponding products with isolated yields between 55% and 60%.

In a subsequent study, three ene-reductases from *P. putida* were used in this cascade but introduced as isolated enzymes (purified enzymes and crude cell-free extracts).<sup>293</sup> Cyclohexenol, cyclohexanone, and cyclopentenone were investigated as substrates, leading to  $\delta$ -valerolactone or  $\epsilon$ -caprolactone as

product. At a substrate concentration of 3 mM, conversions ranging from 19% to 99% were obtained within 1 h.

To gain as much redox equivalents as possible out of a single molecule for regeneration of cofactors, the three-step oxidation of methanol to carbon dioxide was realized by the combined use of alcohol dehydrogenase from yeast, formate dehydrogenase from *C. boidinii*, and formaldehyde dimutase from *P. putida* (Scheme 65).<sup>294</sup> The latter enzyme catalyzes the (redox-neutral) dimutation of formaldehyde into methanol and formic acid and hence provides a link between the ADH- and FDH-catalyzed steps. The overall cofactor recycling cascade, which produces three molecules of NADH from NAD<sup>+</sup> for each molecule of methanol that is oxidized, was coupled on one hand to an oxyfunctionalization catalyzed by a monooxygenase and on the other hand to a C=C reduction catalyzed by an ene-reductase. In both cases, significantly higher amounts of methanol than the theoretically expected one-third equivalent were required to attain (near-)complete conversion: 500 mM MeOH was used for the transformation of 60 mM substrate. This limitation was attributed to the exceedingly high *K<sub>m</sub>* of yeast ADH for methanol (>300 mM).

**Scheme 66.** Three Redox Steps Transforming Methanol to CO<sub>2</sub> for Generation of 3 Equiv of Hydrogen Peroxide**Scheme 67.** In Situ Generation of Dihydroxyacetone Phosphate (DHAP) via a Kinase Followed by Aldolase-Catalyzed C–C Coupling and Phosphatase Hydrolysis

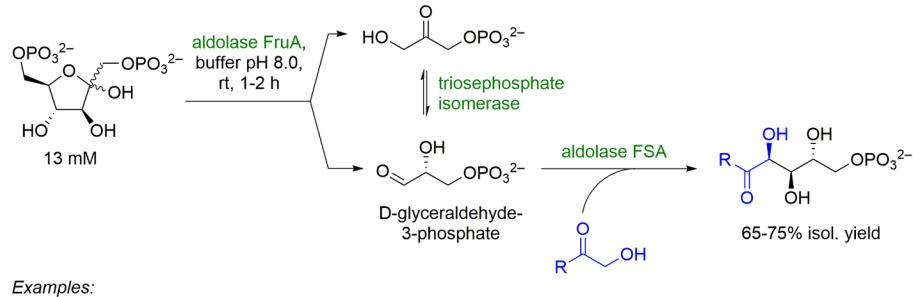
In a related study, three redox equivalents of the enzymatic oxidation of methanol to CO<sub>2</sub> have been exploited for the formation of 3 equiv of hydrogen peroxide, which in turn was used in situ as oxidant in a peroxygenase-catalyzed hydroxylation (Scheme 66).<sup>295</sup> Formaldehyde dismutase takes a central role also in this system as it links the first oxidation step—catalyzed by an alcohol oxidase from *Pichia pastoris*—with the oxidation of formate to CO<sub>2</sub>, which was realized by a two-enzyme combination of formate dehydrogenase and a flavin-dependent monooxygenase employed as NADH oxidase. In contrast to the methanol-promoted NADH-regeneration system discussed above, the H<sub>2</sub>O<sub>2</sub> production system followed the theoretically expected stoichiometry: When methanol was the limiting reagent, the complete system formed 3 equiv of product, while only two equivalents were formed using the alcohol oxidase and formaldehyde dismutase and only one equivalent using the alcohol oxidase alone.

A dihydroxyacetone phosphate (DHAP)-dependent aldolase catalyzing C–C bond formation and a phytase or phosphatase for phosphate hydrolysis were coupled for the synthesis of

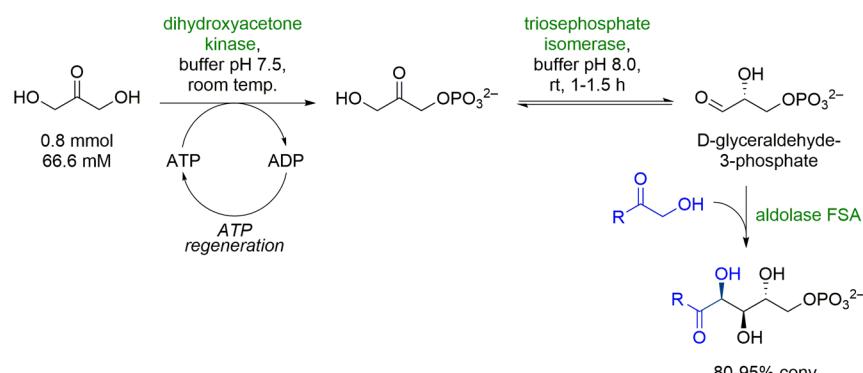
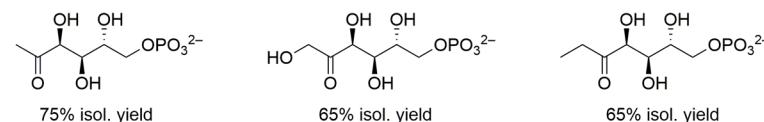
nitrocyclitols (Scheme 67), whereby the final ring closure occurs spontaneously.<sup>284–286</sup> In a similar fashion, D-fructose-6-phosphate aldolase from *E. coli* (FSA) accepting nonphosphorylated aldol donors such as dihydroxyacetone has been employed.<sup>266</sup> Actually, these two-step cascades involving two enzymes were extended by preparing the phosphorylated donor DHAP in situ from dihydroxyacetone employing an ATP-dependent dihydroxyacetone kinase (DHAK) together with acetate kinase for ATP regeneration.<sup>287</sup> Choosing either L-fuculose-1-phosphate aldolase (FucA) or L-rhamnulose-1-phosphate aldolase (RhuA) for the enzymatic carboligation step, nitrocyclitols with different absolute configurations at the C2- and C3-carbons were obtained. While FucA provided the expected (2R,3S)-configuration in most cases, RhuA was much less reliable for the expected (2R,3R)-configuration, hence leading to product mixtures. The Henry reaction gave reliably a trans relative configuration for the hydroxymethyl moiety and the nitro group.

The in situ formation of another common aldolase substrate, glyceraldehyde-3-phosphate, and its further conversion by D-fructose-6-phosphate aldolase from *E. coli* (FSA) has been

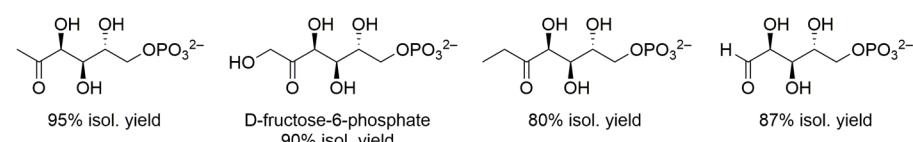
Scheme 68. In Situ formation of D-Glyceraldehyde-3-phosphate as Substrate for Aldol Reactions



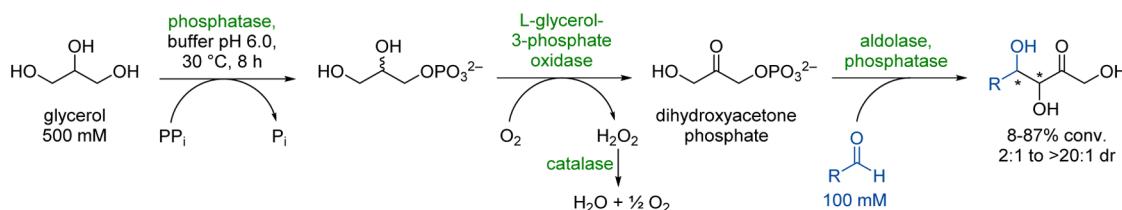
Examples:



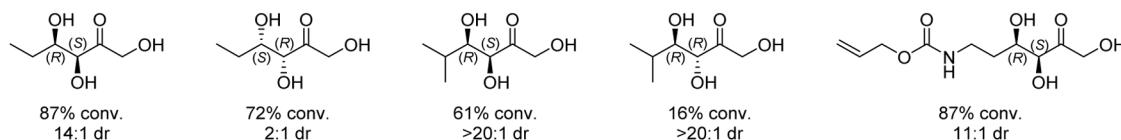
Examples:



Scheme 69. Four-Step, Three-Enzyme Cascade in the Linear Sequence Incorporating in Situ Formation of Dihydroxyacetone Phosphate (DHAP) from Glycerol via Phosphorylation and Oxidation and Followed by Aldol Coupling and Phosphate Hydrolysis



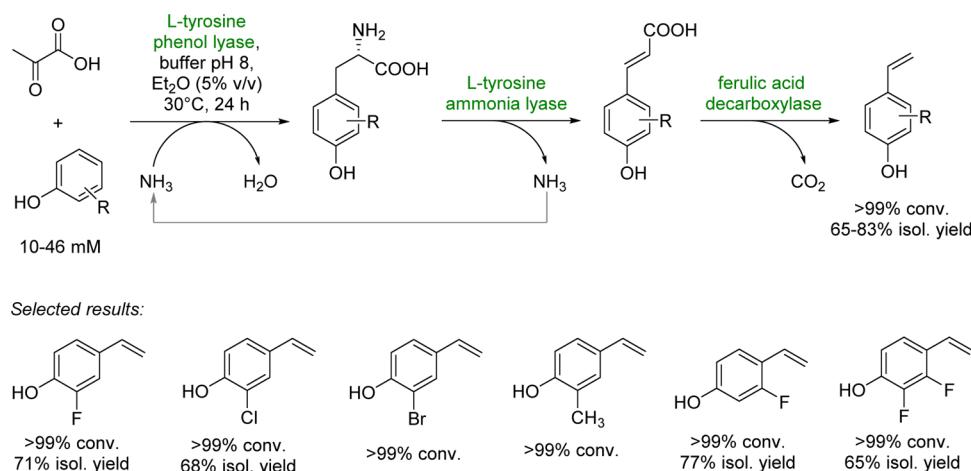
Selected examples:



reported in various studies, whereby some focus on the D-enantiomer and others on its L-counterpart (for L-isomer see sequential cascades, Scheme 86). In the first case, D-fructose-1,6-bisphosphate was cleaved by D-fructose-1,6-bisphosphate aldolase, and the resulting 1:1 mixture of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate was constantly equilibrated by triosephosphate isomerase (Scheme 68, top).<sup>296,297</sup> Both

reactions are well known from the glycolysis pathway. D-Glyceraldehyde-3-phosphate thus formed was reacted with dihydroxyacetone, hydroxyacetone, or 1-hydroxybutan-2-one as aldol donor to afford D-fructose-6-phosphate, 1-deoxy-D-fructose-6-phosphate, and 1,2-dideoxy-D-arabinose-7-phosphate, respectively, in 65–75% isolated yield.<sup>297</sup> Alternatively, D-glyceraldehyde-3-phosphate was obtained from

**Scheme 70.** Coupling C–C Bond formation, NH<sub>3</sub> Elimination, and Decarboxylation To Prepare Substituted Hydroxy Styrenes from Phenols and Pyruvate



dihydroxyacetone via phosphorylation catalyzed by dihydroxyacetone kinase and isomerization catalyzed by triosephosphate isomerase (Scheme 68, bottom). The isolated yields of the final aldol products were higher in this case when performed in sequential mode, ranging from 80% to 95%.<sup>297</sup> The implementation of the same cascade using coimmobilized enzymes in a simultaneous fashion has also been reported, whereby the enzymes were coembedded in layered double-hydroxide nanoplatelets. This two-dimensional structure was expected to offer a suitable microenvironment favoring a high enzyme loading and increased stability. By testing different matrix compositions and enzyme to nanomaterial ratios, 66.6 mM dihydroxy acetone was successfully converted to D-fructose-6-phosphate within 1 h, giving 90% conversion for the product.<sup>104</sup>

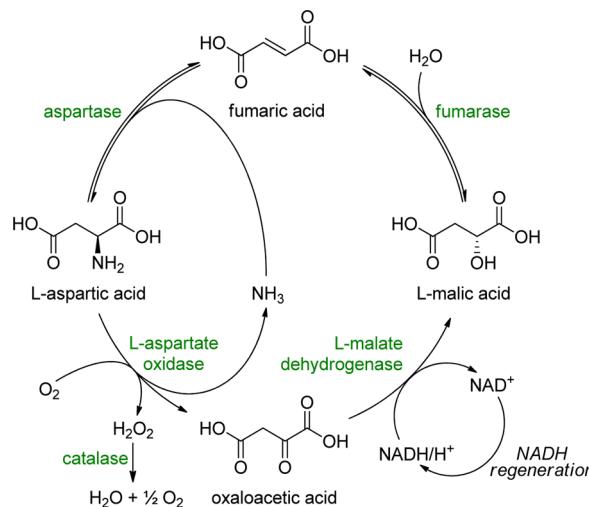
To generate DHAP *in situ* from glycerol, L-glycerol-3-phosphate oxidase (GPO) was introduced upstream and applied in an extension of the reaction system of Scheme 54 (Scheme 69).<sup>298</sup> Phosphorylation of glycerol by PhoN-Sf led to a racemic mixture of glycerol-3-phosphate, out of which only the L-enantiomer was oxidized to DHAP by GPO. It was suggested that the remaining D-glycerol-3-phosphate was constantly racemized via phosphatase-catalyzed phosphate transfer to glycerol. An enzymatic aldol reaction and the dephosphorylation of the aldol product complete the four-step cascade with three enzymes. The extended reaction system was tested with three aliphatic aldehydes and four DHAP-dependent aldolases and afforded aldol products in up to 87% conversion and moderate to excellent diastereomeric purities (dr = from 2:1 to >20:1).

The para vinylation of substituted phenols leading to *p*-hydroxy styrene derivatives just required phenol and pyruvate as reagents (Scheme 70).<sup>299</sup> The transformation was realized by performing a three-step cascade comprising a C–C bond formation step, ammonia elimination, and decarboxylation. The ammonia introduced in the first step was eliminated in the second step, allowing an internal recycling. The enzymes used were a tyrosine phenol lyase from *C. freundii*, a tyrosine ammonia lyase from *Rhodobacter sphaeroides*, and a ferulic acid decarboxylase from *Enterobacter* sp. By optimizing several reaction parameters, such as pH, cosolvent, and ammonium chloride, the transformation of a variety of substituted phenols went to completion with high isolated yields (65–83%).

**3.1.4. Four Catalysts in the Linear Sequence.** An artificial cascade consisting of four reaction steps was designed to

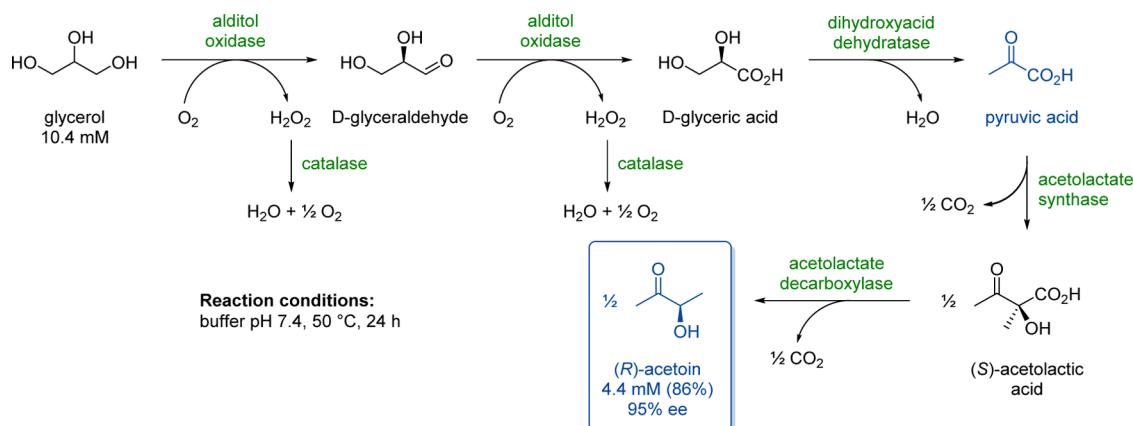
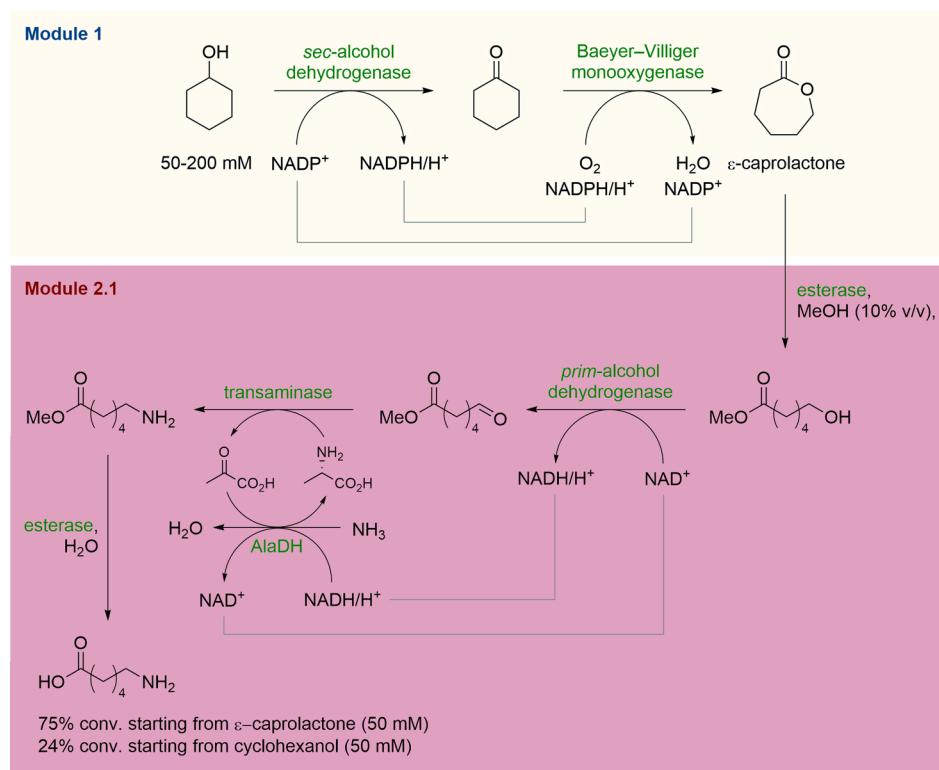
demonstrate the possibility of functional group interconversion, whereby the product of the fourth step is again the substrate for the first step (Scheme 71).<sup>300</sup> This cyclic *in vitro* pathway

**Scheme 71.** Four-Step Cycle Applied to the Interconversion of Functional Groups



comprised four enzymes: an aspartase from *Bacillus* sp., a fumarase from porcine heart, a L-malate dehydrogenase from bovine heart, and a very recently identified L-aspartate oxidase from *Sulfolobus tokodaii*.<sup>196</sup> The combination of these four enzymes allowed the successful establishment of a functional catalytic cycle when a NADH cofactor regeneration system was applied using either 10 mM fumaric acid or 10 mM L-malic acid. By replacing L-specific malate dehydrogenase by its D-selective counterpart, the cascade could be applied to the synthesis of D-malate.

In a cell-free multienzyme cascade, glycerol was transformed to pyruvate with only three enzymatic steps that do not require any cofactor regeneration, Scheme 72.<sup>301</sup> The sequence comprised a double oxidation of glycerol to glyceric acid catalyzed by alditol oxidase from *S. coelicolor*, concomitantly forming hydrogen peroxide as coproduct, which was decomposed into water and molecular oxygen by catalase. Conversion of glyceric acid into pyruvic acid was accomplished using dihydroxyacid dehydratase

**Scheme 72.** Conversion of Glycerol into (R)-Acetoin via a 5-Step 4-Enzyme Cascade**Scheme 73.** Modularized Six-Enzyme In Vitro System for the Synthesis of 6-Aminohexanoic Acid from Cyclohexanol

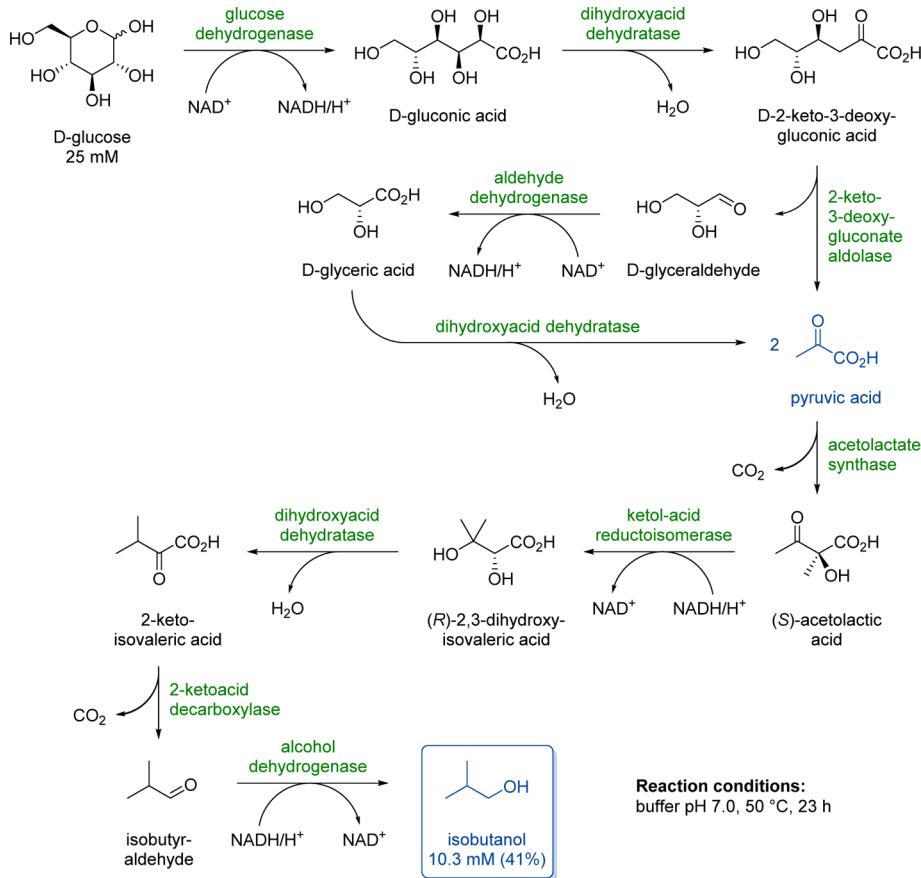
from *Sulfolobus solfataricus*. Starting from 10 mM glycerol, 9.3 mM pyruvate was produced within 24 h and no formation of side products was detected. By extending the cascade with two additional enzymes— $\alpha$ -acetolactate synthase and  $\alpha$ -acetolactate decarboxylase from *B. licheniformis*—the pyruvate thus produced was converted further into (R)-acetoin, formed in 86% analytical yield (4.4 mM) and 95% ee from 10.4 mM of glycerol.

**3.1.5. Six and More Catalysts in the Linear Sequence.** The transformation of cyclohexanol into 6-amino-6-hydroxyhexanoic acid, the building block of nylon-6, comprised steps involving six different enzymes (Scheme 73).<sup>173</sup> The sequence was subdivided into two “modules”: Module 1 comprised the redox self-sufficient (hydrogen-borrowing) two-step oxidation of cyclohexanol to  $\epsilon$ -caprolactone by a sec-alcohol dehydrogenase and a Baeyer–Villiger monooxygenase; this sequence is also described in Scheme 20 and has been intensively investigated.<sup>169,170,289</sup> Module 1 was successfully run on its own at 200 mM substrate

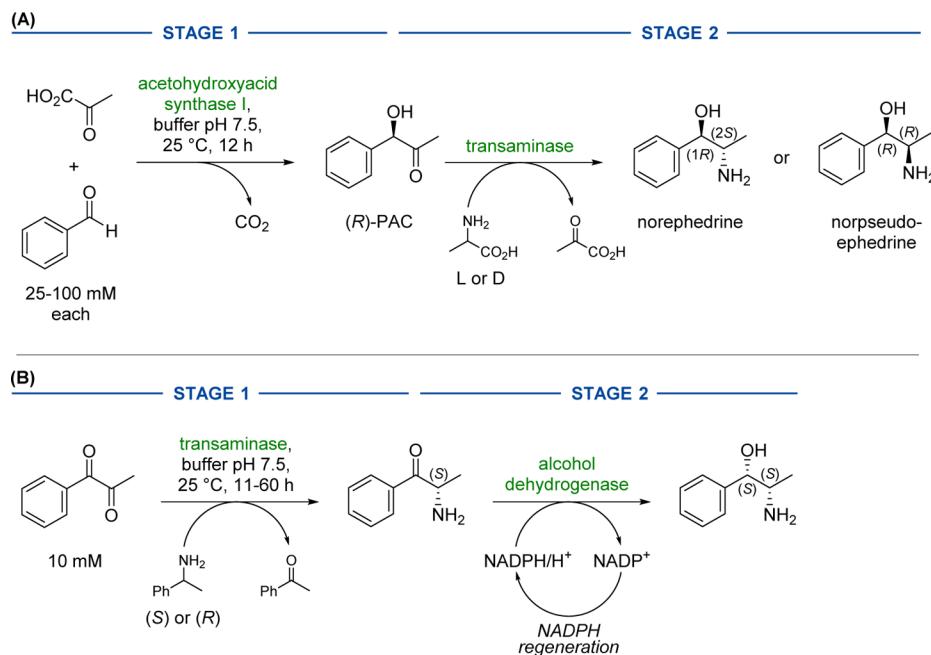
concentration, leading to 96% lactone within 20 h.<sup>173</sup> Module 2.1 involved the opening of the lactone to the corresponding  $\omega$ -hydroxy methyl ester by a horse liver esterase, followed by amination via the well-established sequence alcohol oxidation to the aldehyde and amination (see, e.g., hydrogen-borrowing section 3.1.2.1.). The opening of the lactone to the ester was required, because the corresponding free carboxylic acid was not a substrate for the prim-alcohol dehydrogenases investigated but rather an inhibitor; therefore, this concept termed *in situ* “capping” of the carboxylic acid moiety allowed one to circumvent an inhibiting intermediate. The second module (Module 2.1), run on its own, allowed one to transform  $\epsilon$ -caprolactone (50 mM) to the final product in 75% conversion. Running both modules simultaneously resulted in the formation of 24% of the final product from 50 mM cyclohexanol.

