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Enzymatic Asymmetric Synthesis of All Stereoisomers of Aliphatic, Vicinal Diols in Conventional and Non-Conventional Media

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Abstract: Chiral, vicinal diols are of high interest for academic research and industrial applications. For synthesizing chiral diols, enzymes are important catalysts due to their high selectivity and ability to work under tolerable temperature and no pressure. In this study, two consecutive enzyme-catalyzed steps were used for the asymmetric synthesis of aliphatic, vicinal diols with high product concentrations and chiral purity. The reaction comprised a ligation step employing lyases and a subsequent reduction step using oxidoreductases. Either in an aqueous buffer or an organic solvent, the potentially biobased aldehydes acetaldehyde, propanal, butanal, and pentanal were used as substrates. Here, all possible stereoisomers of 2,3-butanediol, 3,4-hexanediol, 4,5-octanediol, and 5,6-decanediol were produced with *isomeric content* values between 72% and >99%, and concentrations between 4.1 and 115 mM. This work shows how four symmetric, chiral, vicinal diols can be synthesized by combining enzymes in a modular way, including exemplarily scaling.

Keywords: Aliphatic vicinal diols; Asymmetric synthesis; Carboligases; Enzymatic cascade; MARS; Oxidoreductases

Introduction

Aliphatic, vicinal diols are a group of valuable fine chemicals, with 2,3-butanediol as one of the most prominent members. It is currently produced based on butene of fossil origin in a non-stereoselective manner.[1] 2,3-Butanediol can also be produced using microorganisms in fermentation starting from feedstock, such as sugar or starch, as raw materials.[1] Besides biofuels, 2,3-butanediols are applied as food

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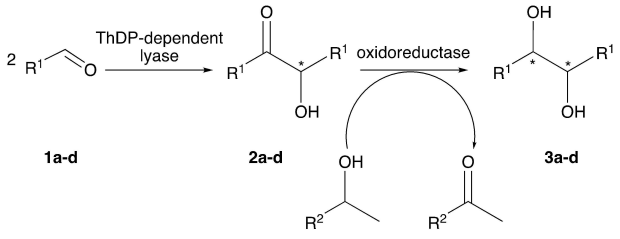
additives, printing inks, and pharmaceuticals.[2] Moreover, 1,2-hexanediol