In recent years, several complex multienzyme cascades were developed for the conversion of carbohydrates into a variety of

Scheme 74. Conversion of D-Glucose into Isobutanol via a 10-Step, 8-Enzyme Cascade

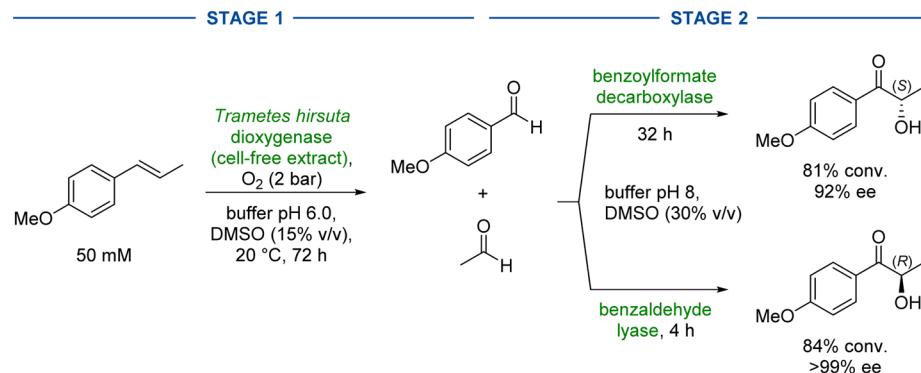


Scheme 75. Synthesis of Norephedrine and Norpseudoephedrine Isomers via (A) C–C Coupling and Reductive Amination or (B) Reductive Amination and Carbonyl Reduction

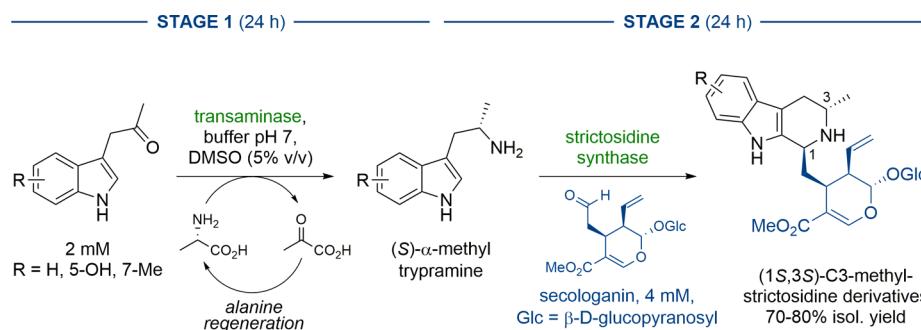


products. While many of these systems break down carbohydrates into building blocks that also occur in natural metabolism, they usually reach these intermediates via non-natural pathways. For instance, ethanol and isobutanol have been produced from D-

glucose via the common intermediate pyruvic acid, which was obtained from glucose in only 5 enzymatic reactions (Scheme 74),<sup>302</sup> in contrast to the 10 steps that are required in natural glycolysis. Subsequent conversion of pyruvic acid into ethanol

Scheme 76. Oxidative C=C Cleavage of *trans*-Anethole and Subsequent Carboligation To Produce (*R*)- and (*S*)-Hydroxyketones

Scheme 77. Amination Coupled with C–C Bond Formation in Simultaneous or Sequential Mode, Whereby the Sequential Mode Performed Better



was achieved in two enzymatic reactions identical to natural ethanol fermentation, while formation of isobutanol required a non-natural sequence of five additional steps from pyruvic acid (Scheme 74). A key enzyme in the overall system is dihydroxyacid dehydratase (DHAD) from *S. solfataricus*, which catalyzes two reactions in the streamlined glycolysis sequence, namely, the conversions of gluconic acid into 2-keto-3-deoxygluconic acid and of glyceric acid into pyruvic acid. The same enzyme also performs the dehydration of 2,3-dihydroxyvaleric acid to 2-ketoisovaleric acid in the isobutanol-forming pathway. Starting from 25 mM D-glucose, the target alcohols were formed in concentrations of 28.7 mM (ethanol; theoretical yield, two molecules per molecule of glucose) and 10.3 mM (isobutanol) within 19–23 h.

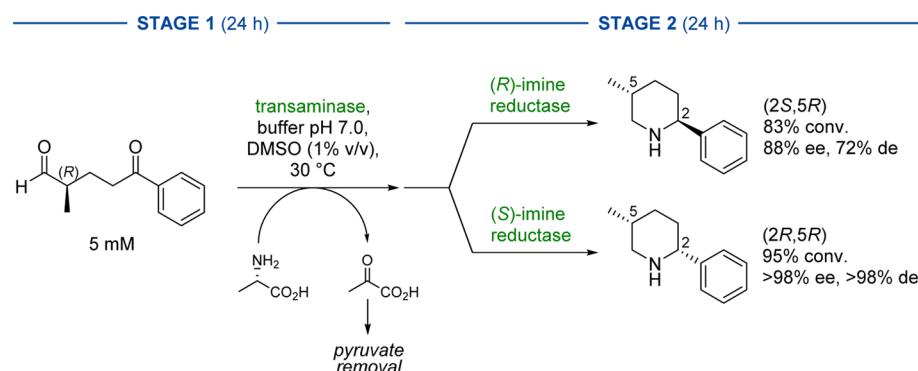
### 3.2. Sequential Mode

**3.2.1. Two Catalysts in the Linear Sequence.** A biocatalytic carbon–carbon bond formation and transamination were performed in sequential mode to achieve the asymmetric synthesis of norephedrine and norpseudoephedrine from pyruvate and benzaldehyde (Scheme 75A).<sup>303</sup> For the C–C coupling step, ThDP-dependent acetohydroxyacid synthase I (AHAS-I) from *E. coli* was used, which produced intermediate (*R*)-phenyl acetyl carbinol (PAC) in >98% optical purity. This intermediate was reductively aminated by the (*S*)-selective transaminase from *C. violaceum* to afford (1*R*,2*S*)-norephedrine or by the (*R*)-selective transaminase from *Aspergillus terreus* to give (1*R*,2*R*)-norpseudoephedrine, both compounds being obtained as single stereoisomers. L-Alanine was chosen as amino donor, affording pyruvate as coproduct of the transamination step. As a consequence, the second reaction in the cascade replenishes the substrate of the first reaction, leading to the term “recycling cascade” for processes of this kind.<sup>304</sup>

However, a simultaneous coupling of both biotransformation steps turned out to be problematic when the transaminase from *C. violaceum* was used, since this enzyme converted benzaldehyde more efficiently than (*R*)-PAC. The cascade process hence accumulated benzylamine as an undesired side product while forming norephedrine in only 2% conversion. A sequential one-pot setup (i.e., adding the transaminase after 1 h, once benzaldehyde had been completely consumed) worked much better, giving norephedrine in 78% conversion.<sup>303</sup> The reaction using *A. terreus* transaminase produced 96% of norpseudoephedrine. In both cases, subsequent addition of another equivalent of benzaldehyde and fresh AHAS-I led to further product formation, demonstrating that pyruvate recycling was indeed taking place. In further work, the authors not only increased the space–time–yield of their process by raising the initial benzaldehyde concentration from 10 to 100 mM but also sought to access the opposite enantiomers of both products by employing an (*S*)-selective variant of pyruvate decarboxylase from *Acetobacter pasteurianus* (ApPDC-E469G) for the C–C coupling step. However, the stereoselectivity of ApPDC-E469G proved insufficient under the investigated reaction conditions, as the diastereomeric excess reached only 70%.<sup>305</sup> One of these stereoisomers was finally obtained in high optical purity via an alternative sequential one-pot process with two sequences in which 1-phenyl-1,2-propanedione was subjected to reductive amination by suitable transaminases and subsequent carbonyl reduction catalyzed by alcohol dehydrogenase from *L. brevis* (Scheme 75B). This alternative cascade required pH-induced deactivation of the transaminase after completion of the first step to minimize side-product formation and afforded the (1*S*,2*S*)-amino alcohol in 80% conversion, >99% ee, and >98% de.<sup>305</sup>

Benzaldehyde derivatives required for the C–C bond-forming benzaldehyde lyase (BAL) or benzoylformate decarboxylase

**Scheme 78.** Two Sequential Enzymatic Reduction Steps (reductive amination, imine reduction) To Access Substituted Piperidines<sup>a</sup>



<sup>a</sup>For intermediates please refer to Scheme 108.

(BFD) were obtained by cleavage of the C=C double bond of *trans*-anethole by cell-free extract of dioxygenase-containing white rot fungus *Trametes hirsuta* at the expense of molecular oxygen (Scheme 76).<sup>306</sup> Subsequent transformation by either BAL or BFD generated the (*R*)- or (*S*)-enantiomer of the hydroxyketone. This cascade had to be performed in a sequential mode due to inhibition of the carboligation step by *trans*-anethole and divergent pH optima of the two steps. Furthermore, additional 60 mM acetaldehyde in the BAL-catalyzed reaction and 500 mM in the BFD-catalyzed reaction had to be added due to the enzyme high  $K_M$  value. Starting from *trans*-anethole, an overall conversion of 81% to the (*S*)-enantiomer was observed, showing a good ee of 92%, while the (*R*)-enantiomer was obtained with 84% conversion and a perfect ee of >99%.

Asymmetric reductive amination of a ketone followed by C–C bond formation was realized in the following cascade. Strictosidine synthase (STR), a key C–C-bond-forming enzyme of the monoterpenoid indole alkaloid biosynthetic pathway, was combined with a transaminase, whereby each biocatalyst controlled the outcome of one stereogenic center (Scheme 77).<sup>307</sup> Transaminases from *Arthrobacter* sp. and *Silicibacter pomeroyi* were used to generate either the (*R*)- or the (*S*)-enantiomer of a series of  $\alpha$ -methyltryptamine derivatives by reductive amination of the substrate ketones. The amines were then subjected to enzymatic Pictet–Spengler reaction catalyzed by STR from *Ophiorrhiza pumila* using the natural aldehyde substrate secologanin as second reaction partner. Thus, (1*S*,3*R*)- and (1*S*,3*S*)-isomers of C3-methyl-substituted strictosidine derivatives were obtained in stereoisomerically pure form. The reaction sequence was performed both in a simultaneous fashion—with all enzymes and starting materials present from the outset—and in sequential mode, whereby the transamination was run to completion first and the transaminase whole-cell biocatalyst was removed before addition of the strictosidine synthase. The sequential mode led to improved conversions compared to the simultaneous mode (from 56% to >99% vs 31% to 97%), which was attributed to a potential side reaction of secologanin with the transaminase. The optical purity was excellent in both cases, as all products were obtained as single stereoisomers. Isolation and purification of the products required column chromatography and preparative HPLC and led to 70–80% isolated yield.

For the synthesis of disubstituted piperidines and pyrrolidines, a cascade was designed by coupling regioselective monoamination of diketones or keto aldehydes by transaminases with

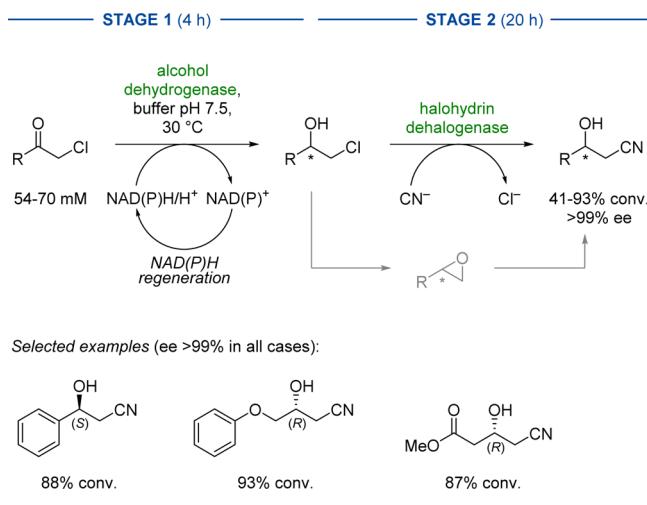
subsequent stereoselective reduction of the generated cyclic imines by imine reductases (Scheme 78).<sup>308</sup> Initial results suggested that the use of imine reductase-containing whole cells in a concurrent cascade inhibited conversion of the starting diketone by the transaminase. Therefore, the procedure was optimized to allow complete consumption of the diketone starting material by the transaminase first, before addition of the imine reductase whole-cell biocatalyst after 24 h in the same pot. A stereogenic center in the  $\alpha$  position of the aldehyde was largely retained under the reaction conditions and had no influence on the stereopreference of the imine reductase. In contrast, if the final reaction product was a 2,6- or 2,4-disubstituted piperidine, the trans isomers were preferentially formed irrespective of the inherent stereopreference of the imine reductase.

Several keto aldehydes were prepared *in situ* from corresponding keto acids by biocatalytic reduction employing a carboxylic acid reductase from *Mycobacterium marinum*, thus extending the cascade by one step to a three-enzyme cascade. Since the carboxylic acids were reduced with resting cells, taking advantage of cofactor recycling of the cell metabolism, the three-step cascade is discussed in the *in vivo* section 5.

Attempts to combine zinc-dependent ADH-catalyzed reduction of  $\alpha$ -haloketones and halohydrin dehalogenase-catalyzed epoxide opening with cyanide in a simultaneous fashion led to inhibition and undesired side reactions; therefore, the cascade was performed in sequential fashion (Scheme 79).<sup>224</sup> Inhibition and side product formation were avoided by adding sodium cyanide and halohydrin dehalogenase after a reaction time of 4 h, once reduction of the chloroketone was complete. In this way, both enantiomers of four different hydroxynitriles were obtained in 41–92% conversion and excellent enantiomeric purity (ee > 99%). Interestingly, the same reaction sequence was reported in a simultaneous fashion using a single-cell catalyst coexpressing both alcohol dehydrogenase and halohydrin dehalogenase HheC and—if required—an additional enzyme for cofactor regeneration (Scheme 41).<sup>225</sup> Neither enzyme inhibition by cyanide nor accumulation of cyanoketone was observed in this study.

Although ketone reduction and nitrile hydrolysis have been combined in simultaneous fashion (Scheme 42), sequential cascades were also reported, such as the sequence leading to both enantiomers of 3-aryl-3-hydroxypropionic acids (Scheme 80).<sup>309</sup> In a first step, 3-oxo-3-phenylpropanenitrile derivatives were reduced to corresponding (*R*)- or (*S*)-hydroxynitriles using a carbonyl reductase from *Candida magnoliae* or an alcohol dehydrogenase from *S. cerevisiae* in combination with glucose

**Scheme 79. Sequential Combination of Alcohol Dehydrogenase and Halohydrin Dehalogenase for the Synthesis of  $\beta$ -Hydroxynitriles from  $\alpha$ -Chloroketones**



dehydrogenase for cofactor regeneration. After reduction was complete, the nitrile intermediates were hydrolyzed to the acids by addition of a nitrilase either from *Synechocystis* sp. or *Bradyrhizobium japonicum*. The resulting hydroxy acids were obtained in 90–95% isolated yield and 98–99% ee, which represents an up to 1.4-fold yield improvement over a conventional process in which the intermediate was isolated.

A sequential cascade of ketone reduction and nitrile hydrolysis was also implemented for the synthesis of (1*S*,2*S*)-2-hydroxycyclopentanecarboxylic acid (**Scheme 81**)<sup>229</sup> using a commercial alcohol dehydrogenase and whole cells of *R. rhodochrous* as discussed for **Scheme 41**, where this cascade was performed in a simultaneous fashion; however, in contrast to the products reported there, the preparation of (1*S*,2*S*)-2-hydroxycyclopentanecarboxylic acid required a sequential setup due to incompatible pH optima of the reduction and hydrolysis steps.

In the cascade consisting of three reactions, (i) hydroxylation in allylic position, (ii) alcohol oxidation, and (iii) enantioselective

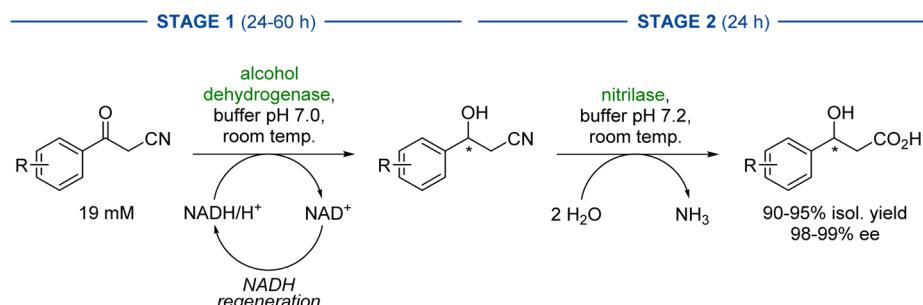
C=C reduction, *E. coli* cells containing the heterologously expressed enzymes were employed (**Scheme 82**).<sup>310</sup> Two catalysts were active in the linear three-step cascade, while one enzyme was responsible for NADPH recycling (glucose dehydrogenase). The two enzymes in the linear sequence performed three reaction steps, since the first enzyme, a P450-BM3 monooxygenase, hydroxylated the substrate in allylic position and performed the oxidation of the alcohol thus obtained to the ketone. Since a chemo- and regioselective oxidation had to be performed, a triple mutant (V78L/A82F/F87A) of the monooxygenase was generated via directed evolution and gave the best results under the investigated conditions. For the stereoselective reduction by an ene-reductase, two enantiocomplementary YqjM mutants were evolved by iterative saturation mutagenesis.

To establish a functional cascade, three different approaches were investigated: (i) sequential use of two different *E. coli* cells, expressing the enzymes separately, (ii) a single *E. coli* host coexpressing all desired proteins, and (iii) cells harboring the YqjM gene inserted into the genome with the P450 remaining on a plasmid.

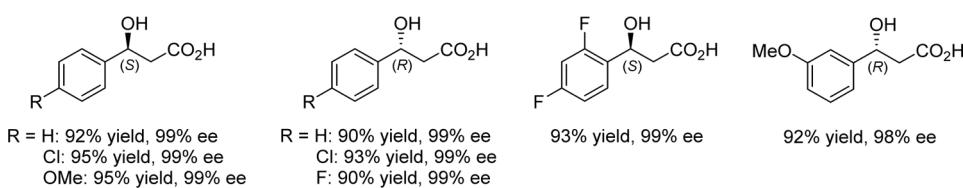
Comparing the three techniques, the first (sequential) option proved to be the most suitable one. Using 7.3 mM starting material, 72% and 75% of the (*R*)- and (*S*)-enantiomers, respectively, were obtained in perfect ee's (99%) and with an isolated yield of 69% in both cases.

The combination of linolenic acid oxidation to a peroxide with subsequent C–C cleavage led to formation of 9-oxononanoic acid (**Scheme 83**).<sup>311</sup> The bienzymatic cascade required a lipoxygenase from *Solanum tuberosum* and a hydroperoxide lyase from *Cucumis melo* or *Cucumis sativus*. When this cascade was performed in a simultaneous mode, conversions to the 9-oxo fatty acid were generally low (ca. 20%). Changing the setup to a sequential fashion dramatically increased the product yield to 72–76% of 9-oxononanoic acid. In a follow-up study the formed 9-oxononanoic acid was successfully further transformed into azelaic acid, making use of an endogenous *E. coli* oxidoreductase.<sup>312</sup> With a substrate loading of 1 mM linoleic acid, 29

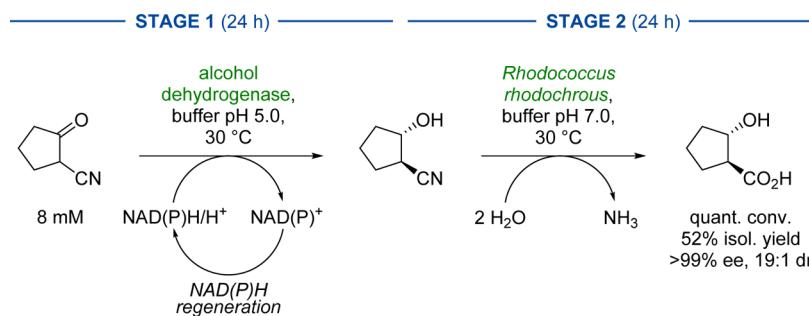
**Scheme 80. Sequential Cascade of Biocatalytic Ketone Reduction and Nitrile Hydrolysis for the Synthesis of 3-Aryl-3-hydroxypropionic Acids**



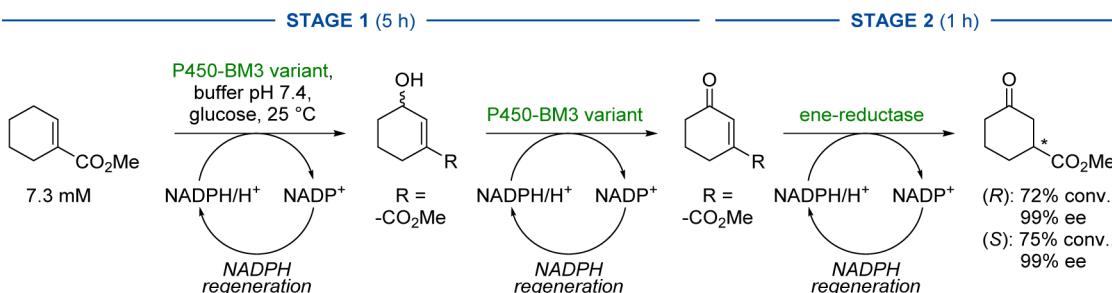
Examples (all yields refer to pure, isolated product):



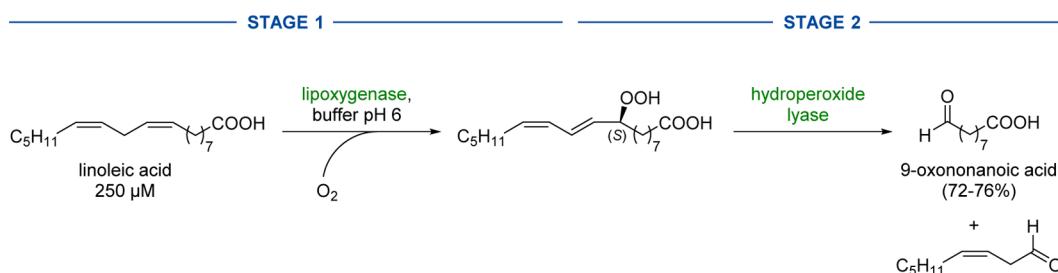
**Scheme 81.** Synthesis of Cyclic 2-Hydroxycarboxylic Acids via Sequential Biocatalytic Reductive Dynamic Kinetic Resolution and Nitrile Hydrolysis



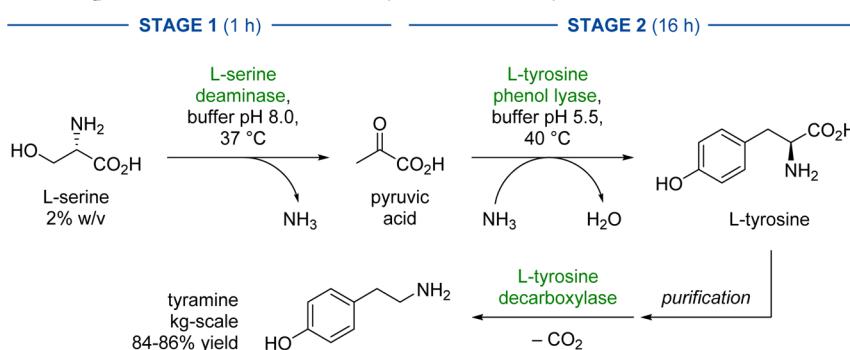
**Scheme 82.** Three-Step Two-Enzyme Cascade Comprising Hydroxylation, Alcohol Oxidation, and C=C Reduction



**Scheme 83.** Sequential Lipoxygenase and Hydroperoxide Lyase-Mediated Cascade for the Synthesis of 9-Oxononanoic Acid



**Scheme 84.** Sequential Two-Step Cascade from Serine to Tyramine via L-Tyrosine

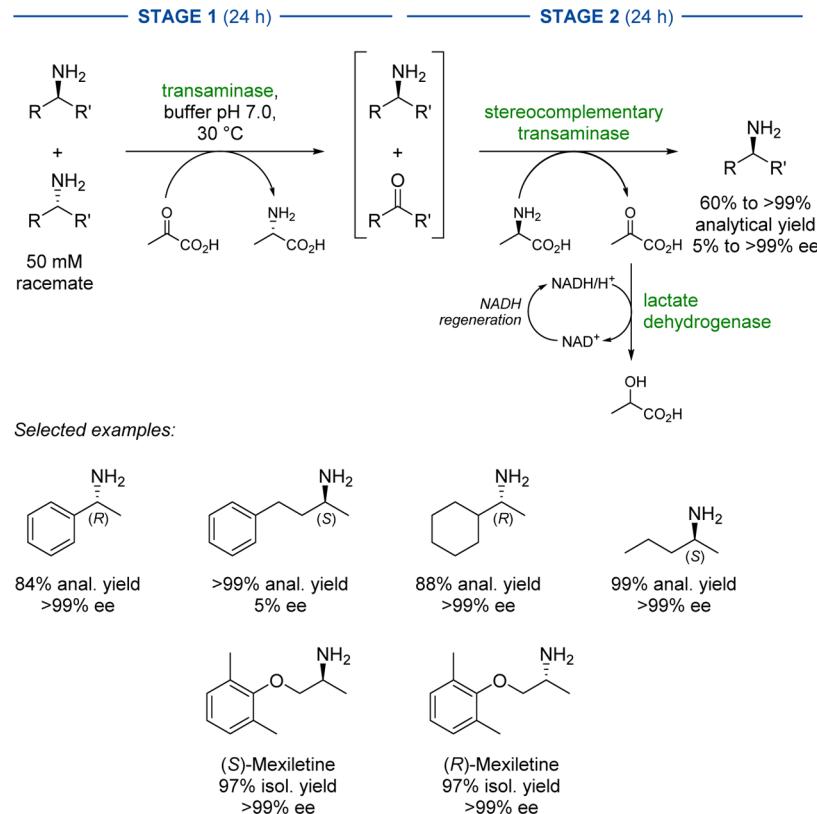


mg/L azaleic acid was obtained which corresponds to an overall yield of 16%.

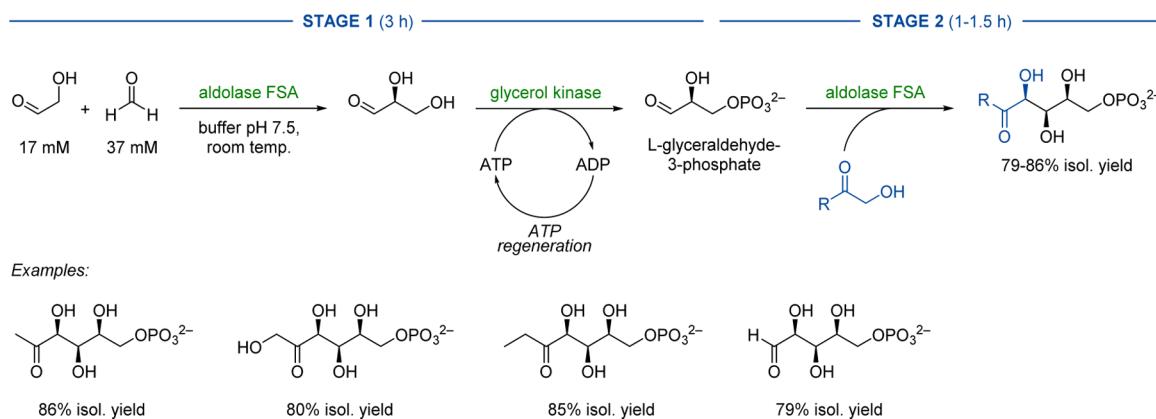
Multienzyme processes enabled the synthesis of tyramine from crude biomass.<sup>313,314</sup> Tyramine was formed by decarboxylation of L-tyrosine by tyrosine decarboxylase from *L. brevis* after a purification step of tyrosine. In the actual cascade, pyruvate was formed in situ from L-serine present in keratin acid hydrolysis wastewater by a serine deaminase from *E. coli* (Scheme 84).<sup>314</sup> Pyruvate was subsequently transformed to L-tyrosine by tyrosine phenol lyase from *Citrobacter koseri* using ammonium and phenol

as cosubstrates. After thorough optimization, the process was successfully performed on a kilogram scale and tyramine was isolated in good yield by crystallization of its hydrochloride salt (84% from crude L-serine).

The deracemization of  $\alpha$ -chiral primary amines was achieved via a sequential sequence of enantioselective deamination and stereoselective amination catalyzed by stereocomplementary transaminases (Scheme 85).<sup>315</sup> To this end, the racemic amine was first subjected to a kinetic resolution catalyzed by one transaminase, ideally resulting in a 1:1 mixture of the desired

Scheme 85. Deracemization of  $\alpha$ -Chiral Primary Amines Using Two Stereocomplementary Transaminases in a Sequential Cascade

Scheme 86. Two-Catalyst, Three-Step Cascade Including Two C–C Bond-Forming Steps and Phosphorylation for in Situ Formation of L-Glyceraldehyde-3-phosphate and Further Transformation to Sugar Phosphates



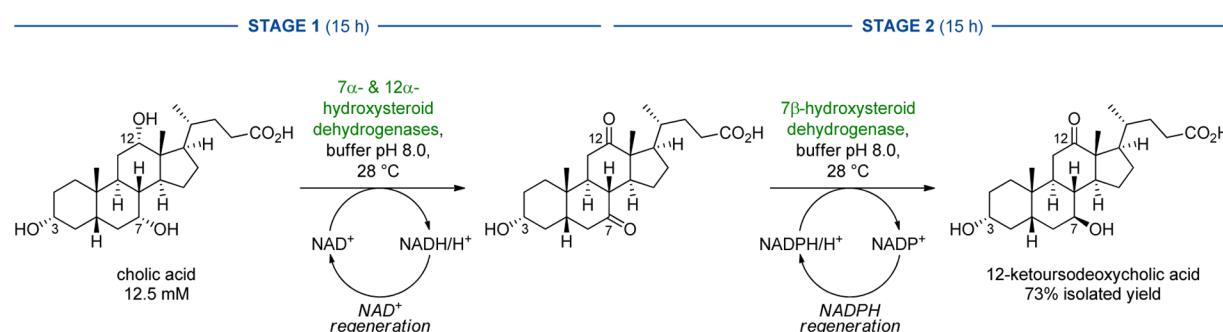
amine enantiomer and the corresponding ketone. The addition of stoichiometric amounts of pyruvate as cosubstrate was sufficient for this transformation, but pyruvate regeneration using an amino acid oxidase could also be envisaged in case an additional driving force for the reaction was desired.<sup>316</sup> In a second step, the intermediate ketone was reductively aminated by the second transaminase, supported by one of the established systems for driving the transamination equilibrium (e.g., alanine as amino donor, LDH and GDH for pyruvate removal). A proof of concept for this process has been established using commercial transaminases ATA-113 and ATA-117 from Codexis. Since cross-reactivity of the two transaminases initially resulted in limited optical purities (ee = 29–88%), the first transaminase had to be deactivated by heat treatment before addition of the second one. Alternatively, immobilized transaminases can be employed

and the first enzyme can thus be removed after completion of the kinetic resolution step.<sup>317</sup> Both methods raised the enantiomeric purity of the product amines to excellent levels (ee >99%). The two-step deracemization protocol was applied to a wide range of chiral amines, including the antiarrhythmic agent mexiletine.<sup>316</sup>

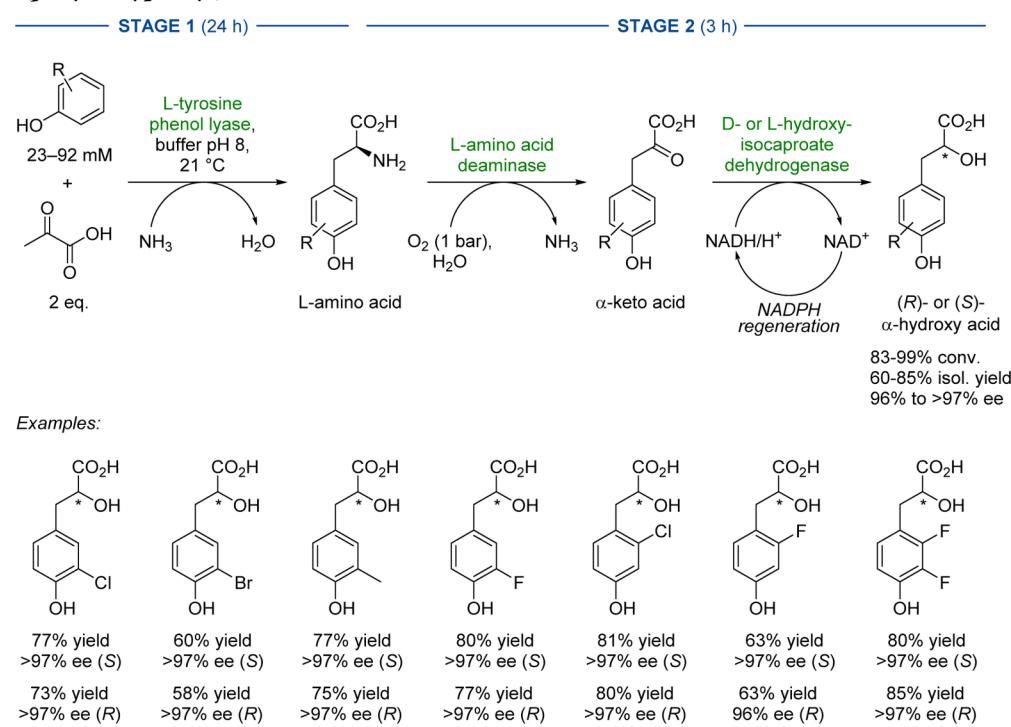
Simultaneous deracemization of  $\alpha$ -chiral primary amines was only achieved using stereocomplementary transaminases<sup>187</sup> and amine oxidases (Scheme 27).

In a cascade involving L-glyceraldehyde (for simultaneous cascades with D-glyceraldehyde see Scheme 68), D-fructose-6-phosphate aldolase FSA from *E. coli* catalyzed two aldol reactions. In the first reaction, coupling of glycolaldehyde and formaldehyde afforded L-glyceraldehyde, which was then phosphorylated by glycerol kinase at the expense of ATP (Scheme 86).<sup>318</sup> The latter was regenerated in situ from

**Scheme 87. Multienzyme Synthesis of 12-Ketoursodeoxycholic Acid from Cholic Acid Using Sequential Oxidation and Reduction Steps**



**Scheme 88. Sequential Cascade Encompassing One C–C Bond Formation Step and Two Redox Steps for the Preparation of Optically Pure 3-(*p*-Hydroxyphenyl) lactic Acids**



phosphoenolpyruvate by pyruvate kinase. L-Glyceraldehyde-3-phosphate was isolated with 72% yield by precipitation as its barium salt but could also be converted further into more complex carbohydrates by addition of an aldol donor suitable for FSA. To circumvent selectivity issues, the second aldol reaction was initiated once glyceraldehyde-3-phosphate formation was completed. Dihydroxyacetone, hydroxyacetone, and 1-hydroxybutan-2-one were investigated as aldol donors, and corresponding sugar phosphates L-sorbose-6-phosphate, 1-deoxy-L-sorbose-6-phosphate and 1,2-dideoxy-1-xylohept-3-ulose-7-phosphate were obtained in isolated yields of 80–86% (82–87 mg).

**3.2.2. Three Catalysts in the Linear Sequence.** The transformation of cholic acid into 12-ketoursodeoxycholic acid has been achieved using three different hydroxysteroid dehydrogenases (HSDHs) and independent regeneration systems for NAD<sup>+</sup> and NADPH.<sup>319</sup> In a first step, 7α- and 12α-HSDHs were employed for oxidation of the respective hydroxy groups of cholic acid (Scheme 87). The ketone moiety at C7 was then reduced to the hydroxy group with opposite absolute configuration by 7β-HSDH. Initially, the authors

attempted a simultaneous cascade in which all three HSDHs and both cofactor regeneration systems operated concurrently. However, insufficient substrate specificity of the glucose dehydrogenase used for NADPH recycling led to an undesired “back-reduction” of C12 upon extended reaction times. To circumvent this problem, the oxidation and reduction steps had to be separated in time, which was realized via two approaches: (i) Compartmentalization of the enzymes in dialysis bags that were applied sequentially or (ii) compartmentalization in two distinct membrane reactors. The second option allowed shorter reaction times, as complete conversion of cholic acid (12.5 mM) into 12-ketoursodeoxycholic acid was achieved within 2 × 15 h. The reaction mixtures of six consecutive cycles (using the enzymes retained in the reactors) were combined, and the product was isolated in 73% yield.

A tyrosine phenol lyase was employed to extend the two-step cascade shown in Scheme 31 to the transformation of para unsubstituted phenols to 3-(*p*-hydroxyphenyl) lactic acid derivatives (Scheme 88).<sup>320</sup> In a first step, a tyrosine derivative was formed employing tyrosine phenol lyase (TPL) from *C.*

*freundii* by C–C coupling between phenol derivatives and pyruvate in the presence of ammonia. Once the C–C bond formation was finished (24 h), oxidative deamination and subsequent reduction were started. The sequential mode was essential, since even small amounts of the final product inhibited the tyrosine phenol lyase in the first step. As an example, phenol ( $R = H$ ) was converted in a range of 23–46 mM with excellent conversions (93–97%) and ee (>99%) to both lactic acid enantiomers. Other phenol substrates were accepted as well at concentrations up to 92 mM with conversions up to >99% and perfect ee (>97% by HPLC). A preparative-scale reaction of 56.6 mg of phenol afforded the enantiopure (S)-3-(*p*-hydroxyphenyl) lactic acid with 97% conversion and 77% isolated yield (ee > 97%).

### 3.3. Multienzyme Cascades in Flow

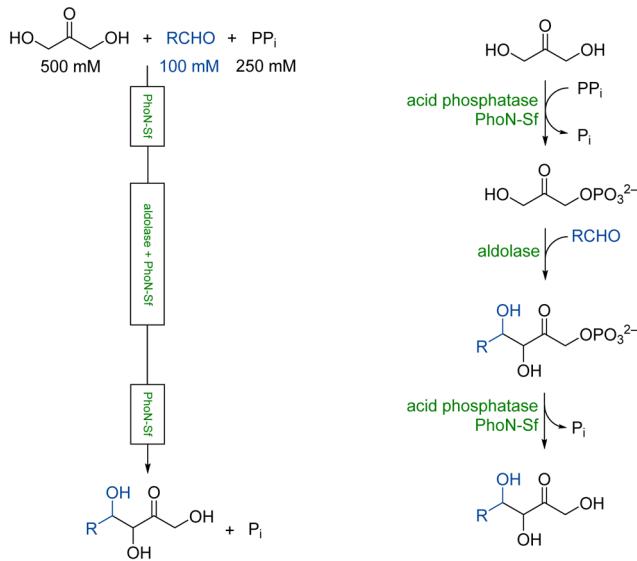
The implementation of flow reactors in synthesis instead of standard batch reactors benefits from multiple advantages, including improved heat and mass transfer resulting in improved energy consumption, safer operating conditions, straightforward combination with other enabling technologies, easier and more reliable scale-up, all of which render continuous flow methodologies highly attractive for the preparation of fine chemicals.<sup>321,322</sup> Advances in protein immobilization and entrapment techniques<sup>323</sup> have enabled the successful implementation of biocatalysts in flow setup; however, so far, the majority of cases reports on single-enzymatic step transformations<sup>324–327</sup> and examples of multi- or chemoenzymatic cascades (see section 4.1) in flow for synthesis are scarce.

Initial developments were conducted with enzyme membrane reactors (EMR)<sup>59,60,328</sup> and allowed combination of enzymes in cascades,<sup>59,60,329</sup> in which the substrate is pumped through the reactor at a constant rate (translating in constant resident time) and the product is collected at the outlet.<sup>50</sup>

A packed bed continuous flow reactor was designed for the synthesis of carbohydrates through a bienzymatic cascade combining a phosphatase and an aldolase and starting from an aldehyde, dihydroxyacetone, and pyrophosphate (Scheme 89).<sup>298</sup> The sequence was conducted in three coupled HPLC columns filled with immobilized enzyme preparation and flushed with a mixture of the three reagents. It started by activation of dihydroxyacetone through monophosphorylation using acid phosphatase in the first reactor. The intermediate reactor was packed with both acid phosphatase—to keep the amount of phosphorylated substrate high—and aldolase, which catalyzed the C–C bond formation of the activated dihydroxyacetone with the aldehyde. Finally, the last reactor contained the acid phosphatase, which catalyzed the deprotection of the alcohol group by removing the phosphate moiety, thus freeing the final aldol products. The major advantage of the setup was the suppression of retro-aldolase activity of the immobilized aldolase and the possibility to perform continuous synthesis of carbohydrate analogues over several days due to increased enzyme stability.

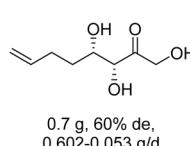
Successful combination of seven enzymes immobilized on agarose beads was demonstrated for the production of uridine diphosphate galactose (UDP-galactose) (Scheme 90).<sup>330</sup> The major advantage of this technique resulted from the increased stability of the immobilized enzymes, which allowed the process to run through the superbead column for 48 h and led to 50% conversion of uridine monophosphate (UMP) into UDP-galactose. Molar equivalent amounts of UMP and galactose were injected into the system, and only catalytic amounts of

**Scheme 89. Continuous Flow Synthesis of Carbohydrates through a Phosphorylation–Aldol Condensation–Dephosphorylation Sequence Catalyzed by Acid Phosphatase PhoN-Sf and an Aldolase, Fructose-1,6-diphosphate aldolase (RAMA), or Rhamnulose 1-Phosphate Aldolase (RhuA from *Thermotoga maritima*)<sup>a</sup>**

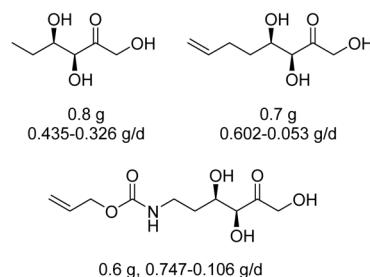


Examples:

with rhamnulose 1-phosphate aldolase:



with fructose-1,6-diphosphate aldolase:



<sup>a</sup>Space time yields given were obtained after a time period of 1–5 days ( $g\ d^{-1}$ ).

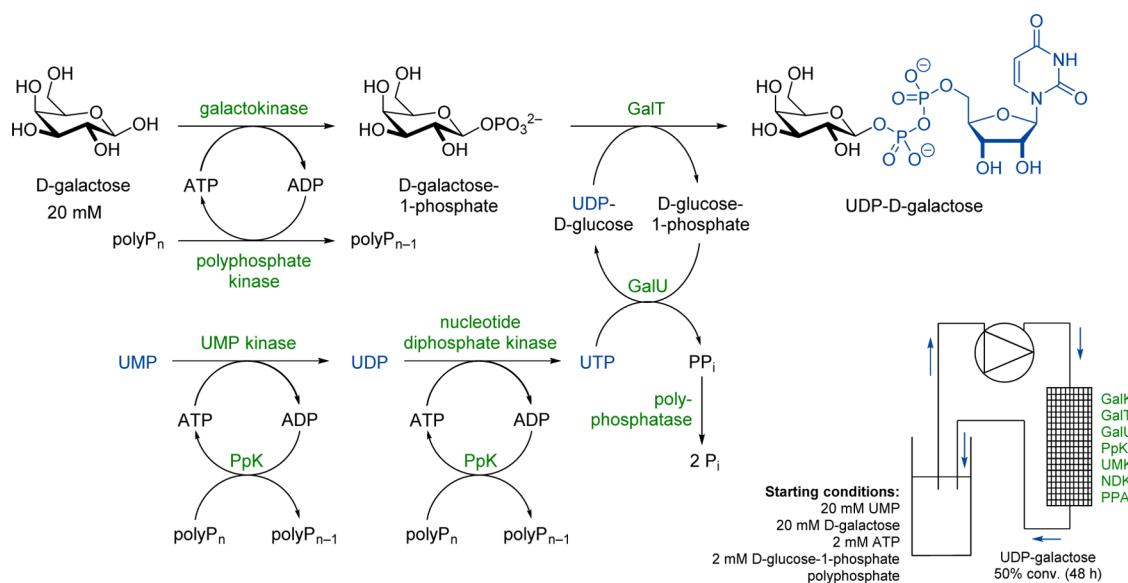
adenosine triphosphate (ATP) and glucose 1-phosphate were necessary since part of the pathway included an ATP regeneration system to alleviate cost. Polyphosphate was used as the energy source (phosphate donor), and inorganic phosphate was generated as only byproduct. The process was designed as a closed recirculating reaction, which allowed the reaction mixture to flow through the column packed bed reactor multiple times. In this configuration (continuous closed circuit), the product required purification from unreacted substrate and byproducts.

## 4. COMBINATIONS OF ENZYMES WITH NON-NATURAL CATALYSTS OR CHEMICAL STEPS

### 4.1. Simultaneous Mode

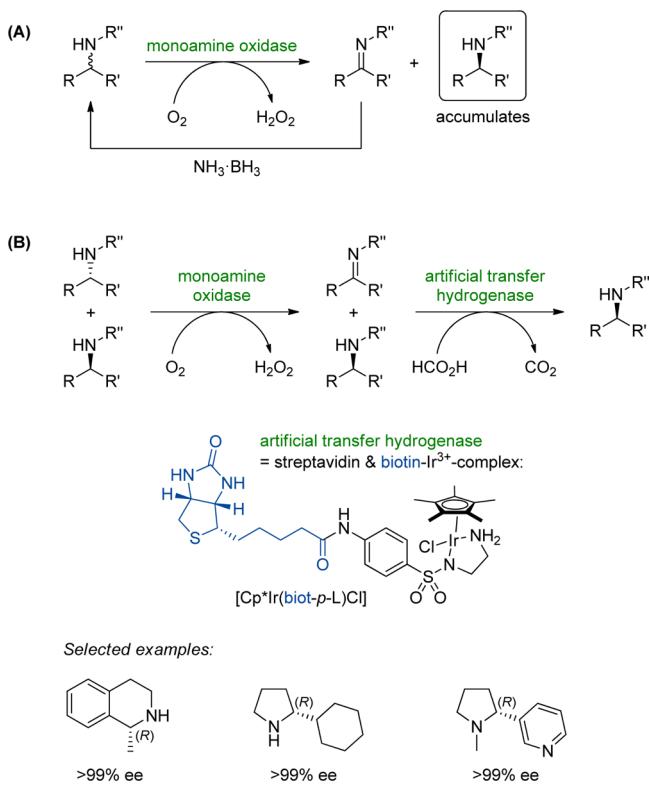
**4.1.1. Combinations with Chemical Reagents or Artificial Metalloproteins.** Deracemization of *rac*-amines by monoamine oxidase from *A. niger* (MAO-N) and non-stereoselective reduction of the resulting intermediate imine or iminium ion by ammonia–borane is a well-established method for structurally diverse primary, secondary, and tertiary amines (Scheme 91).<sup>12,20,21,331–334</sup> The ee of the product depends on

**Scheme 90.** Conversion of Uridine Monophosphate (UMP) into Uridine Diphosphate Galactose (UDP-Galactose) through Multienzymatic Cascade in a Column Packed Bed Reactor<sup>a</sup>



<sup>a</sup>GalT: galactose 1-phosphate uridylyltransferase. GalU: UDP-glucose pyrophosphorylase. PpK: polyphosphate kinase. polyP: polyphosphate. PPA: pyrophosphatase. PP<sub>i</sub>: pyrophosphate. Pi: monophosphate.

**Scheme 91.** Deracemization of  $\alpha$ -Chiral Amines by Enantioselective Oxidation by Monoamine Oxidase (MAO) and (A) a Nonstereoselective Chemical Reduction or (B) Asymmetric Reduction Using an Ir-Dependent Artificial Transfer Hydrogenase (ATHase)



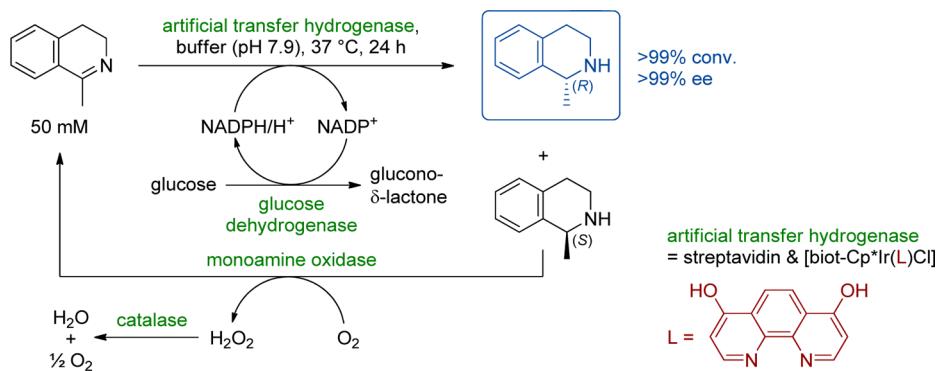
one hand on the turnover of the redox cycle, and on the other hand, its maximum is determined by the enantioselectivity of the amine oxidase.<sup>335</sup> With the development of artificial transfer hydrogenases, the reduction can also be performed stereo-

selectively (**Scheme 91B**), in which both catalysts contribute to the overall stereoselectivity of the process (see also the deracemization with enzymes section 3.1.2.1.2). This was demonstrated by combining engineered variants of MAO-N with an artificial transfer hydrogenase based on streptavidin ligated with the biotinylated iridium(III) complex (**Scheme 91B**).<sup>336</sup> While the free complex and MAO-N suffered from mutual inactivation, the complex embedded in the streptavidin binding site was compatible with the oxidase. Deracemization of two chiral secondary amines was achieved as well as the stereoinversion of a third model compound; in all three cases the (R)-enantiomers were obtained with quantitative conversion and >99% ee.

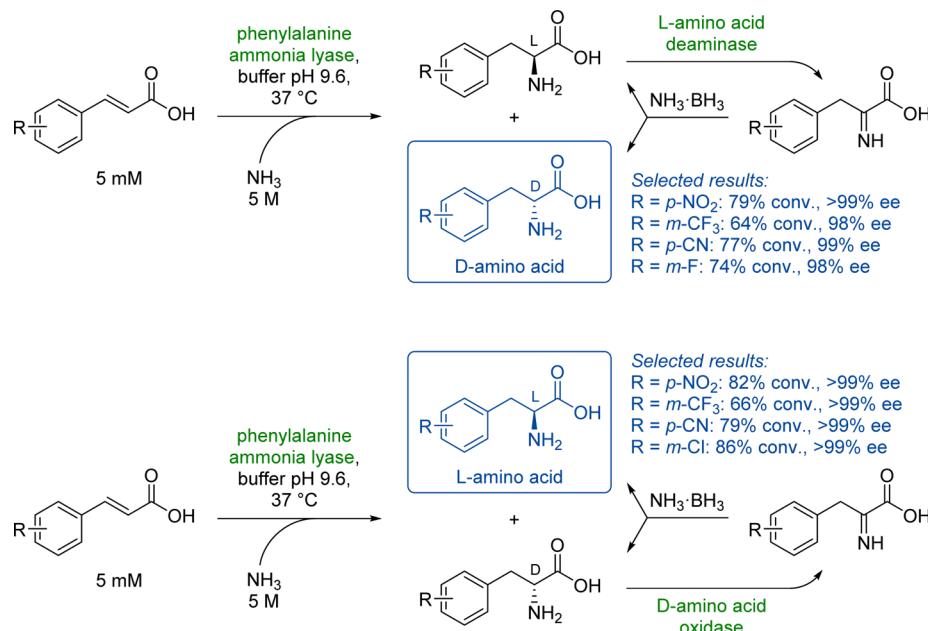
In a subsequent study, the formate-dependent artificial transfer hydrogenase (ATHase) was replaced by a NAD(P)H-dependent ATHase and combined to NAD(P)H recycling based on glucose/glucose dehydrogenase (GDH) (**Scheme 92**).<sup>337</sup> The NAD(P)H-dependent ATHase was obtained by incorporating a Cp\*Ir cofactor possessing a biotin moiety and bidentate ligand 4,7-dihydroxy-1,10-phenanthroline into streptavidin. Reduction of cyclic imines to enantiopure amines was successfully realized through a four-enzyme cascade relying on (i) reduction of 1-methyl-3,4-dihydroisoquinoline (MDQ) to corresponding amine by NADPH-dependent ATHase with low stereoselectivity [(R)-methyl-1,2,3,4-tetrahydroisoquinoline with ee 13%], (ii) enantioselective oxidation of (S)-amine by MAO releasing H<sub>2</sub>O<sub>2</sub>, (iii) concomitant degradation of H<sub>2</sub>O<sub>2</sub> by catalase, and (iv) in situ regeneration of NADPH via oxidation of glucose by GDH. Quantitative conversion to enantiopure (R)-methyl-1,2,3,4-tetrahydroisoquinoline was achieved with 2 equiv of glucose.

Several D- and L-phenylalanine derivatives were successfully prepared via alkene amination followed by deracemization employing a two-enzyme/one-reducing agent chemoenzymatic simultaneous cascade.<sup>338</sup> The phenylalanine ammonia lyase (PAL) from *Anabaena variabilis* was selected as catalyst for the amination of the cinnamic acid starting material, producing the D- and L-enantiomer of the amino acid, which were then

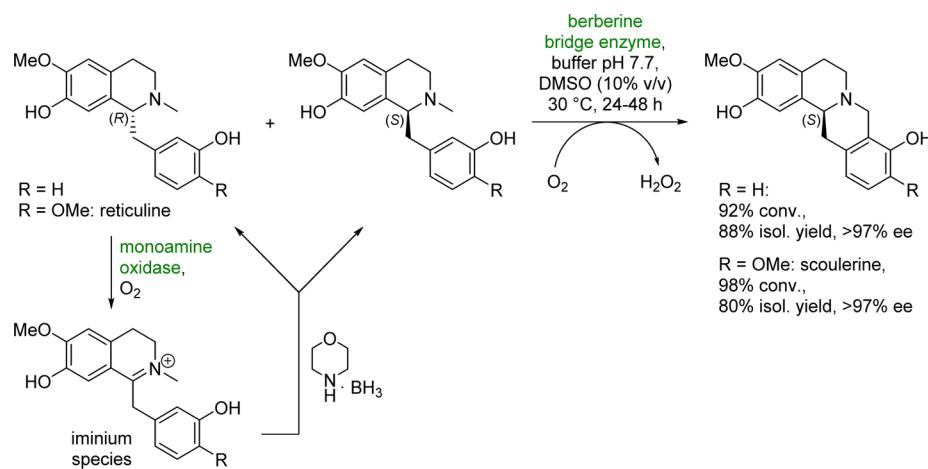
**Scheme 92.** Combination of Artificial Transfer Hydrogenase (ATHase), Monoamine Oxidase (MAO), Catalase, and Glucose Dehydrogenase (GHD) for Reduction of 1-Methyl-3,4-dihydroisoquinoline to (R)-Methyl-1,2,3,4-tetrahydroisoquinoline



**Scheme 93.** Amination/Deracemization for the Synthesis of Enantiomerically Pure D- or L-Phenylalanines



**Scheme 94.** Combination of Two Enzymes and a Chemical Reduction for the Transformation of *rac*-benzylisoquinolines into Optically Pure (S)-Berkines



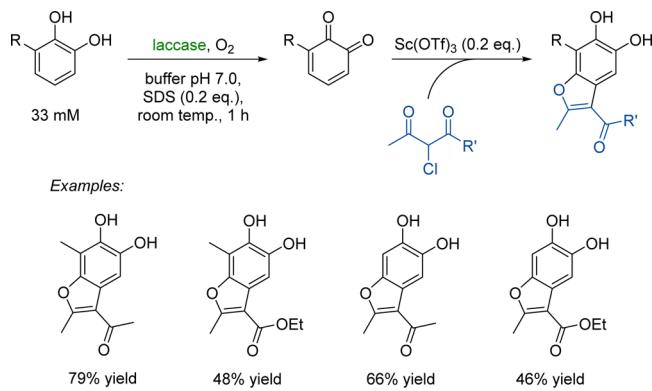
stereoselectively oxidized to the imino acid by either an L-amino acid deaminase (LAAD) or an D-amino acid oxidase (DAAO). The imino acid, on the other hand, could then be nonselectively

reduced to produce optically pure D- or L-phenylalanines with good conversions (62–80%) and perfect ee's (from 97% to >99% (Scheme 93)).

The monoamine oxidase/borane system was also coupled with an oxidative C–C bond-forming enzyme, namely, the berberine bridge enzyme (BBE) (Scheme 94).<sup>339</sup> BBE is a flavin-dependent oxidase involved in plant secondary metabolism that converts the benzylisoquinoline alkaloid (*S*)-reticuline into berbine alkaloid (*S*)-scoulerine. BBE has been shown to transform a range of racemic benzylisoquinoline derivatives in a kinetic resolution with excellent enantioselectivity, affording optically pure (*S*)-berbines and (*R*)-benzylisoquinolines as products.<sup>340–343</sup> On the basis of this kinetic resolution, a chemobioenzymatic cascade process was developed in combination with a borane reduction transforming racemic benzylisoquinolines into (*S*)-berbines with near-quantitative conversion and excellent enantiomeric excess.<sup>339</sup> The system combines a kinetic resolution (BBE) with a stereoinversion ( $\text{MAO}/\text{BH}_3$ ). A variant of the monoamine oxidase from *A. niger* was used, which was engineered for conversion of sterically demanding substrates (MAO-N D11). Morpholine–borane had to be used as reducing agent to enable compatibility with the C–C bond-forming step. The cascade was carried out on preparative scale (0.5 mmol) with two substrates, and the corresponding (*S*)-products were isolated in 88% and 80% yields, respectively, and in optically pure form (ee > 97%).

The use of laccases allows the employment of molecular oxygen as benign oxidant and produces water as byproduct, thus not impacting further reactions in the sequence. The laccase-catalyzed oxidation of catechols to *o*-quinones was combined to the Michael addition of 1,3-dicarbonyl compounds mediated by scandium trifluoromethanesulfonate ( $\text{Sc}(\text{OTf})_3$ ) as Lewis acid (Scheme 95).<sup>344</sup> Subsequent intramolecular cyclization led to the

**Scheme 95. Synthesis of Benzofurans through Laccase Oxidation/Michael Addition Cascade Mediated by Lewis Acid  $\text{Sc}(\text{OTf})_3$**

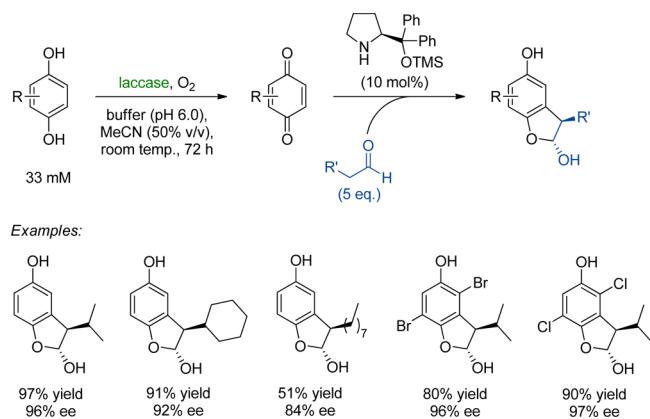


regioselective formation of benzofurans as final products. The simultaneous cascade was performed in the presence of surfactant (SDS) and applied to a range of catechols and 1,3-dicarbonyl compounds. Catechols which were transformed to *o*-quinones with moderate reactivity led to the highest yield of final benzofuran derivatives, which precipitated during the reaction and thus allowed straightforward isolation of the products. Isolated yields up to 79% could be achieved after 1 h reaction time. The catalytic system laccase/ $\text{Sc}(\text{OTf})_3$  could be recycled for three cycles.

A laccase is also part of the next cascade. The biooxidation of 1–4-dihydroxybenzene derivatives to corresponding quinones by a laccase was combined to the secondary amine-catalyzed enantioselective  $\alpha$ -arylation of aldehydes. The one-pot one-step reaction was conducted in a 1:1 mixture MeCN/aqueous buffer

in the presence of 10 mol % (*S*)-2-[diphenyl(trimethylsilyloxy)methyl]pyrrolidine as organocatalyst, yielding  $\alpha$ -arylated aldehydes, which cyclized to the corresponding hemiacetals (Scheme 96).<sup>345</sup> Due to the reversibility of the cyclization, ee values of the

**Scheme 96. Enantioselective Synthesis of  $\alpha$ -Arylated Aldehydes (as hemiacetals) through Laccase Oxidation and Asymmetric Organocatalysis**



products slightly decreased over prolonged reaction time. The aldehyde was provided in excess (5 equiv), and the reaction was applied to a combination of various hydroxyquinones and aldehydes. Corresponding products were isolated in good to high yields and excellent ee values.<sup>345</sup>

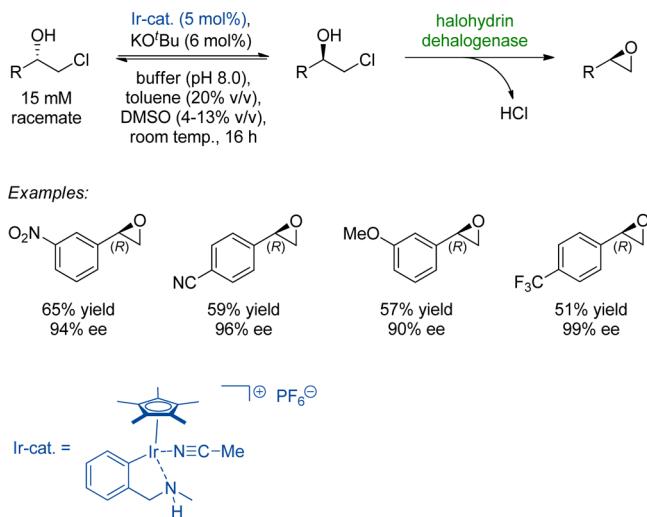
**4.1.2. Combinations with Metal Catalysts.** The successful design of cascades combining metal catalysis and biocatalysis offers new perspectives in terms of “doable” chemistry with the promise to harvest the strength of both disciplines, in particular, in terms of reaction scope and selectivity. Several reviews and book chapters were published recently, reflecting on one hand the interest of the community in merging these two fields and on the other the terrific progress made in designing such cascades. In the next schemes, recent examples which are starting to define new grounds and offer perspectives but also inspiration will be presented.<sup>26–31</sup>

Lipases belong by far to some of the most investigated and employed enzymes in chemistry, mostly owing to their robustness and tolerance toward high temperature and organic solvents. They have been used in multiple synthetic routes and were pioneering biocatalysts in chemoenzymatic processes running in organic media. This is one of the reasons why lipases were among the first enzymes to be coupled to metal-catalyzed reactions in one pot, because both reactions were compatible in nonaqueous media. Pioneering works were conducted in the field of dynamic kinetic resolutions (DKRs) relying on transition-metal-catalyzed racemization and are now being applied to (functionalized) alcohols, amines, or allylic acetates (other available racemization techniques include base-catalyzed racemization, racemization via formation of Schiff base, or via  $\text{S}_{\text{N}}2$  displacement).<sup>32,33</sup> Notably, the first published example of one-pot chemoenzymatic DKR reported a process running in aqueous buffer.<sup>346</sup>

The DKR-transforming racemic  $\beta$ -haloalcohols to epoxides was designed based on the combination of enantioselective haloalcohol dehalogenase-catalyzed conversion of  $\beta$ -chloroalcohols to corresponding epoxides through asymmetric ring closure and racemization of haloalcohols by an iridacycle catalyst. The reaction was performed in a biphasic system consisting of toluene

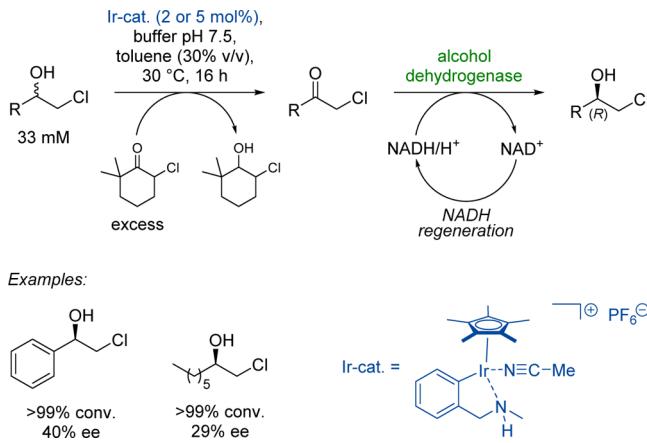
and buffer in the presence of DMSO as cosolvent (5 vol %) using an engineered protein to ensure highest enantioselectivity and stability (HheC C153S W249F originating from *A. radiobacter* AD1, supplemented with BSA for stabilization). Thus, enantioenriched (*R*)-epoxides were obtained as final products in excellent ee values and good yields in a two-step simultaneous cascade (Scheme 97).<sup>347</sup>

**Scheme 97. Dynamic Kinetic Resolution of Racemic  $\beta$ -Chloroalcohols to Epoxides Using Iridacycle Catalyst and Haloalcohol Dehalogenase**



The same iridacycle was used in a oxidation–reduction deracemization sequence of chlorohydrins (Scheme 98).<sup>348</sup> This

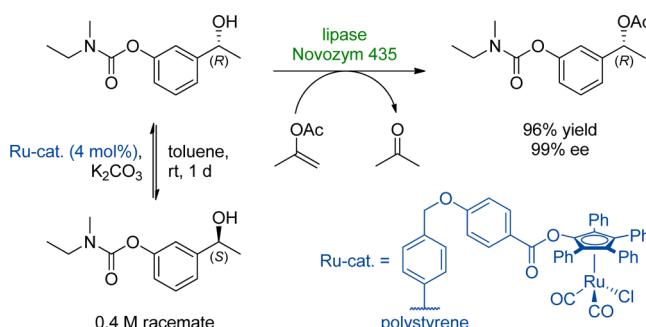
**Scheme 98. Deracemization of Chlorohydrins through Concurrent Iridium-Catalyzed Oxidation/Enzymatic Reduction Cascade**



was achieved by coupling nonstereoselective iridium-catalyzed oxidation to stereoselective reduction catalyzed by an alcohol dehydrogenase. The metal and biocatalyst were shown to be compatible thanks to the identification of orthogonal reagents for each step: a specific hydrogen acceptor for the oxidation in the form of 6-chloro-2,2-dimethylcyclohexanone and a specific hydride donor for the reduction in the form of formate (used in the recycling of the nicotinamide hydride shuttle through formate dehydrogenase FDH). Since the iridacycle catalyst racemized the secondary alcohol, ee values were only moderate.

A precursor of rivastigmine, a cholinergic agent used to treat symptoms of dementia such as Alzheimer's and Parkinson's diseases, was prepared through DKR of the respective secondary alcohol (Scheme 99).<sup>349</sup> For this purpose, the racemic substrate

**Scheme 99. Dynamic Kinetic Resolution of *rac*-sec-Alcohol Using Ru-Based Racemization and Lipase-Catalyzed Enantioselective Acylation<sup>a</sup>**



<sup>a</sup>Novozym 435: immobilized lipase B from *C. antarctica*.

was mixed with the lipase Novozym 435 (immobilized lipase B from *C. antarctica*) and isoprenyl acetate (acyl donor) in toluene in the presence of a base (K<sub>2</sub>CO<sub>3</sub>, 1 equiv) and a Ru-based racemization catalyst, which was used as a polymer-bound preparation. The immobilized catalytic system could be easily recycled five times with conserved efficiency provided fresh K<sub>2</sub>CO<sub>3</sub> was added after few cycles.<sup>349</sup>

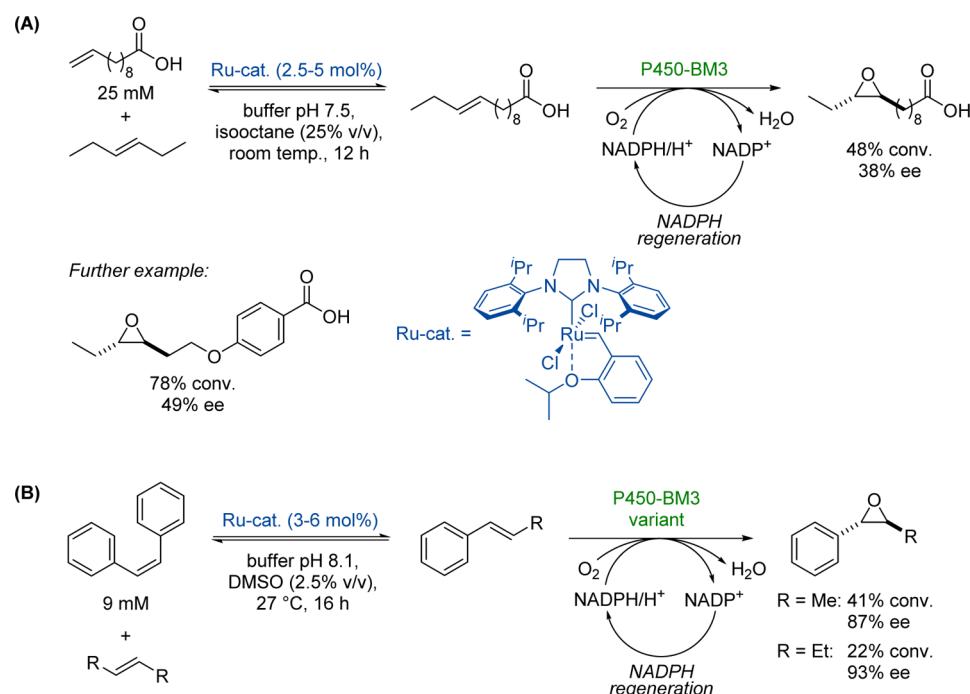
A metathesis/epoxidation cascade combining a Ru-based catalyst and a metalloprotein was performed in a biphasic system and applied to the synthesis of enantioenriched epoxides (Scheme 100). Cross-metathesis between an unsaturated carboxylic acid and an alkene was followed by selective epoxidation catalyzed by a P450 enzyme. The reaction could be performed in one step owing to the nonreactivity of both carboxylic acid and alkene starting materials toward P450 BM3 (P450 from *B. megaterium*) and the use of P450-compatible organic cosolvent (Scheme 100A).<sup>350</sup> The protocol was further applied to the conversion of stilbenes to chiral aryl epoxides using engineered P450, which ensured higher enantioselectivity in the epoxidation reaction (Scheme 100B).

The Ru-catalyzed isomerization of allylic alcohols was combined in a simultaneous fashion to the stereoselective enzymatic reduction of ketones, thus offering an approach for the formal reduction of allylic alcohols to secondary alcohols (Scheme 101), a chiral variant of a previously described protocol.<sup>352</sup> The required nicotinamide cofactor for the alcohol dehydrogenase (commercial ketoreductase P1-A04) was provided in catalytic amounts and recycled through a substrate-coupled approach using 2-propanol as cosubstrate.<sup>353</sup>

The simultaneous cascade for the transformation of racemic styrene oxides to (*R*)-hydroxy triazoles was designed by combining the enzymatic enantioselective azidolysis of epoxides by halohydrin dehalogenase with subsequent ligation of 1,2-azido alcohols with alkynes in a copper-catalyzed [3 + 2] cycloaddition (“click” chemistry) (Scheme 102).<sup>354</sup> A copper-free procedure was developed for cyclic alkynes. Since the first step catalyzed by halohydrin dehalogenase is a kinetic resolution, conversions do not exceed 50%.

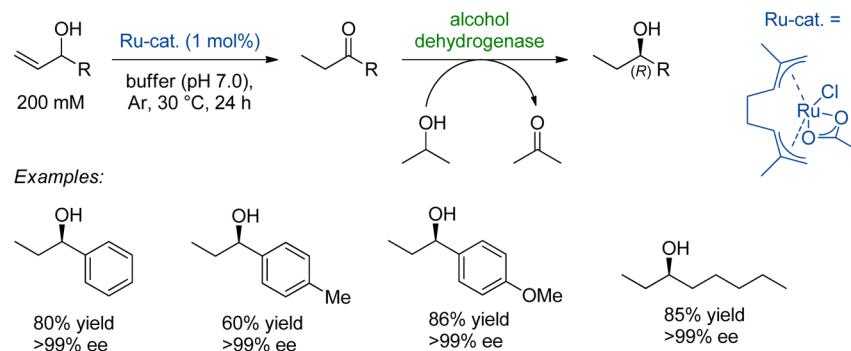
A similar strategy was applied but in a sequential chemoenzymatic transformation using whole cells as biocatalysts, whereby enantiopure epoxides were generated in situ by the

**Scheme 100. Simultaneous Olefin Metathesis and Enzymatic Epoxidation in Biphasic System for the Synthesis of Alkyl (A) And Aryl (B) Epoxides<sup>a</sup>**

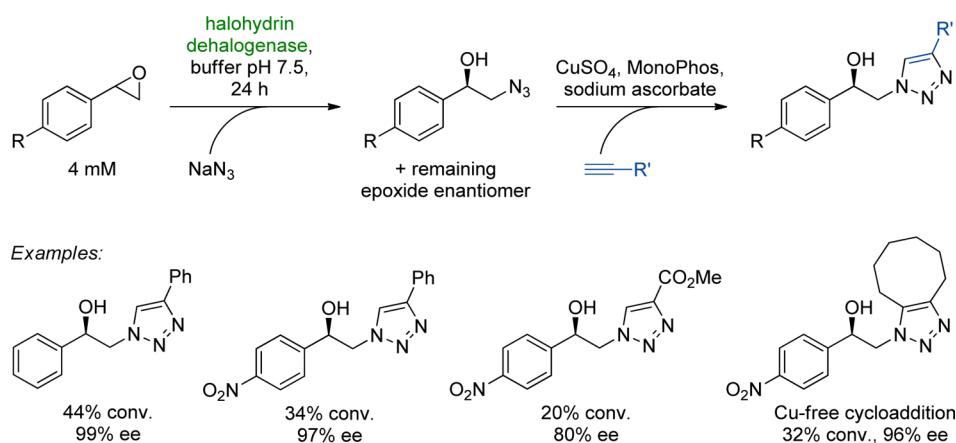


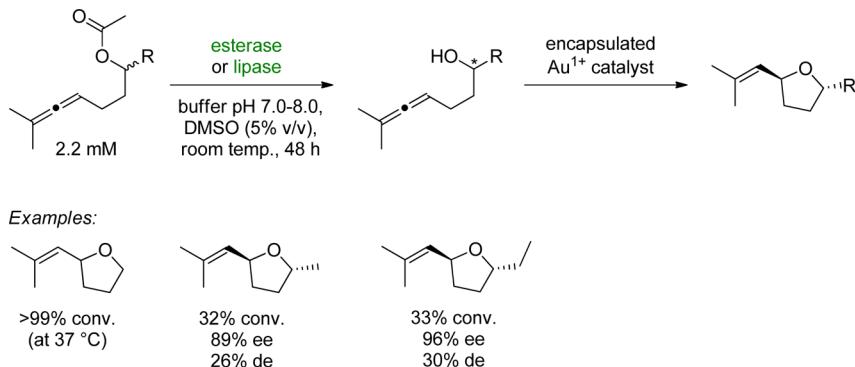
<sup>a</sup>Self-metathesis products—mostly nonreactive—not displayed for clarity.

**Scheme 101. Simultaenous Isomerization/Reduction Cascade for the Formal Reduction of Allylic Alcohols to Chiral Alcohols**

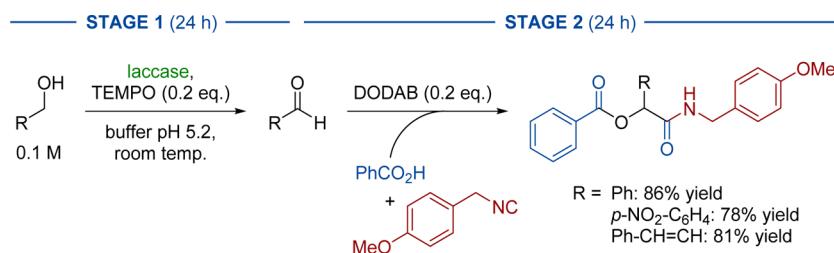


**Scheme 102. One-Step One-Pot Synthesis of Chiral Hydroxy Triazoles through Enzymatic Azidolysis/“Click” Chemistry Sequence**

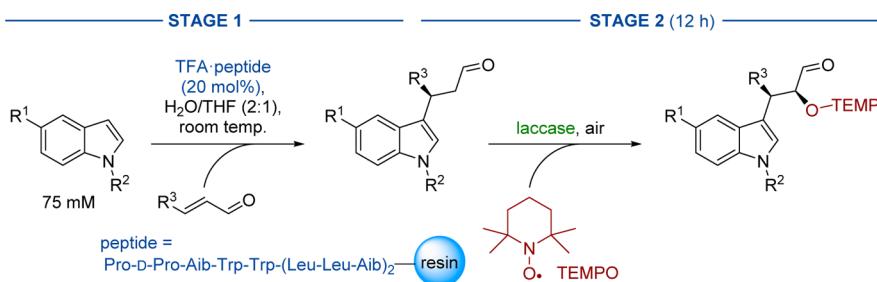


Scheme 103. One-Pot One-Step Enzymatic Hydrolysis/Au-Catalyzed Cyclization to Tetrahydrofuran Derivatives<sup>a</sup>

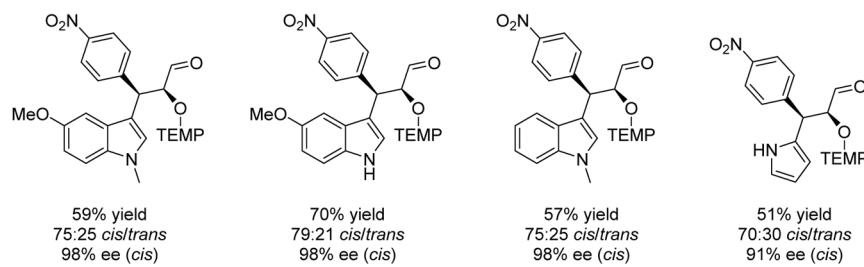
<sup>a</sup>Esterase from rabbit liver; lipase PS from Amano. Au(I)-catalyst: chloro(trimethylphosphine) gold(I) encapsulated in tetrahedral Ga<sub>4</sub>L<sub>6</sub> (L = N,N'-bis(2,3-dihydrobenzoyl)-1,S-diaminonaphthalene) cluster.

Scheme 104. Synthesis of  $\alpha$ -Acyloxy Carboxamides through Laccase/TEMPO Oxidation System/Passerini-Three Component Reaction Cascade<sup>a</sup>

<sup>a</sup>DODAB: dioctadecyldimethylammonium bromide.

Scheme 105. Sequential Friedel–Crafts-Type Asymmetric Alkylation/ $\alpha$ -Oxyamination Cascade

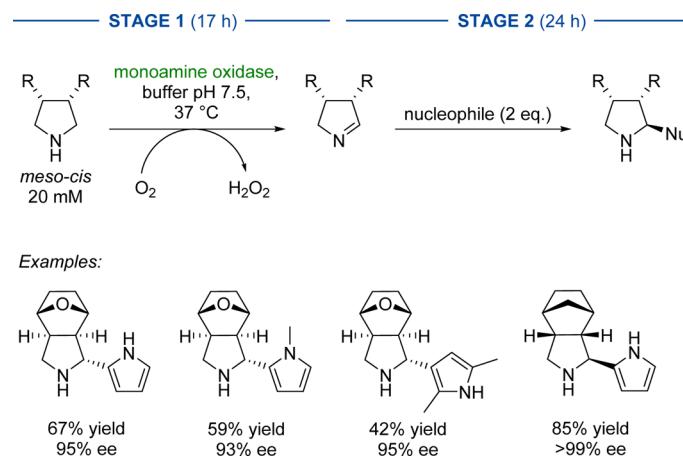
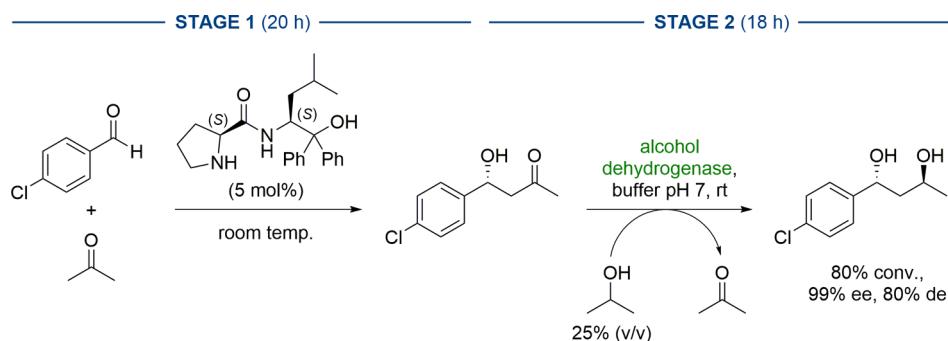
Examples:



bienzymatic transformation of  $\beta$ -halo ketones. The whole cells coexpressed the halohydrin dehalogenase (Hhe) together with an alcohol dehydrogenase, which first reduced the keto substrates stereoselectively to the corresponding alcohols, which were further converted by Hhe to epoxides (the starting material for the cascade in Scheme 102) and eventually to  $\beta$ -hydroxy triazoles. By matching the stereoselectivity of the two enzymes, conversions were not limited to 50% anymore and both

enantiomers could be obtained.<sup>355</sup> A variant HheC C153S of the halohydrin dehalogenase from *A. radiobacter* was employed.

A unique example of combining gold catalysis with biocatalysis demonstrated the possibility to run such chemoenzymatic processes simultaneously (Scheme 103).<sup>352</sup> The gold catalyst was encapsulated in a tetrahedral supramolecular cluster to prevent deactivation of the biocatalyst, thus allowing the process to run in one stage. This supramolecular encapsulation was

Scheme 106. Stereoselective Monoamine Oxidase (MAO)-Mediated Oxidative Aza-Friedel–Crafts Reactions of *meso*-PyrrolidinesScheme 107. Sequential Organo-/Biocatalyzed Cascade to 1,3-Diols<sup>a</sup>

<sup>a</sup>Alcohol dehydrogenase from *Rhodococcus* sp.

applied to the enzymatic hydrolysis of allenic acetylated substrates and further cyclization of intermediate alcohols to yield substituted tetrahydrofurans in aqueous buffer. A tandem enzymatic kinetic resolution/cyclization was successfully carried out on racemic substrates. This strategy was also implemented in the Ru-catalyzed isomerization/enzymatic reduction of allyl alcohol to furnish propanal.

#### 4.2. Sequential Mode

**4.2.1. Combinations with Chemical Reagents.** The laccase/TEMPO-catalyzed oxidation of alcohols to aldehydes was combined with a Passerini three-component reaction (P-3CR) with isocyanides and carboxylic acids to furnish  $\alpha$ -acyloxy carboxamides as final products (Scheme 104).<sup>356</sup> The reaction was performed in aqueous buffer in sequential mode and required a surfactant (DODAB = dioctadecyltrimethylammonium bromide) for improved yield of the P-3CR. The final products  $\alpha$ -acyloxy carboxamides precipitated and were obtained in pure form through filtration and purification by column chromatography (up to 86% yield after 48 h total reaction time). The catalytic system laccase/TEMPO and DODAB could be recycled three times through filtration with a moderate to significant drop in final reaction yields over time (from 86% to 67% to 19%).<sup>356</sup>

The sequential Friedel–Crafts-type asymmetric alkylation/ $\alpha$ -oxyamination was realized by combining peptide-catalyzed alkylation of  $\alpha,\beta$ -unsaturated aldehydes with peptide/laccase-catalyzed oxyamination.<sup>357</sup> A range of oxygen-functionalized indole derivatives was obtained employing this protocol with a

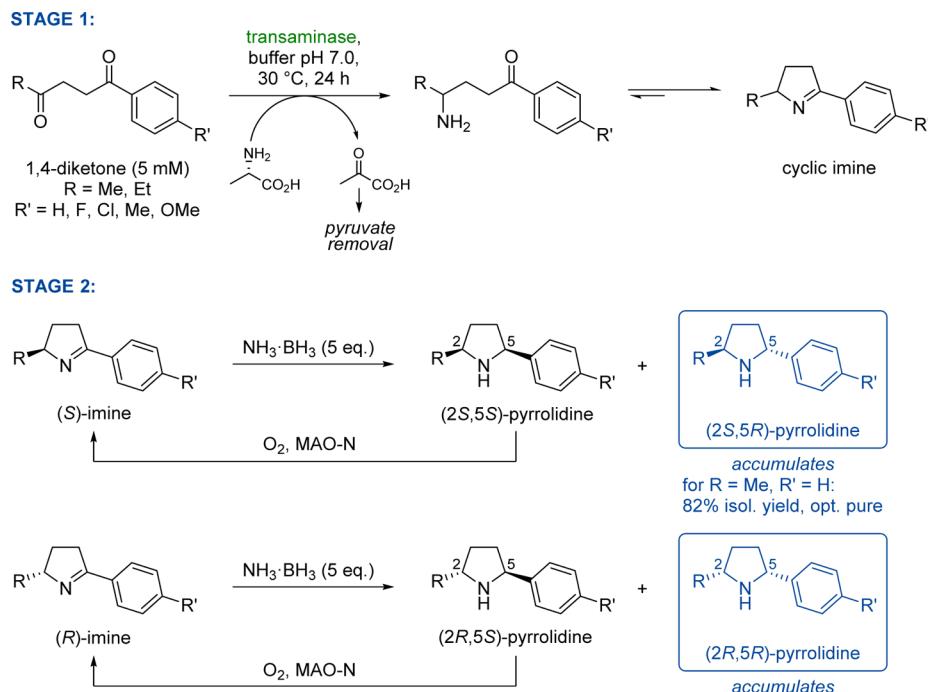
resin-supported peptide catalyst in H<sub>2</sub>O/THF 2:1 (Scheme 105).<sup>358</sup>

A sequential chemoenzymatic oxidative aza-Friedel–Crafts reaction was developed by using monoamine oxidase (MAO-N DS) for oxidation of *exo*-configured *meso*-pyrrolidines, which afforded reactive imines that reacted further in an aza-Friedel–Crafts reaction, furnishing 2-substituted pyrrolidines as single diastereoisomers with overall high ee values (Scheme 106). Several C-nucleophiles were combined with diverse pyrrolidines, and the process was run in two stages to ensure high conversion and ee values.<sup>359</sup>

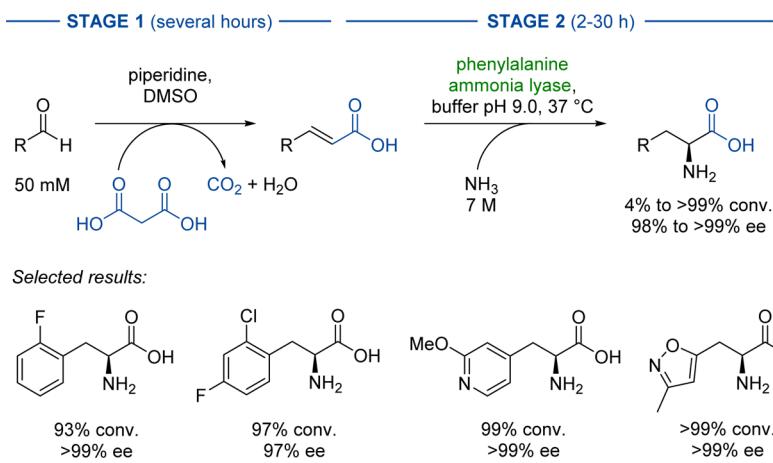
Chiral 1,3-diols were obtained through combination of organo- and biocatalysts. Organo-catalyzed aldol reactions of aldehydes with ketones furnished enantiopure  $\beta$ -hydroxyketones, which were further reduced by stereoselective alcohol dehydrogenases (ADHs) to the final diol products. Due to the modular character of the sequence, it was possible to get access to all four stereoisomers in enantiomerically and diastereoisomerically pure form by combining both stereoselective catalysts in complementary fashion in each step. The one-pot synthesis of (1*R*,3*S*)-1-(4-chlorophenyl)butane-1,3-diol was accomplished by combining 4-chlorobenzaldehyde, acetone, and the proline derivative under solvent-free conditions for 20 h, followed by mixing with aqueous buffer, ADH, and nicotinamide recycling system (NAD<sup>+</sup> and 2-propanol). Column chromatography purification afforded the final product (99% ee and 80% de) in 80% conversion (Scheme 107).<sup>360</sup>

This protocol was further optimized to allow the use of reduced amount of organocatalyst (0.5 mol %). This was

**Scheme 108. Sequential Combination of a Transaminase-Catalyzed Amination and a Reduction/Deracemization Using Borane/Monoamine Oxidase To Get Access to 2,5-Disubstituted Pyrrolidines**



**Scheme 109. Combination of a Knoevenagel–Doebner Condensation with an Enzymatic Hydroamination to L-3-(Hetero)arylalanine Derivatives**

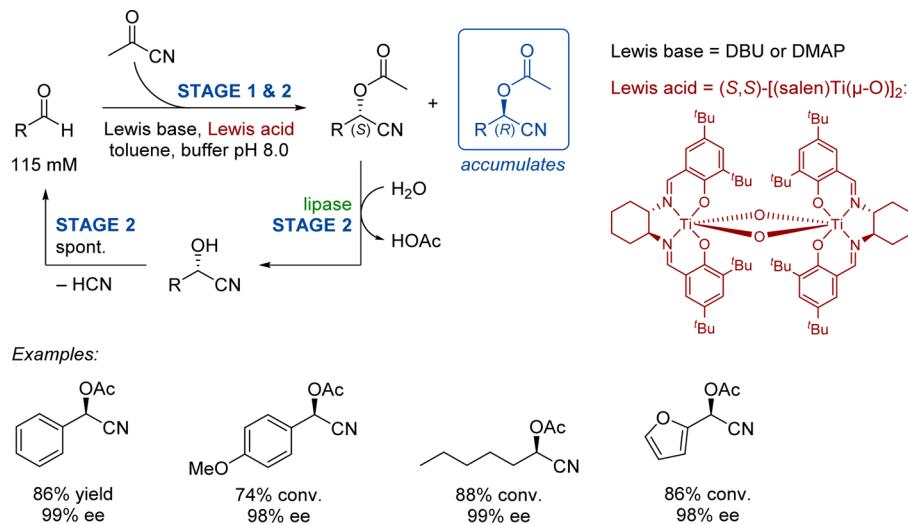


accomplished by supplementing the initial solvent-free system with a saturated solution of NaCl. The optimized methodology was applied in combination with two stereodivergent enzymes for the synthesis of two diastereomers of 1,3-diol.<sup>361</sup> Finally, the whole sequence was performed using both immobilized organocatalyst and biocatalyst in organic media. Superabsorber-based coimmobilization of alcohol dehydrogenase with its cofactor NAD<sup>+</sup> allowed recycling of the biocatalyst over four cycles without significant loss of efficiency.<sup>362</sup>

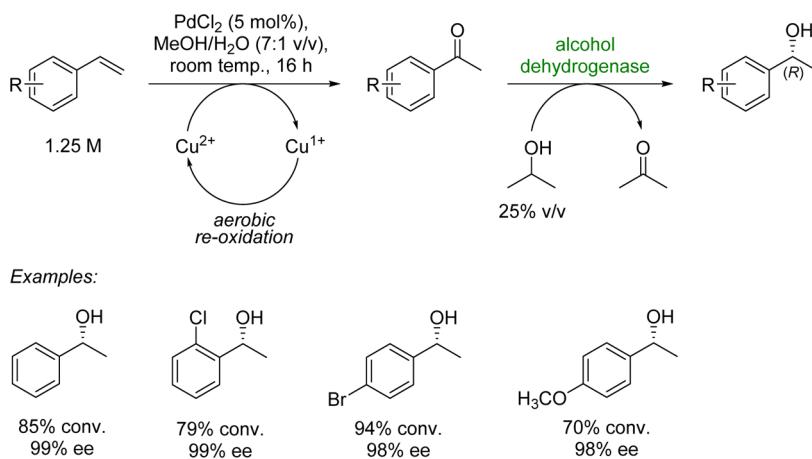
Transaminases have been combined with engineered variants of monoamine oxidase from *A. niger* (MAO-N variants D5 and D9) and ammonia–borane as reducing agent for the enantio- and diastereoselective synthesis of 2,5-disubstituted pyrrolidines (Scheme 108).<sup>363</sup> Thereby, prochiral 1,4-diketones were first selectively monoaminated by commercial transaminases ATA-113 or ATA-117 at the sterically more accessible carbonyl group. The resulting aminoketones underwent spontaneous cyclization

to imines, which in a second step were converted into the target pyrrolidines by the combined action of MAO-N and NH<sub>3</sub>·BH<sub>3</sub>. This formal asymmetric C=N reduction is based on the principle that borane reduces the imine to a mixture of *cis*- and *trans*-pyrrolidine (with a slight excess of the *cis* diastereomer), while MAO-N oxidizes exclusively the (*SS*)-isomer of the pyrrolidine. Hence, the (*SR*)-isomer accumulates in the reaction mixture over several reduction/oxidation cycles. Nevertheless, a simultaneous bienzymatic cascade was not possible, since NH<sub>3</sub>·BH<sub>3</sub> reduced the starting material, diketone. The sequential combination of the two sequences worked well, though providing access to both *cis*- and *trans*-pyrrolidines in >99% de and with quantitative conversion. A preparative-scale reaction using 1-phenyl-1,4-pentandione (R = Me, R' = H; 220 mg) as starting material and transaminase ATA-113 for the first step afforded (2*S*,5*R*)-pyrrolidine (R = Me, R' = H) in 82% isolated yield and stereoisomerically pure form.

**Scheme 110. Minor Enantiomer Recycling Protocol Applied to the Preparation of Acylated Cyanohydrins through Lewis-Base–Lewis-Acid-Catalyzed Acylcyanation of Aldehydes Combined to Lipase (*C. antarctica* lipase B)-Catalyzed Hydrolysis**



**Scheme 111. Formal Asymmetric Hydration of Styrene Derivatives through Sequential Wacker Oxidation and Enzymatic Ketone Reduction Performed in Water<sup>a</sup>**



<sup>a</sup>Cu provided as CuCl; the Wacker oxidation took place in a polydimethylsiloxane thimble immersed in the reaction vessel; components for the second step were added in a sequential mode.

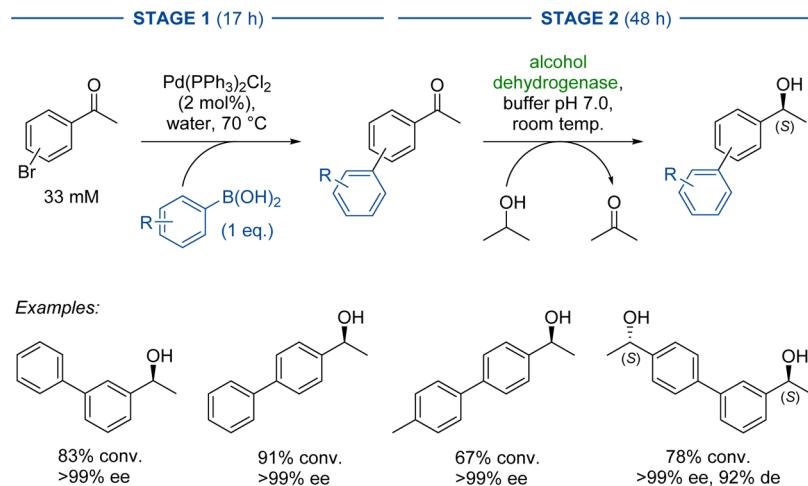
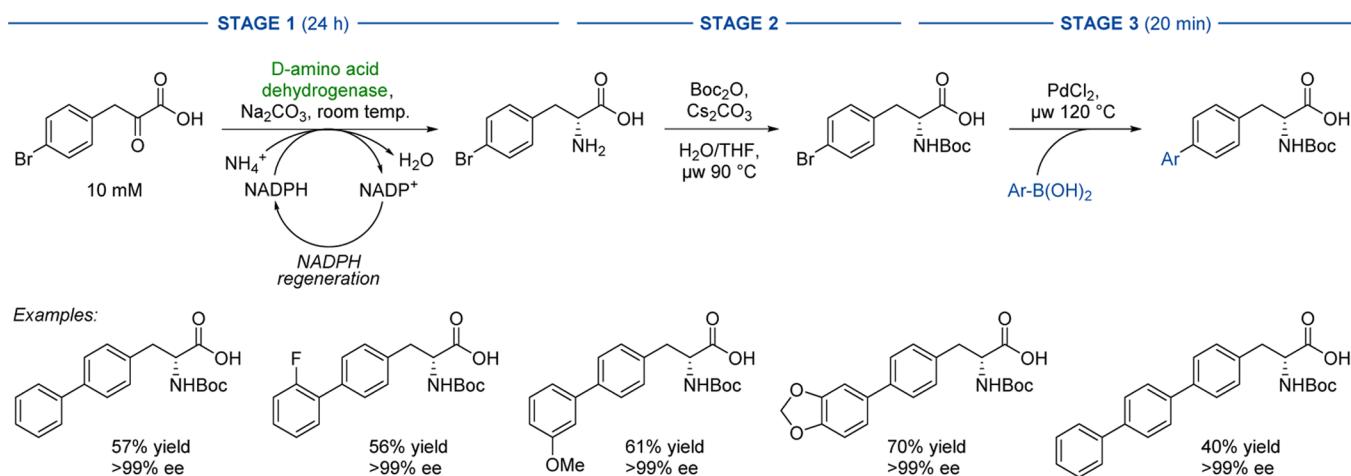
L-Phenyl- and L-pyridyl-alanine derivatives have been prepared by combination of a Knoevenagel–Dobner condensation followed by the hydroamination catalyzed by a phenylammonia lyase from *A. variabilis* (Scheme 109).<sup>364,365</sup> The C=C bond formation product was not isolated but directly transformed in the subsequent biocatalytic step in the same reaction vessel. In addition to benzaldehyde and the different pyridine aldehydes, other products with heterocyclic ring systems such as isoxazole, thiophene, and quinoline were prepared and obtained with high conversion and perfect ee's.

**4.2.2. Combinations with Metal Catalysts.** The concept of minor enantiomer recycling aims at recycling an unwanted enantiomer by transforming it into the desired isomer. A one-pot procedure was developed in a biphasic system to allow the continuous regeneration of an aldehyde starting material through use of sequential stereoselective transformations: the formation of acylated cyanohydrins by acylcyanation of  $\alpha$ -ketonitriles and aldehydes catalyzed by a Lewis-acid–Lewis-base proceeded with imperfect stereoselectivity, resulting in the formation of unwanted enantiomer, which was enantioselectively hydrolyzed

by CALB (*C. antarctica* lipase B) to the unprotected cyanohydrin, the latter spontaneously hydrolyzing back to the aldehyde upon release of HCN (Scheme 110). The aldehyde was thus regenerated and could enter the next cycle of acylcyanation, justifying the need for an excess of  $\alpha$ -ketonitriles (added over time). Although both steps were not perfectly stereoselective, their combination in one pot afforded products in excellent yields and enantiopurities. By using catalysts with opposite stereopreference, (S)-acylated cyanohydrins were obtained.<sup>366</sup> Additional studies were subsequently reported based on this concept.<sup>367–369</sup>

Relying on Pd/Cu-catalyzed Wacker reaction, a chemoenzymatic approach was designed to mimic the asymmetric hydration of styrene derivatives in a cascade. In the first step, Pd/Cu-catalyzed Wacker oxidation using molecular oxygen as oxidant formed intermediate carbonyl compounds, which were subsequently reduced by an alcohol dehydrogenase to chiral secondary alcohols (Scheme 111). Compartmentalization of the two steps was necessary to allow both reactions to proceed in water in one pot. To that end, a polydimethylsiloxane (PDMS)

Scheme 112. Suzuki Cross-Coupling Preceding Enzymatic Ketone Reduction for the Synthesis of Enantiopure Biaryl Alcohols

Scheme 113. Three-Step Sequential Synthesis of Protected D-Amino Acids through a Reductive Amination–Protection–Coupling Cascade<sup>a</sup>

<sup>a</sup>D-Amino acid dehydrogenase engineered from a *meso*-diaminopimelate dehydrogenase from *C. glutamicum*.

thimble was immersed in the reaction vessel, which permitted isolation of the Wacker oxidation components inside the thimble, while formed intermediate ketones could flux through the membrane and diffuse in the vessel, later containing the enzyme. The protocol was run in a sequential mode, with ADH and cofactor being added after completion of the oxidation.<sup>370</sup> This method was a major improvement compared to the initial system, which relied on a stoichiometric amount of benzoquinone as oxidizing agent.<sup>371</sup> The Wacker oxidation dual catalyst could be recycled over 16 cycles without substantial loss of activity.

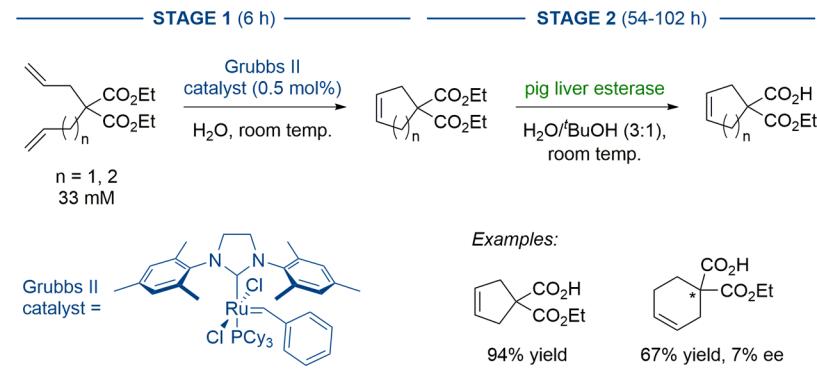
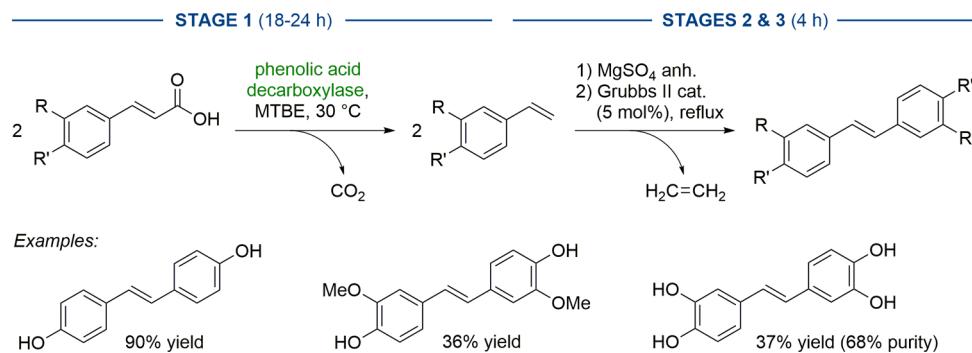
Suzuki cross-coupling preceded the asymmetric enzymatic reduction of a ketone by an alcohol dehydrogenase in aqueous media for the preparation of chiral biaryl alcohols in one pot (Scheme 112). The key point was to identify compatible conditions for the two steps to circumvent isolation of the intermediate. Consequently, the cascade had to be run in sequential mode since the enzyme could not tolerate the presence of the components of the first step, in particular, the boronic acid. Simple pH adjustment was required before addition of the biocatalyst. A range of enantiopure products was synthesized in excellent conversion levels, and biaryl diols could also be obtained with high de value.<sup>372</sup> A phosphine-free

variant was soon after developed, utilizing Pd nanoparticles stabilized within the cavity of a thermostable DNA binding protein, which combined to stereoselective alcohol dehydrogenase (ADH) affording a range of biaryl alcohols in enantiopure form.<sup>373</sup> This strategy was also adapted to biphasic systems containing ionic liquids, which resulted in faster reaction rates and allowed easy recycling of both catalysts (four runs without loss of efficiency); in particular, the biocatalyst was used in the form of whole cells expressing the recombinant ADH.<sup>374</sup>

In a related fashion, a Suzuki cross-coupling reaction in aqueous media was exploited for the chemoenzymatic preparation of biarylalanines in a sequential fashion (Scheme 113).<sup>375</sup> The reaction consisted of three steps: reductive amination of  $\alpha$ -ketoacids to  $\alpha$ -amino acids by D-amino acid dehydrogenase, subsequent protection of the amino group, and Pd-catalyzed cross-coupling to boronic acids. The last two steps were conducted under microwave irradiation. A panel of enantiopure-protected D-amino acids could thus be obtained.

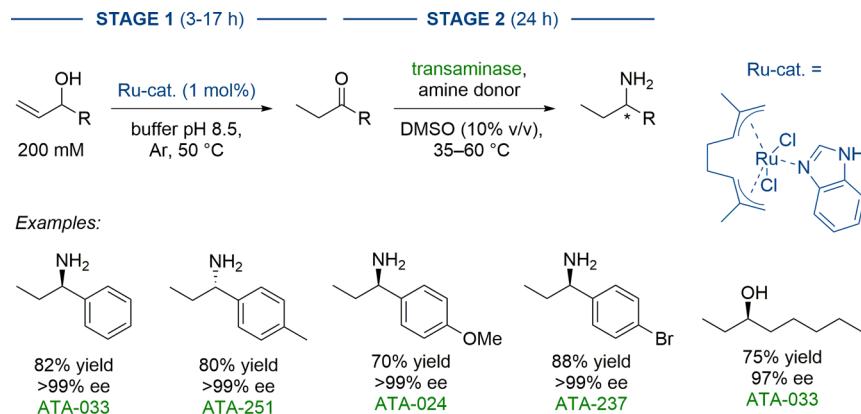
A recent report showed how the enzymatic halogenation and Suzuki–Miyaura coupling could be combined for the regioselective arylation of aromatics in a simultaneous fashion.<sup>376</sup> Flavin-dependent halogenase-catalyzed regioselective halogenation to aryl halides were further subjected to Suzuki–Miyaura coupling

Scheme 114. Two-Step One-Pot Synthesis Combining Olefin Metathesis and Enzymatic Hydrolysis in Aqueous Media

Scheme 115. One-Pot Sequential Synthesis of Stilbene Derivatives by Enzymatic Decarboxylation/Ru-Catalyzed Self-Metathesis Sequence<sup>a</sup>

<sup>a</sup>Acid decarboxylase from *B. subtilis* trapped in a poly(vinyl alcohol)/poly(ethyleneglycol) (PVA/PEG) cryogel.

Scheme 116. Chemoenzymatic Sequential Cascade To Convert Allylic Alcohols to Chiral Amines



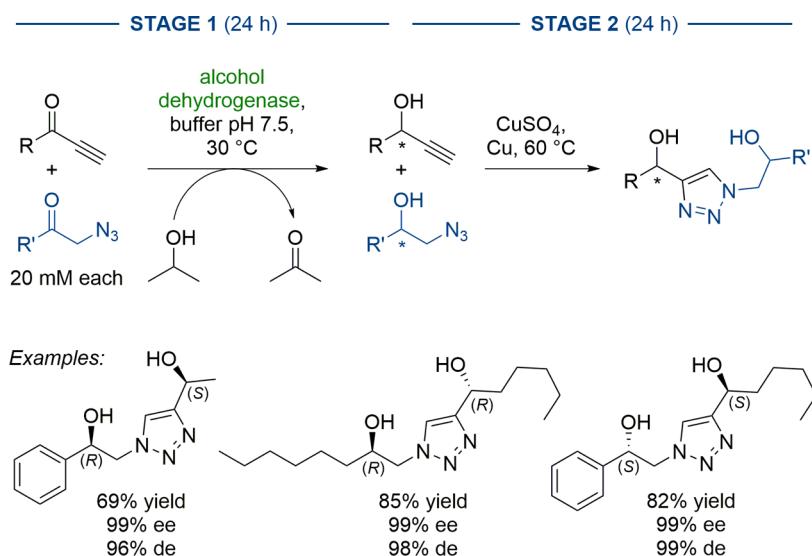
conditions using a variety of boronic acids. Key was the compartmentalization of the enzymatic reaction, since the presence of protein affected the efficiency of the coupling reaction. This was achieved by use of a polydimethylsiloxane (PDMS) thimble inside the reaction vessel, which functioned as a membrane and inside of which the cross-coupling reaction was taking place (see Wacker oxidation/enzymatic reduction cascade;<sup>370</sup> Scheme 112). Several halogenase enzymes with varying regioselectivity were employed, which allowed development of a regiodivergent catalytic platform.<sup>376</sup>

The one-pot synthesis of cyclic malonic acid monoesters was designed over a two-step sequential cascade, involving transition-metal-catalyzed olefin metathesis followed by regioselective enzymatic hydrolysis using pig liver esterase (Scheme 114).<sup>377</sup> A water-compatible version of the Grubbs Ru catalyst allowed the

use of aqueous media for both stages. The reaction gave access to five-membered ring and six-membered ring products; however, the enzymatic reaction with larger rings proceeded slower, and the enzyme could not differentiate between the two enantiomers, yielding the final product with low ee value (symmetric five-membered rings yield nonchiral products).

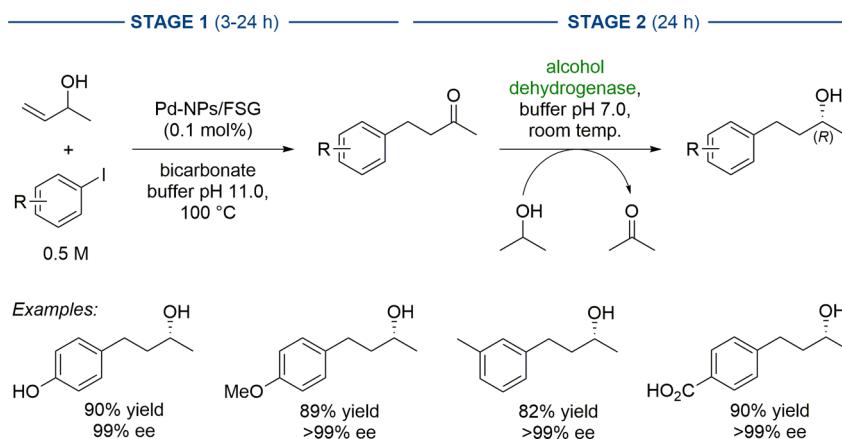
Olefin metathesis and enzymatic decarboxylation are the reaction steps leading to polysubstituted stilbene derivatives in a sequential cascade (Scheme 115).<sup>98</sup> Encapsulation of the decarboxylase in a cryogel was required to allow the presence of organic solvent necessary for good efficiency of the metathesis reaction while at the same time providing an aqueous environment for the enzyme. Although the reaction was performed in one pot, it required an intermediate drying step,

**Scheme 117. Sequential Cascade Comprising Enzymatic Ketone Reduction and Cu-Catalyzed Click Chemistry Leading to 1,2,3-Triazole-Derived Diols<sup>a</sup>**



<sup>a</sup>(R,S)- and (R,R)-isomers were obtained using ADH-A from *R. ruber*; (S,S)-isomers were obtained using LBADH from *L. brevis*.

**Scheme 118. Heck Reaction Followed by Enzymatic Reduction Leading to (R)-Rhododendrol Derivatives<sup>a</sup>**



<sup>a</sup>Pd<sub>np</sub>/FSG: perfluoro-tagged palladium nanoparticles immobilized on fluorous silica gel through fluorous–fluorous interactions. Alcohol dehydrogenase from *L. brevis*.

which was performed by addition of anhydrous salt before supplementing the ruthenium catalyst.

Ru-catalyzed isomerization of allylic alcohols was combined to enzymatic reductive transamination employing a transaminase, offering a sequential approach in aqueous media to chiral amines. The isomerization step was performed under inert atmosphere (argon) at high substrate loading and followed by dilution to reach operative concentration of the ketone for the enzymatic step (Scheme 116).<sup>378</sup>

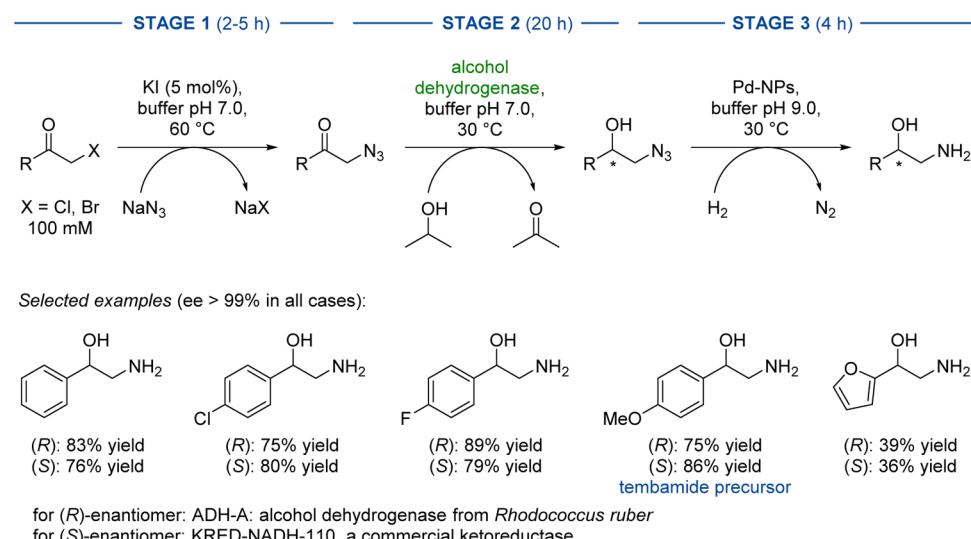
Copper was already mentioned as catalyst for click chemistry in a simultaneous cascade leading to triazol formation (Scheme 102) as well as in a sequential cascade starting from haloketones.<sup>355</sup>

Starting directly from  $\alpha$ -azido ketones and alkynes, an alcohol dehydrogenase (ADH) was employed for the stereoselective conversion of both ketones to the two corresponding chiral alcohols, which reacted subsequently in copper-catalyzed [3 + 2] cycloaddition to give the 1,2,3-triazole-derived diols (Scheme 117).<sup>379</sup> The final configuration of the product was

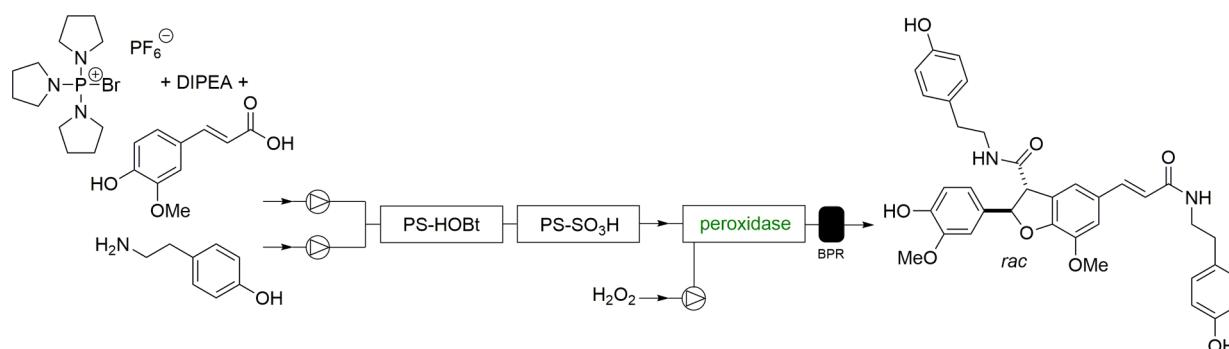
controlled by the biocatalyst, and (R,S), (R,R) and (S,S)-isomers were obtained in high yields and optical purity.

The Pd-catalyzed Heck reaction of aryl iodides with allylic alcohols was successfully linked to the enzymatic reduction of intermediate ketones in aqueous medium in a sequential fashion in one pot and applied to the synthesis of (R)-(-)-rhododendrol and derivatives thereof (Scheme 118).<sup>380</sup> The Heck reaction was catalyzed by Pd nanoparticles immobilized on fluorous silica gel (allowing their recycling) under aerobic conditions in water and followed by pH adjustment and addition of the alcohol dehydrogenase.

Pd was also used in the following cascade but this time for reduction of an azide moiety to the corresponding amino functionality. The synthesis of chiral 1,2-amino alcohols was performed in three steps/stages starting from 2-halo ketones by integrating in situ formation of azido ketones, subsequent enzymatic reduction to azido alcohols by alcohol dehydrogenase, and Pd-catalyzed hydrogenation to amino alcohols in a one-pot sequential approach (Scheme 119).<sup>381</sup> The hydrogenation was performed employing lignin-stabilized nanoparticles. The choice

Scheme 119. Synthesis of Enantiopure 1,2-Amino Alcohols in Three-Step One-Pot Reaction<sup>a</sup>

<sup>a</sup>Pd–NP: palladium nanoparticles.

Scheme 120. Synthesis of Grossamide by Continuous Flow Process through Amide Coupling Followed by Peroxidase-Catalyzed Oxidative Dimerization and Intramolecular Cyclization<sup>a</sup>

<sup>a</sup>PS-HOBt: polymer-supported hydroxybenzotriazole. PS-SO<sub>3</sub>H: sulfonic acid resin. Peroxidase: immobilized horseradish peroxidase on silica. BPR: back-pressure regulator.

of biocatalyst could be adapted to afford both enantiomers of the final product. The protocol was applied to the gram-scale synthesis of (S)-tembamide in a four-stage sequence, whereby the fourth stage was the benzylation of the amine, and afforded 0.98 g of the product in 73% yield and enantiopure form.

#### 4.3. Chemoenzymatic Cascades in Flow

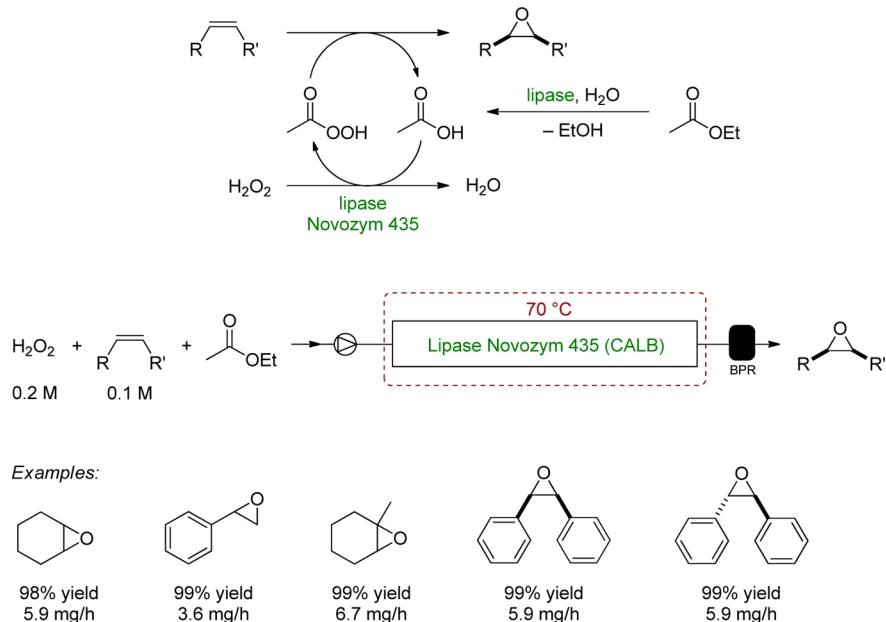
An introduction to cascades in flow has been given in section 3.3. Here the focus is on cascades encompassing an enzymatic and at least one chemical step.

The continuous flow synthesis of neolignan grossamide, a natural product, paved the way in demonstrating the possibility to perform a total synthesis in a fully automated and scalable flow reactor (Scheme 120).<sup>382</sup> To that end, three types of reactors were employed. The first two columns contained a polymer-supported hydroxybenzotriazole whereby the third reactor was packed with an immobilized peroxidase and flushed with hydrogen peroxide. This setup allowed in sequence the amide coupling of tyramine with ferulic acid; thereby ferulic acid was activated in the first column to an activated ester which reacted with the amine to the amide. The latter underwent in the third column oxidative dimerization and subsequent intramolecular cyclization to the final racemic trans product. An intermediate

column filled with sulfonic acid resin was used to remove unreacted amine by trapping.

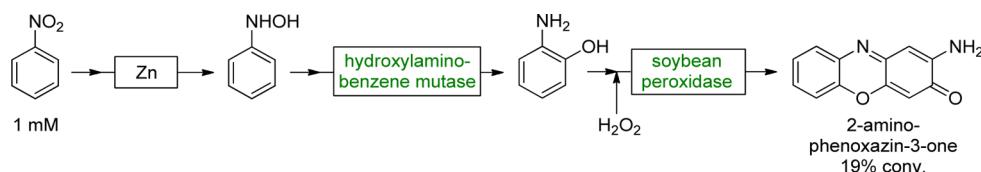
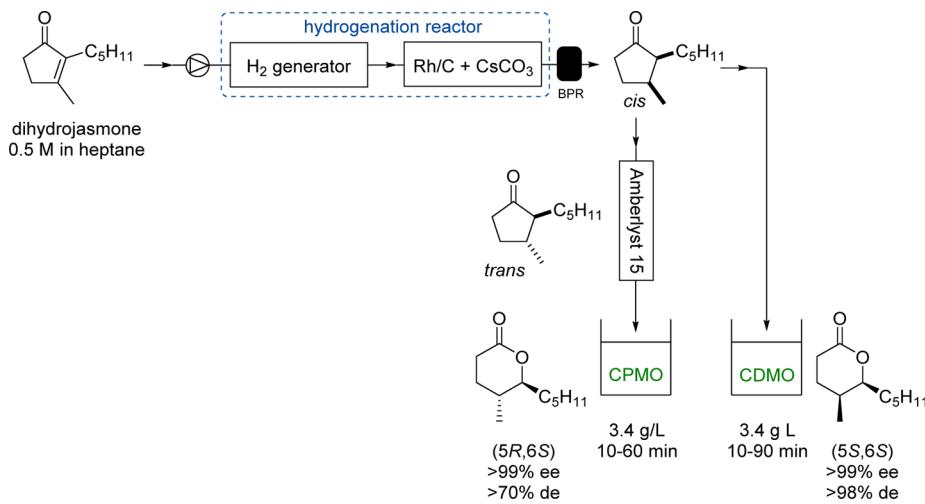
The lipase-mediated oxidation of unactivated alkenes to their corresponding epoxides was transferred to continuous flow reactor (Scheme 121).<sup>383</sup> The motivation was to reduce reaction time and at the same time allow the use of higher amounts of hydrogen peroxide without concomitant loss of enzymatic activity. The epoxidation of alkenes can be performed using catalytic amounts of carboxylic acids, which undergo lipase-catalyzed perhydrolysis in the presence of hydrogen peroxide to form the corresponding peroxycarboxylic acids, the ultimate oxidizing agent. Ethyl acetate was used as both solvent and reagent, which upon lipase-catalyzed hydrolysis furnished the required carboxylic acid. Several alkenes were successfully converted in a packed bed reactor consisting of a borosilicate glass column packed with Novozym 435 (immobilized *C. antarctica* lipase B). Compared to batch reactions, reaction times were dramatically reduced (up to 381-fold).<sup>384</sup>

The synthesis of 2-aminophenoxyazin-3-one (APO) from nitrobenzene was performed in a three-step sequence relying on three distinct microfluidic chips connected in series (Scheme 122).<sup>385</sup> The first chip contained Zn, which catalyzed the reduction of nitrobenzene to hydroxylaminobenzene, and was

Scheme 121. Continuous Flow Process for the Lipase-Mediated Oxidation of Alkenes<sup>a</sup>

<sup>a</sup>BPR: back-pressure regulator. Novozym 435: immobilized *C. antarctica* lipase B CALB.

Scheme 122. Chemoenzymatic Conversion of Nitrobenzene to 2-Aminophenoxazin-3-one in a Microfluidic Device Using Silica-Immobilized Enzymes

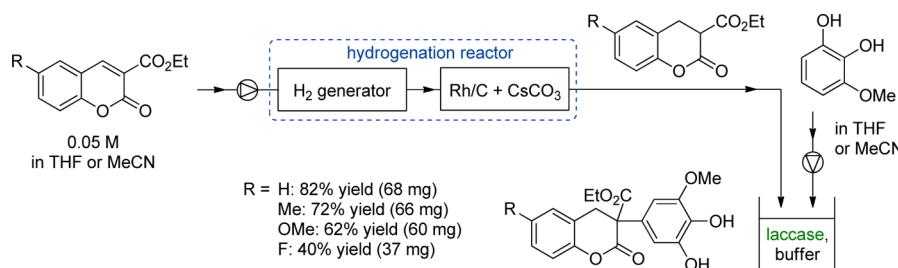
Scheme 123. Stereoselective Synthesis of *Aerangis* Lactones through Continuous Flow Hydrogenation/Enzymatic Oxidation Cascade<sup>a</sup>

<sup>a</sup>BPR: back-pressure regulator. CPMO: cyclododecanone monooxygenase from *R. ruber* SC1. CPMO: cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872.

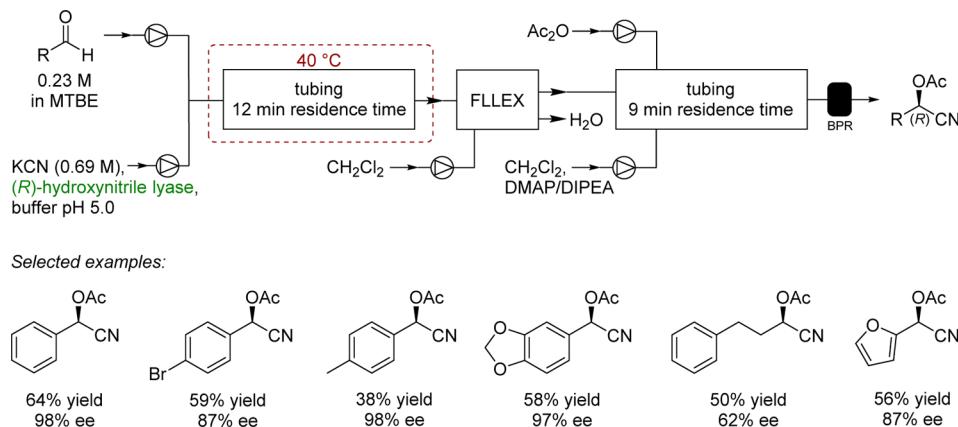
connected to the second chip on which silica-immobilized hydroxylaminobenzene mutase (HAB mutase) produced 2-aminophenol, which eventually dimerized to APO upon the action of soybean peroxidase immobilized on the third chip and hydrogen peroxide. Because of the divergent trend in catalytic

rate of the individual steps and flow rate, only 19% conversion could be obtained at  $150 \mu\text{L h}^{-1}$ ; however, the low efficiency was partly explained by loss of substrate through absorption, especially at lower rates. Overall, this technology offers the

**Scheme 124.** Synthesis of 3,4-Dihydrocoumarins through Continuous Flow Hydrogenation/Enzymatic–Oxidation/Michael Addition Sequence

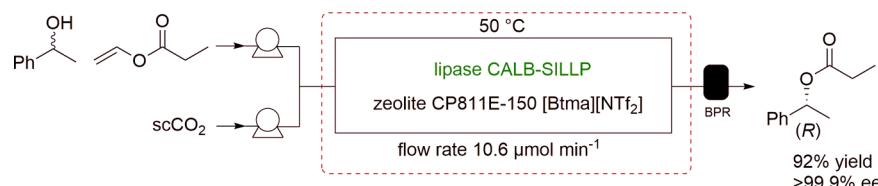


**Scheme 125.** Chemoenzymatic Flow Synthesis of Protected (*R*)-Cyanohydrins<sup>a</sup>



<sup>a</sup>FLLEX: flow liquid–liquid extraction. BPR: back-pressure regulator.

**Scheme 126.** Continuous Dynamic Kinetic Resolution of Racemic Phenylethanol in Supported Ionic Liquid Phase (SILLP)/Supercritical CO<sub>2</sub> (scCO<sub>2</sub>) System through Biocatalytic Esterification and Racemization by a Zeolite<sup>a</sup>



<sup>a</sup>BPR: back-pressure regulator. Btma: butyltrimethylammonium.

possibility of rapid screenings through the use of a “lab-on-a-chip” device.

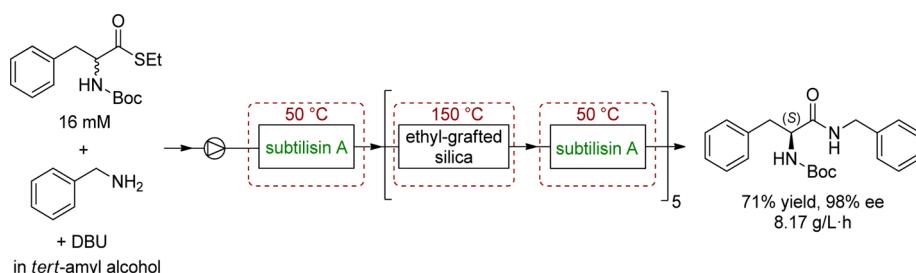
Continuous flow hydrogenation of dihydrojasmine was combined with enzymatic Baeyer–Villiger oxidation for the stereoselective synthesis of *Aerangis* lactones, which are used as aroma compounds (Scheme 123).<sup>386</sup> The first step consisted in *syn*-hydrogenation of the C=C double bond employing a cartridge filled with a heterogeneous catalyst mixture (Rh/C, Cs<sub>2</sub>CO<sub>3</sub>). Molecular hydrogen was furnished through electrolysis of water from a continuous-flow hydrogenation reactor. The system was designed to allow formation of the trans isomer in parallel, which was obtained by epimerization of this *cis*-saturated ketone through a glass column packed with an ion-exchange polymer displaying strong acidic properties (Amberlyst 15). The second step consisted of the kinetic resolution of formed saturated ketones using crude cell extracts of recombinant Baeyer–Villiger mono-oxygenases (BVMOs). This step was performed in a batch reactor flushed with the efflux of the hydrogenation or hydrogenation/epimerization. A BVMO specific for each isomer was identified, which permitted the

synthesis of both (5*S*,6*S*)- and (5*R*,6*S*)-isomers of the final *Aerangis* lactones in excellent enantiopurity (>99% ee) and high diastereoselectivity (70–98% de).

Using a related concept, continuous flow hydrogenation was combined with a laccase-catalyzed oxidation reaction for a consecutive approach toward arylation of dihydrocoumarins based on coumarins and catechols as starting materials (Scheme 124).<sup>387</sup> The system was composed of a continuous-flow hydrogenation reactor equipped with a catalyst cartridge containing Pd(OH)<sub>2</sub>/C. The efflux of the reduction containing the 3,4-dihydrocoumarin intermediate product was directed to a batch reactor containing the laccase, and a catechol solution was pumped in parallel into the reaction mixture, leading to the enzyme-catalyzed formation of *o*-quinones with aerial oxygen and allowing the oxidation/Michael addition sequence to proceed. A range of 3-arylated 3,4-dihydrocoumarins was obtained in good to high yields (40–82%).

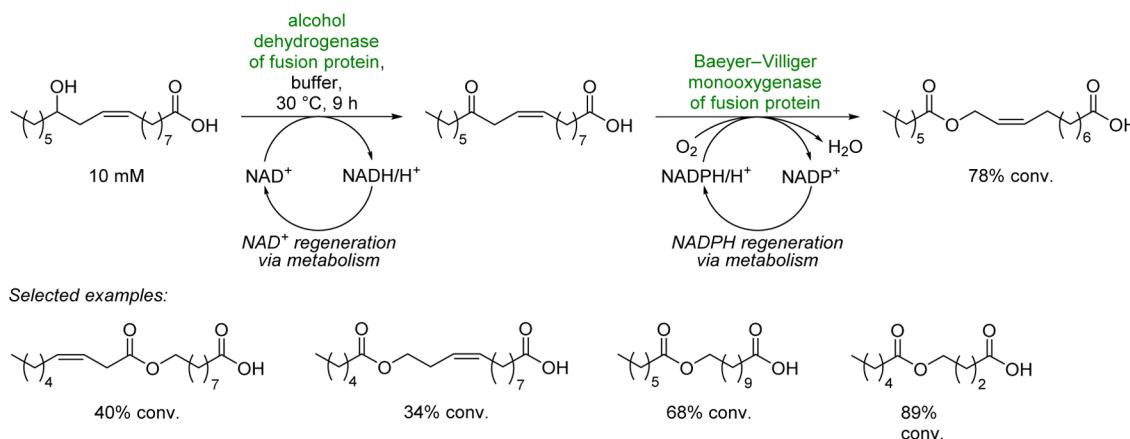
The chemoenzymatic formation of protected cyanohydrins was developed as a continuous flow process, relying on an inline separation module, which allowed the lining up of two

**Scheme 127.** Formal Dynamic Kinetic Resolution of N-Boc-phenylalanine Ethyl Thioester by Serial Enzymatic Kinetic Resolution/Base-Catalyzed Racemization under Continuous Flow Conditions<sup>a</sup>



<sup>a</sup>DBU: 1,8-diazabicycloundec-7-ene.

**Scheme 128.** Single Catalyst Obtained by Fusion of the Two Cascade Enzymes To Transform a Hydroxy Acid to an Ester



incompatible steps (Scheme 125).<sup>388</sup> The motivation was to prevent the spontaneous racemization of enantiopure cyanohydrins, obtained from the stereoselective enzyme-catalyzed reaction of aldehyde and cyanide using (*R*)-selective hydroxymitrile lyase HNL, through protection of the free hydroxy group. Cyanohydrin formation occurred in the first module at 40 °C, followed by pumping through a flow liquid–liquid extraction module (FLLEX) with supply of CH<sub>2</sub>Cl<sub>2</sub>. As water was removed, the efflux was pumped into the last module, together with reagents and solvent for the protection step (acetylation), delivering final products in good yield and ee values up to 98%. This protocol was subsequently extended by introducing *in situ* generation of HCN through lipase-catalyzed hydrolysis of ethyl cyanoformate and making use of immobilized enzymes in packed bed reactors in both steps, thus allowing recycling of the two biocatalysts.<sup>389</sup>

The kinetic resolution of *rac*-phenylethanol catalyzed by *C. antarctica* lipase B (CALB) through enantioselective esterification combined with alcohol racemization by an acidic zeolite (Scheme 126) was adapted to flow process conditions using supercritical CO<sub>2</sub> (scCO<sub>2</sub>).<sup>390,391</sup> To protect and conserve the activity of the enzyme in supercritical fluids, ionic liquids (ILs) were employed as stabilization and immobilization agent, affording supported ionic-liquid phase (SILLP) to be used in the reactor (biphasic IL/scCO<sub>2</sub>). Since the zeolite showed substantial nonselective esterification activity, physical separation of the two catalysts was required for efficient dynamic kinetic resolution (DKR). Therefore, the zeolite catalyst was coated in a different IL than that used with the lipase so that both catalysts could be combined in one reactor. The process was run at 50 °C under 10 MPa scCO<sub>2</sub> and allowed isolation of (*R*)-phenylethyl

propionate in high yield (92%) and excellent enantiopurity (>99.9% ee).

The formal dynamic kinetic resolution (DKR) of racemic N-Boc-phenylalanine ethyl thioester was performed in continuous flow mode through the combination of alternating kinetic resolution and racemization in a sequence of packed bed reactors (Scheme 127).<sup>392</sup> Immobilized alcalase (Subtilisin A) catalyzed the enantioselective amidation of the thioester using benzylamine as nucleophile in the first reactor at 50 °C. Subsequent base-catalyzed racemization of the remaining substrate in an ethyl-grafted silica gel-filled reactor at 150 °C was followed again by kinetic resolution in the next alcalase-containing reactor. In total, a succession of 11 reactors separated in two blocks for control of individual temperature was used to allow serial kinetic resolution/racemization cascade in a multicolumn continuous flow reactor.

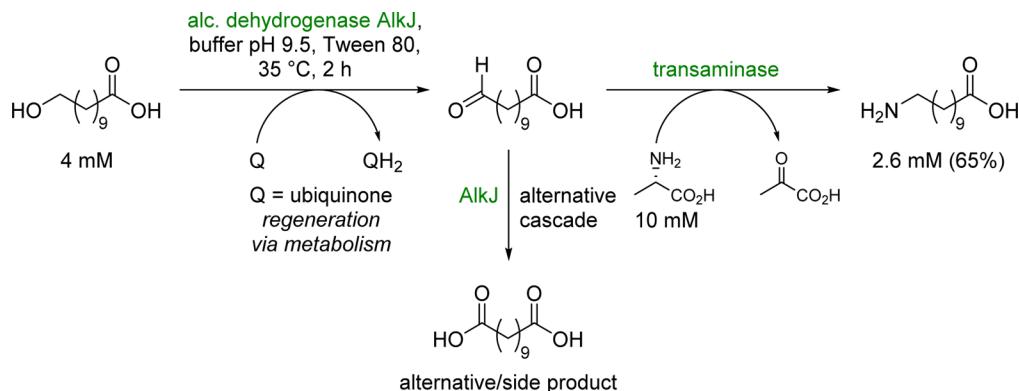
## 5. ARTIFICIAL IN VIVO CASCades

Reasons for using an *in vivo* system can be (i) the recycling of the cofactors for the artificial cascade and then the *in vivo* cascade involves at least one redox step or (ii) the cascade relies on a metabolite as substrate or reagent. In particular, for the incorporation of P450 enzymes in cascades, *in vivo* systems are broadly applied. Only examples of artificial cascades will be discussed here, while the engineering of complete natural pathways will not be discussed here.<sup>393–396</sup>

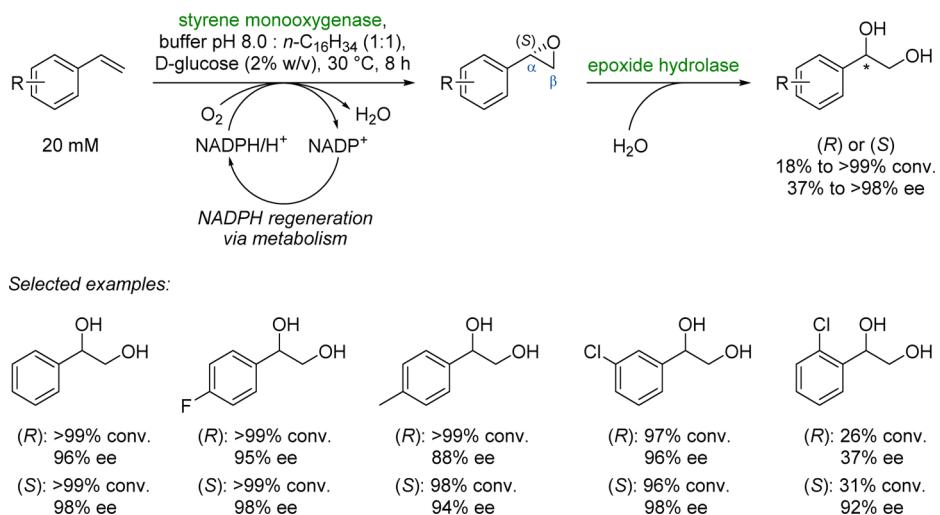
### 5.1. One Catalyst in the Linear Sequence

Formally, a single catalyst with multiple activities may also be obtained by linking of the involved enzymes to one another. For instance, an ADH from *Micrococcus luteus* NCTC2665 was fused with a BVMO from *P. putida* KT2440 via a 12 amino acid

**Scheme 129.** Conversion of an  $\omega$ -Hydroxycarboxylic Acid into the Corresponding  $\omega$ -Aminocarboxylic Acid by Two Redox Steps Using Membrane-Associated Alcohol Dehydrogenase AlkJ and a Transaminase

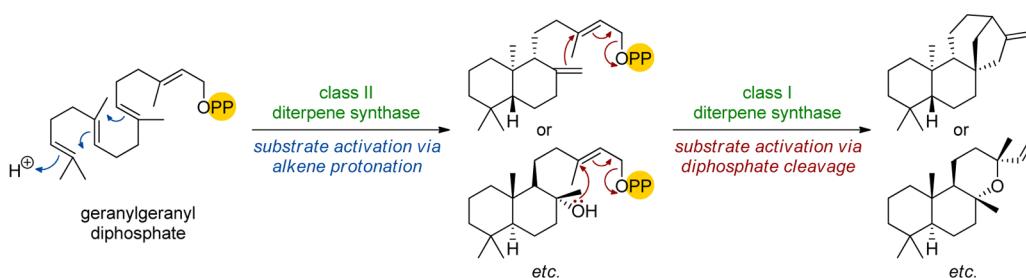


**Scheme 130.** Bienzymatic Cascade Transforming Styrenes to Vicinal Diols via One Redox and One Hydrolytic Step<sup>a</sup>

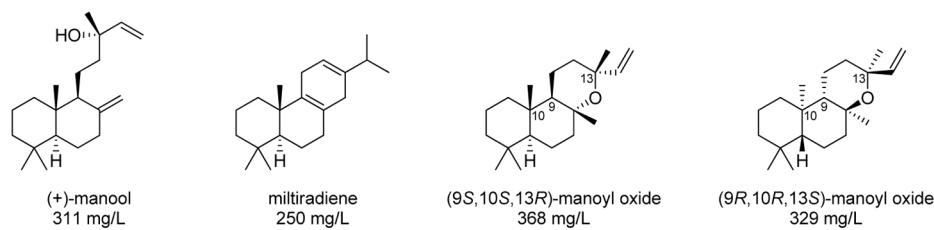


<sup>a</sup>Resting *E. coli* cells (10 g cdw/L) coexpressing both enzymes were employed.

**Scheme 131.** Synthesis of Diterpenes by Combinatorial Biocatalysis



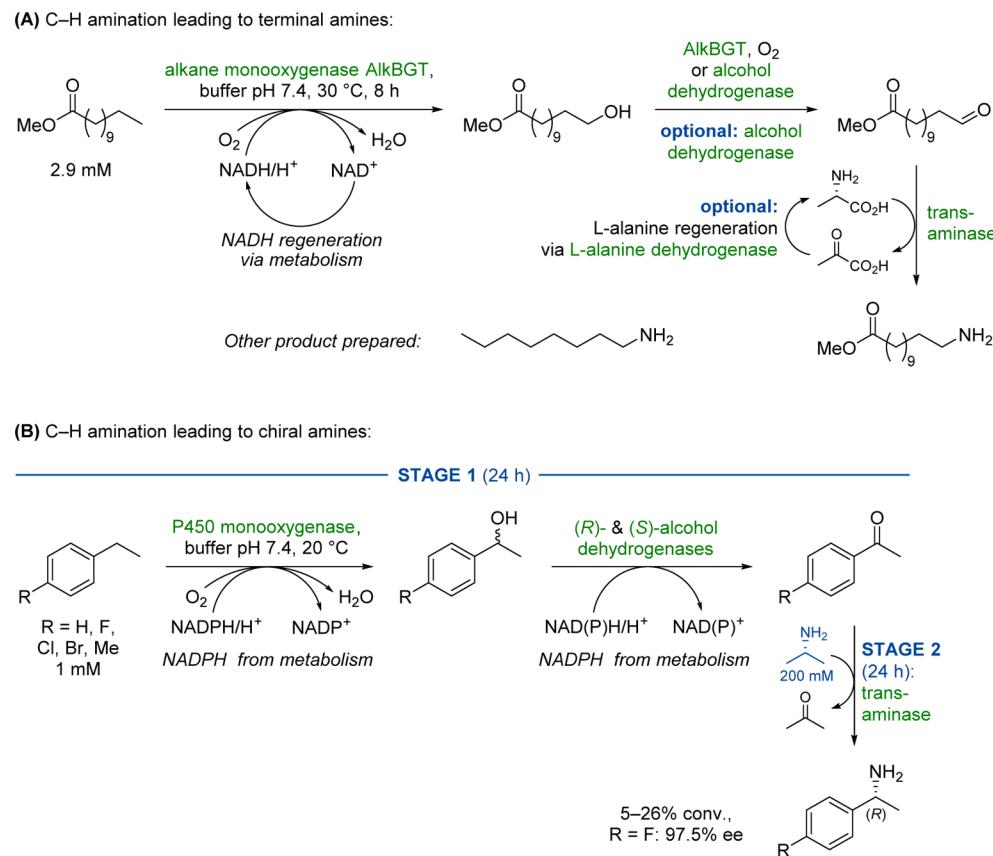
Products obtained by fermentation using engineered *S. cerevisiae*:



-containing glycine-rich linker to achieve the conversion of alcohols into esters (Scheme 128).<sup>145</sup> The fusion was expected to enhance the substrate conversion due to the decreased

theoretical distance between the active sites but also improve soluble protein expression, especially of the BVMO. Applying fermenting *E. coli* cells expressing the ADH-BVMO fusion

**Scheme 132. C–H Amination Leading to Terminal or Chiral Amines via Hydroxylation, Alcohol Oxidation, and Amination Using Transaminases**



protein, 10 mM ricinoleic acid was converted with 78% to the ester within 9 h. Related substrates were transformed with conversions of 34–91% within the same time frame.

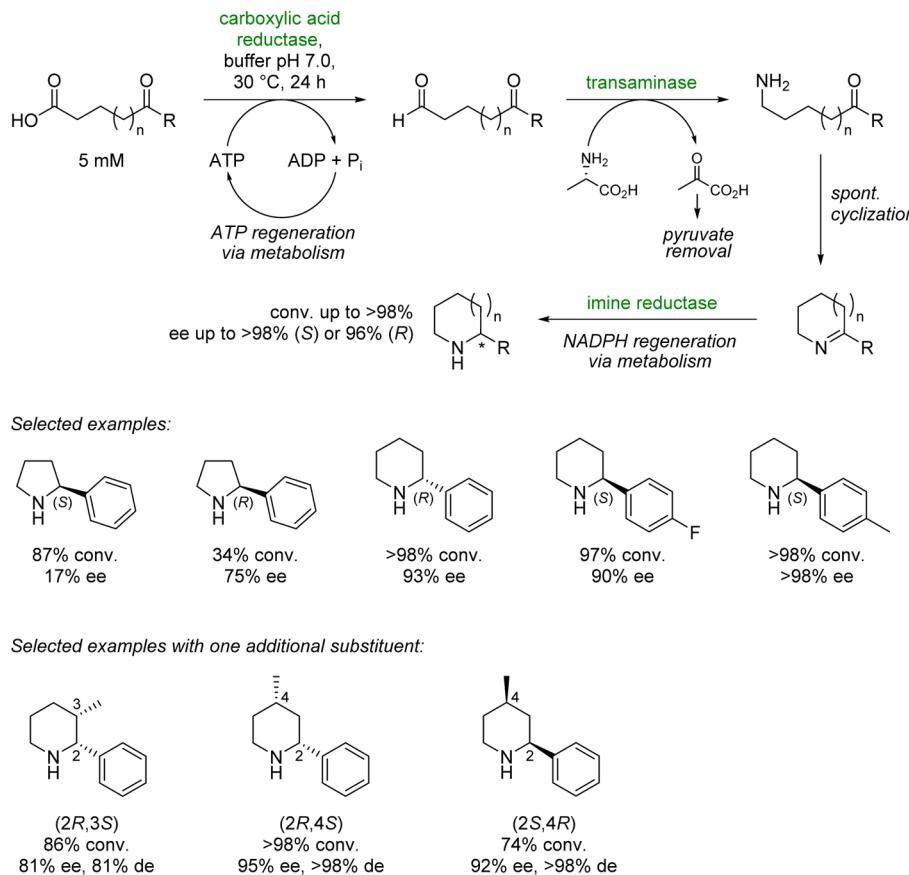
## 5.2. Two Catalysts in the Linear Sequence

**5.2.1. Redox Cascades—2 Redox Steps.** Amination of alcohols has become a broadly investigated cascade (see *in vitro* cascades and *in vivo* cascades with three catalysts). For instance, using the *in vivo* approach with two enzymes, 12-hydroxylodecanoic acid was converted into the corresponding  $\omega$ -amino-carboxylic acid. The alcohol-to-amine interconversion was realized using an *E. coli* whole-cell biocatalyst coexpressing the membrane-associated alcohol dehydrogenase AlkJ from *P. putida* GPo1 and a transaminase from *S. pomeroyi* (Scheme 129).<sup>397</sup> Using 7.2 g/L (cell dry weight) of resting cells, 4 mM substrate was transformed into 2.6 mM (65%) 12-aminododecanoic acid within 2 h. 1,12-Dodecanedioic acid (0.8 mM) was formed as a side product, which was attributed to an overoxidation activity of the alcohol dehydrogenase AlkJ. Exploiting the overoxidation, dicarboxylic acids were obtained for fatty acids bearing 9–13 carbons with 21–46% isolated yield.

**5.2.2. Redox Cascades—1 Redox Step.** The biotransformation of styrene derivatives to diols was performed in a two-phase system consisting of buffer and *n*-hexadecane in a 1:1 mixture using resting *E. coli* whole cells coexpressing the two enzymes of the cascade, namely, a styrene monooxygenase (SMO) from *Sphingomonas* sp. and an epoxide hydrolase (EH) (Scheme 130).<sup>398</sup> By the choice of a suitable epoxide hydrolase, both the (S)-diol as well as the (R)-diol could be prepared from the (S)-epoxide due to complementary regioselectivity. Thus, opening of the (S)-epoxide by attack of water at the  $\alpha$ -carbon

atom led to inversion of configuration and hence afforded the (R)-diol, while epoxide hydrolysis via attack at the  $\beta$ -carbon proceeded with stereochemical retention to give the (S)-diol. Regeneration of the nicotinamide cofactor required for the monooxygenation step relied on cell metabolism, with D-glucose being added as sacrificial cosubstrate. For instance, (S)-1-phenylethane-1,2-diol ( $R = H$ ), a synthetic precursor of the antidepressant (R)-fluoxetine, was obtained with >99% conversion and 98% ee.

Very recently, both natural and non-natural diterpenes were accessed via combinatorial biocatalysis permutating two types of enzyme (Scheme 131).<sup>399</sup> The combinatorial approach is based on class II (set of 11 enzymes) and class I diterpene synthases (set of nine enzymes) from various plant sources. The class II enzymes cyclize geranylgeranyl diphosphate (GGPP) to a variety of bicyclic diphosphate intermediates that all contain a characteristic decalin core structure. These intermediates are substrates for the class I diterpene synthases, which trigger further cyclization, hydration, and rearrangement reactions by cleavage of the diphosphate group. In an initial *in vivo* screening in *Nicotiana benthamiana* the class II and class I enzymes were paired in every possible combination, and the resulting diterpene product profile was determined by GC-MS analysis, which revealed besides the formation of naturally occurring diterpenes also non-natural stereoisomers and entirely unknown structures. The results thus obtained were corroborated using recombinant enzymes expressed and purified from *E. coli*, and finally, a selection of representative reactions was performed by fermentation of engineered *S. cerevisiae* coexpressing the required

Scheme 133. Cascade Comprising a Carboxylic Acid Reductase, a Transaminase, and an Imine Reductase<sup>a</sup>

<sup>a</sup>The carboxylic acid conversion step relies on ATP recycling by the metabolism of the host *E. coli*.

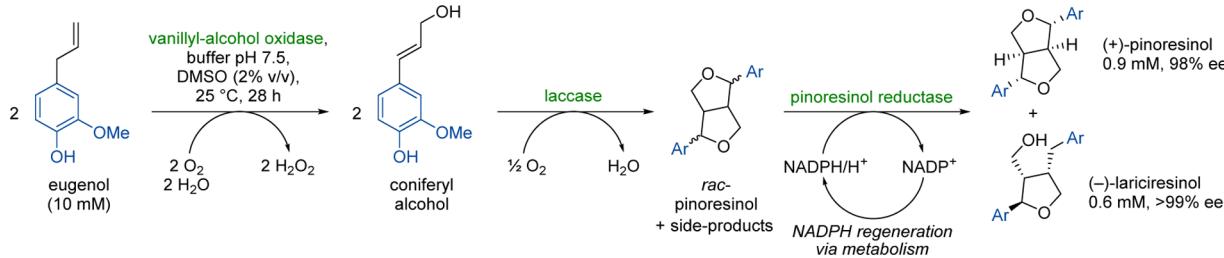
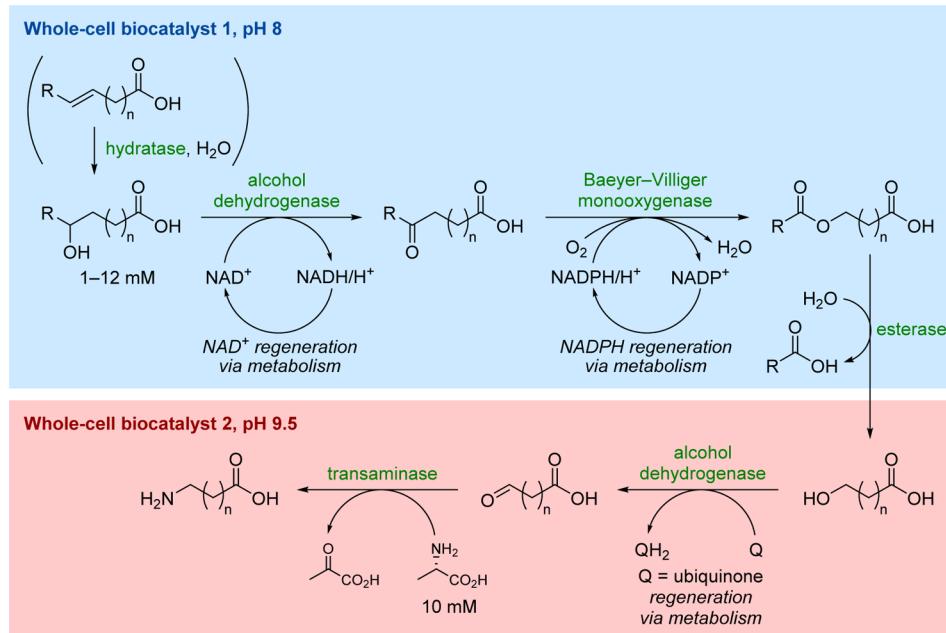
diterpene synthases. The products accumulated to final titers of 250–370 mg/L.

### 5.3. Three Catalysts in the Linear Sequence

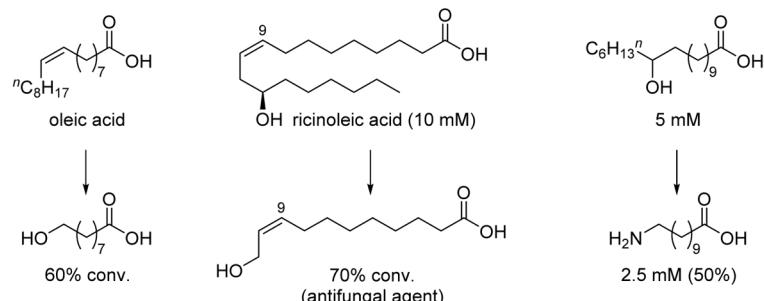
The overall amination of a C–H bond was initiated by a P450 enzyme, leading to the hydroxylated product alcohol, which was followed by alcohol oxidation and amination (for the amination of alcohols see also *in vitro* cascades section 3). For instance, alkane monooxygenase AlkBGT from *P. putida* Gpo1 was coexpressed with a transaminase from *C. violaceum* in *E. coli*, and resting cells of the recombinant biocatalyst were used for the synthesis of 12-aminododecanoic acid methyl ester (Scheme 132A),<sup>400</sup> a polymer building block.<sup>401</sup> The monooxygenase AlkBGT was able to catalyze two subsequent transformations, namely, the hydroxylation in the first step and the alcohol oxidation to the aldehyde in the second step. Additionally, AlkBGT was also responsible for an undesired side reaction, the overoxidation of the aldehyde to the diacid monoester.<sup>402</sup> The cofactor required by the monooxygenase, NADH, was provided via cell metabolism; NADH was also required for removal of the transamination byproduct, pyruvate. However, the amine donor for the transaminase, L-alanine, had to be supplied externally in a concentration of 50 mM. Using 1.4 g/L (cell dry-weight) of the biocatalyst, 0.13 mM (4.5%)  $\omega$ -amino ester was produced from 2.9 mM methyl dodecanoate within 90 min, along with equal levels of the diacid monoester. Extension of the substrate scope to nonfunctionalized hydrocarbons was also possible, as demonstrated by the production of 1-octylamine (0.22 mM) from *n*-octane (1.4 mM).<sup>403</sup> The productivity of the whole-cell biocatalyst was substantially increased by following three

optimization strategies: (i) Introduction of the outer membrane protein AlkL from *P. putida* Gpo1 led to improved substrate uptake by the cells, which in turn increased the reaction rate of all productive biotransformations 6.8–8.1-fold; (ii) coexpression of L-alanine dehydrogenase from *B. subtilis* enabled the biocatalyst to use NH<sub>4</sub>Cl as terminal nitrogen source for the transamination, although the reactions in this case were slower compared to those carried out in the presence of externally added L-alanine; and (iii) introducing an alcohol dehydrogenase (AlkJ from *P. putida* Gpo1), which was linked to the electron transport chain and hence catalyzed irreversible alcohol oxidation, increased the intracellular concentration of the intermediate oxo-ester, and thereby made the transamination step considerably faster. The combined effects of these three adjustments increased the formation rate of product 10-fold and its analytical yield per unit biomass 3.5-fold.

For the amination of ethylbenzene derivatives in benzylic position, a similar concept was applied (Scheme 132B) using resting *E. coli* whole cells coexpressing all used enzymes.<sup>404</sup> In this system, hydroxylation was catalyzed by a self-sufficient P450 monooxygenase variant, P450cam Y96F,<sup>405</sup> while a pair of stereocomplementary alcohol dehydrogenases, (R)-selective ADH from *L. brevis* and (S)-selective ADH from *R. erythropolis*, was responsible for oxidation of the intermediate chiral alcohol to ketone. The final transamination step was catalyzed by an (R)-selective transaminase from *Arthrobacter* sp. 2-Propylamine (200 mM) was used as the amino donor, but since it inhibited the P450 reaction, it was added only after 24 h, once the conversion of the ethylbenzene derivative (1 mM initial concentration) into

Scheme 134. Synthesis of Optically Pure (+)-Pinoresinol Using *E. coli* Containing the Three Coexpressed EnzymesScheme 135. Five/Six-Step Sequence Using Two *E. coli* Hosts for the Transformation of Fatty Acids Bearing a *sec*-Alcohol to  $\omega$ -Amino Carboxylic Acids

Selected examples:



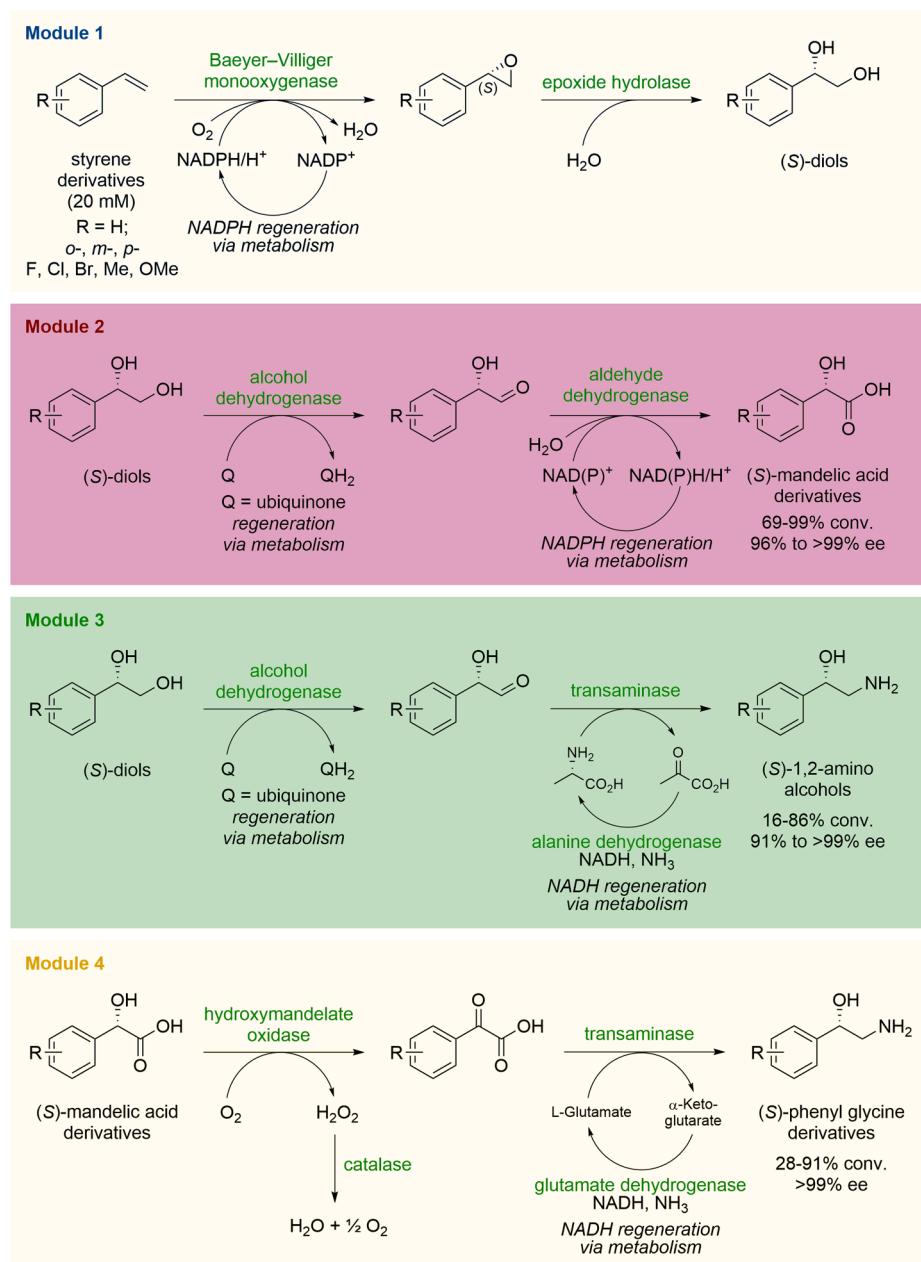
the ketone was completed. Thus, the third reaction step was performed in a sequential manner. After another 24 h, the (*R*)-amines were obtained with 5–26% conversion and in excellent enantiomeric purity (ee for R = F: 97.5%).

$\omega$ -Hydroxy acids have also been obtained by using glucose as starting material, exploiting *E. coli* fatty acid metabolism (reaction scheme not shown).<sup>406</sup> With the focus on medium chain  $\omega$ -hydroxy fatty acids (C12 and C14), selective thioesterases were employed releasing the fatty acids with the specific chain length. The fatty acids were then  $\omega$ -selectively hydroxylated by an engineered cytochrome P450 enzyme from *Marinobacter aquaeolei* which produced 111 mg/L of 12-hydroxydodecanoic acid and 25 mg/L of its C14 counterpart when 367 and 230 mg/L of the free fatty acid were employed, respectively. Starting from

glucose and oxidizing the  $\omega$ -hydroxy acid further to the corresponding dicarboxylic acid allowed one to obtain up to 600 mg/L C12-dicarboxylic acid.

The cascade for the synthesis of disubstituted piperidines and pyrrolidines involving regioselective aldehyde–amination of keto aldehydes catalyzed by transaminases and subsequent stereoselective reduction of the generated cyclic imines by imine reductases (see Scheme 78) has been extended to start from the corresponding carboxylic acids (Scheme 133).<sup>308</sup> The biocatalytic reduction of the carboxylic acids was achieved by a carboxylic acid reductase from *M. marinum* overexpressed in *E. coli*. The metabolism of resting *E. coli* cells was exploited for cofactor recycling (ATP), while the other two steps actually

**Scheme 136. Modularized Multienzyme In Vivo System for the Synthesis of (S)-1,2-Diols, (S)- $\alpha$ -Hydroxy Acids, (S)-1,2-Amino Alcohols, or (S)- $\alpha$ -Amino Acids from Styrene Derivatives**

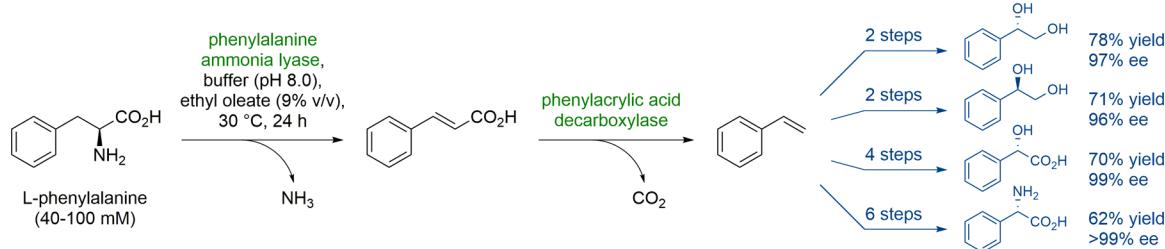


constitute an *in vitro* cascade. This cascade is thus made up of an *in vivo* module as well as an *in vitro* module.

Access to optically enriched pinoresinol was achieved by extending a two-enzyme cascade described for the synthesis of racemic pinoresinol (**Scheme 35**)<sup>209</sup> by a kinetic resolution step (**Scheme 134**). The latter was achieved by a reductive opening of the tetrahydrofuran ring using enantioselective enzymes leading to either (+)- or (-)-pinoresinol.<sup>212</sup> Pinoresinol reductase from *A. thaliana* was employed for the synthesis of (+)-pinoresinol and pinoresinol-lariciresinol reductase from *F. intermedia* for the synthesis of the opposite enantiomer. By performing the cascade in a sequential mode with resting *E. coli* cells as biocatalyst expressing the necessary enzymes, 876  $\mu$ M (+)-pinoresinol with an ee of 98% and 610  $\mu$ M (-)-pinoresinol with an ee > 99% were produced within 28 h starting from 10 mM eugenol.

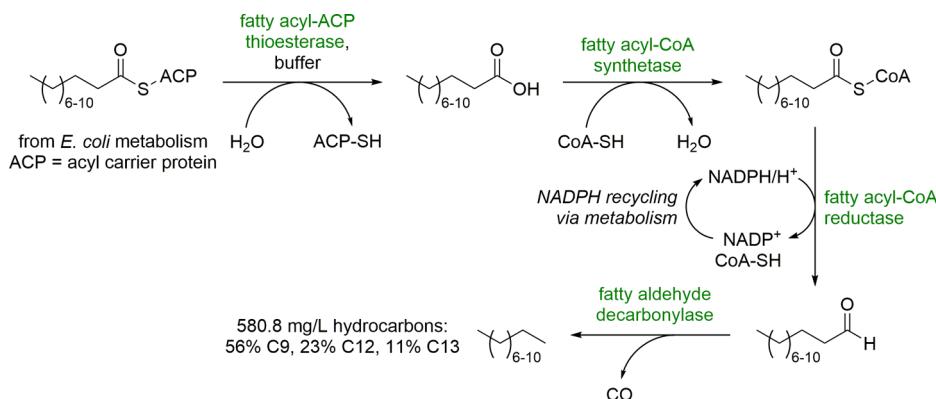
#### 5.4. Four and More Catalysts in the Linear Sequence

Starting from either fatty acids bearing a *sec*-alcohol moiety or unsaturated fatty acids, the substrates were shortened to access terminal hydroxy fatty acids in a three- or four-step cascade, respectively (**Scheme 135**, whole-cell biocatalyst 1).<sup>407</sup> The enzymes required were coexpressed in a whole-cell catalyst. Thus, the sequence starting from unsaturated fatty acids first required hydration to give *sec*-hydroxy fatty acids; *sec*-hydroxy fatty acids were then transformed into the corresponding  $\omega$ -hydroxycarboxylic acids via alcohol oxidation, Baeyer–Villiger monooxygenation, and ester hydrolysis. Conversion ranged from 60% to 70% at 1 mM substrate concentration. Several fatty acids, such as 5-hydroxydecanoic acid, lesquerolic acid, and linoleic acid, were transformed by this cascade in a similar range of conversion. Treating 5 g/L olive oil with lipase generated a hydrolysate containing 11.3 mM oleic acid, and subsequent

Scheme 137. Formation of Styrene from L-Phenylalanine and Further Conversion into 1,2-Difunctionalized Derivatives<sup>a</sup>

<sup>a</sup>See also Scheme 136.

Scheme 138. Degradation of Fatty Acids Obtained from Metabolism for the Production of Short Chain Alkanes



biotransformation yielded 9-hydroxynonanoic acid with 60% conversion. In a follow-up study,<sup>408</sup> the expression protocol and the biotransformation conditions were optimized for the transformation of ricinoleic acid to 11-hydroxyundec-9-enoic acid, which has potential as an antifungal agent.<sup>409</sup> Employing substantially increased concentrations of the substrate ricinoleic acid (10 mM) and the biocatalyst (>10 g cell dry-weight per liter), a final concentration of 4.0 g/L 11-hydroxyundec-9-enoic acid was reached.

In a very similar strategy, 13-hydroxy-*cis*-9-octadecenoic acid was transformed into 12-hydroxy-*cis*-9-dodecanoic acid using resting *E. coli* cells coexpressing an ADH from *Micrococcus luteus* NCTC2665, a Baeyer–Villiger monooxygenase from *P. fluorescens* DSM 50106, and an esterase from *P. fluorescens* SIK WI.<sup>410</sup> After 4 h the product was obtained in 90% conversion starting from 2 mM substrate.

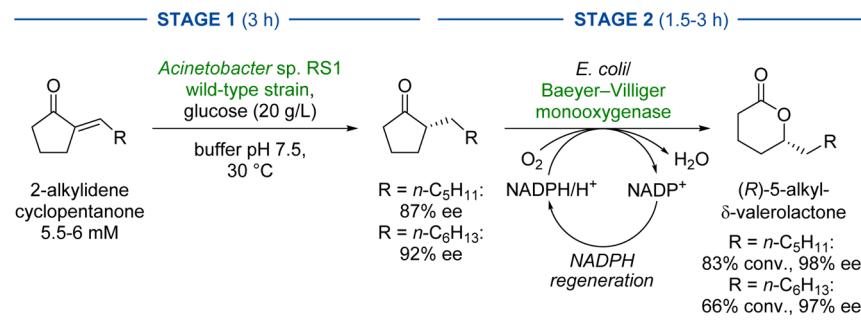
As an extension, the product of the first *E. coli* whole-cell catalyst was transformed further in a second *E. coli* whole-cell catalyst to the  $\omega$ -amino carboxylic acids (Scheme 135, whole-cell biocatalyst 2).<sup>407</sup> This second module was realized as already described in section 4.1.<sup>397</sup> After adjusting the pH of the reaction medium from 8.0 to 9.5, the amination biocatalyst and L-alanine were added. By this sequence, 5 mM 12-hydroxystearic acid was transformed into 2.5 mM 11-aminoundecanoic acid within less than 3 h.

An even more extensively modularized multienzyme system allowed a high flexibility to transform styrene derivatives to diols (Scheme 136, module 1),  $\alpha$ -hydroxy acids (module 2), 1,2-amino alcohols (module 3), as well as  $\alpha$ -amino acids (module 4).<sup>411</sup> For convenience, the genes required for each multienzyme module were encoded on a separate plasmid so that *E. coli* cells could be prepared with two or more plasmids as needed. For the biotransformation, resting *E. coli* cells were employed and all reaction sequences started with the conversion of the styrene

into the vicinal diol via epoxidation by styrene monooxygenase and hydrolysis of the intermediate epoxide by an epoxide hydrolase (module 1). The 1,2-amino alcohols were obtained by combining module 1 with module 3; the latter encoded the already discussed combination of alcohol dehydrogenase, transaminase, and alanine dehydrogenase (module 3). Multienzyme sequences for the transformation of styrenes into  $\alpha$ -hydroxy acids required four enzymes in the linear sequence (module 1 + 2), while the transformation into  $\alpha$ -amino acids (module 1 + 2 + 4) required six enzymes in the linear sequence. For instance, an *E. coli* strain containing modules 1 and 3 converted 60 mM of styrene ( $R = H$ ) into 42 mM (70%) of the corresponding (S)-1,2-amino alcohol ( $R = H$ ) with an ee of 98%. The reactions were demonstrated for a range of aryl-substituted styrene derivatives with good conversions (16–81%) and excellent optical purities (from 91% to >99% ee) at 20 mM substrate concentration.

Very recently, the above-mentioned modular multienzyme pathways were extended by two upstream steps, which generated the starting material styrene from the amino acid L-phenylalanine.<sup>412</sup> Phenylalanine was first deaminated to cinnamic acid by phenylalanine ammonia lyase from *A. thaliana*. Phenylacrylic acid decarboxylase from *A. niger* then converted the cinnamic acid into styrene (Scheme 137). The coexpression of both enzymes using a single plasmid allowed a facile combination of their reactions with modules 1–4 (Scheme 136). Whole-cell biocatalysts harboring 2–4 plasmids, depending on the sequence, afforded (S)-styrene oxide, (S)- or (R)-1-phenylethane-1,2-diol, (S)-mandelic acid, or (S)-phenylglycine from L-phenylalanine in 85–92% conversion, 62–78% isolated yield, and from 96% to >99% enantiomeric excess over 3–8 enzymatic steps. Besides, formation of the same products directly from D-glucose has been achieved using these whole-cell biocatalysts, demonstrating that artificial cascade pathways can also be

**Scheme 139.** Sequential Cascade Using *In Vivo* Cofactor Recycling in the First Step for the Preparation of (*R*)- $\delta$ -Valerolactones Starting from 2-Alkylidene cyclopentanones



coupled to primary metabolism. However, the product titers attained by this approach are still very limited (0.7–1.6 mM).

To access short chain alkanes (C9–C13), which might find application as a petrol source, an engineered *E. coli* strain devoid of  $\beta$ -oxidation was used (Scheme 138).<sup>413</sup> This deletion strain showed an increased production of free fatty acids, which were further processed by an artificial degradation route to yield the desired hydrocarbons. The starting material fatty acyl-ACP was produced from glucose using *E. coli*'s metabolism. The subsequent transformations were performed in a four-step cascade *in vivo* combining a fatty acyl-ACP thioesterase and fatty acyl-CoA synthetase from *E. coli* followed by a fatty acyl-CoA reductase from *Clostridium acetobutylicum* and a fatty aldehyde decarbonylase from *A. thaliana*. In a fed-batch culture, 580.8 mg/L hydrocarbons was obtained composed of 327.8 mg/L nonane, 136 mg/L dodecane, 64.8 mg/L tridecane, 42.8 mg/L 2-methyl-dodecane, and 8.9 mg/L tetradecane. Furthermore, trace amounts of fatty alcohols and aldehydes, such as heptanol, decanol, and decanal, were also found.

**5.4.1. Sequential Mode.** The asymmetric reduction of prochiral  $\alpha,\beta$ -unsaturated ketones catalyzed by ene-reductases was combined with the Baeyer–Villiger oxidation of the resulting saturated ketones leading to chiral lactones (Scheme 139).<sup>414</sup> This concept was applied to the synthesis of 5-alkyl-substituted  $\delta$ -valerolactones, which are valuable flavor and fragrance compounds. The strain *Acinetobacter* sp. RS1 was identified as the best organism (in terms of activity, growth, and enantioselectivity) for the C=C reduction step by screening 200 alkane-, benzene-, or toluene-degrading microbial strains using glucose as final reducing agent (therefore, this cascade is described as *in vivo* cascade, although the second step has a defined cofactor recycling). The reaction reached >99% conversion after 2 h, producing saturated (*R*)-cyclopentanones with 87–92% ee. For the subsequent Baeyer–Villiger oxidation, resting cells of recombinant *E. coli* expressing a cyclohexanone monooxygenase (CHMO) and a glucose dehydrogenase (GDH) were employed. In preliminary experiments, an undesired oxidation activity on the substrates was observed, and consequently, the cascade was performed in a sequential mode. The improved enantiomeric excess of the final lactones compared to that of the intermediate cyclopentanones was shown to be the consequence of lactone hydrolysis catalyzed by the *Acinetobacter* cells, whereby the (*S*)-enantiomers of lactones were preferentially converted (*E* = 8–11). Hence, the optical purity of the desired products increased over time due to degradation of the minor enantiomer. Preparative-scale transformations of 40 mg of substrates were performed, and the corresponding lactones were isolated in 56% and 41% yield (98% and 97% ee), respectively.

## 6. OUTLOOK

Looking at the year of publication of the papers on artificial cascades in this review, it becomes apparent that there was a constant increase in the number of publications per year during the past decade (2007, ~8; 2010, ~20; 2013, ~50; 2016, ~60). Already between submission and revision of this review numerous further papers on cascades have been published.<sup>415–429</sup>

It can be assumed that this is a result of enzymes becoming more easily accessible, improved enzyme engineering methods, as well as improved expression tools. As a consequence, one can expect the number of artificial cascades to increase further and cascades to become more complex. At the moment, the majority of cascades have two reaction steps in the linear sequence. Complexity can easily be achieved by combining or extending these two reaction step cascades. Additionally, as more and more different types of enzymes become available, more cascades become feasible, leading to an exponential growth of cascade designs. Also, in addition to sugars, novel activated (e.g., phosphorylated) substrates may become more easily accessible, e.g., by novel/improved methods for ATP regeneration,<sup>430</sup> thereby opening a new avenue of possibilities.<sup>431</sup> Additionally, the number of cascades combining enzymes with established chemical reactions or organocatalysts and metal catalysts can be expected to grow. The review shows that in contrast to the large number of cascades already available, the number of biocascades performed in flow is still rather low; so, there is definitely potential.

Another opportunity to enlarge the field of artificial cascades may be to start from different feedstocks. Enzyme cascades may have advantages when renewable feedstocks are applied due to their high chemo-, regio-, and enantioselectivity transforming out of many functional group in the renewable substrate only a single functionality. Glucose may serve as the starting material for fermenting organisms providing metabolites, which will be further upgraded artificial cascades. Glucose is the most obvious feedstock, whose carbon atoms may also be rearranged in non-natural pathways, leading to valuable products.<sup>432</sup> However, also other feedstocks may become of interest, like methanol,<sup>433</sup> or fossil feedstocks, like butane.<sup>434</sup> In this recent study, butane was used for the preparation of so-called biosurfactants, namely, rhamnolipids, which possess 12 stereocenters. The overall reaction encompassed 25 steps and was demonstrated in one pot. This seems to indicate that the length, complexity, and virtuosity of artificial cascades is almost unlimited; what might be limiting for academia is the amount of funding for the projects; for industry the time/investment for development might be limiting. Thus, one may hope that funding agencies will

recognize the potential of this powerful tool and provide sufficient fuel for its growth.

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### Notes

The authors declare no competing financial interest.

### Biographies

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Stefan Velikogne, born 1988, completed the B.Sc. program of Chemistry at the University of Graz, Austria, in 2012. He then got trained in toxicology at the Charité Berlin for 6 months. After his return to Graz he enrolled in a Master's program in Biochemistry and Molecular Biomedicine, which he finished in 2015. Currently he is working on his Ph.D. thesis under the supervision of Prof. Wolfgang Kroutil at the University of Graz, focusing on biocatalytic oxime functionalization.

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## ABBREVIATIONS

ADH	alcohol dehydrogenase
Ar	argon atmosphere
ATP	adenosine triphosphate
BPR	back-pressure regulator
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DMAP	4-(dimethylamino)pyridine
DODAB	dioctadecyltrimethylammonium bromide
FLLEX	flow liquid–liquid extraction
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
Pd-NP	palladium nanoparticles
PLP	pyridoxal 5'-phosphate
PS-HOBt	polymer-supported hydroxybenzotriazole
PS-SO <sub>3</sub> H	sulfonic acid resin
rt	room temperature
μw	microwave

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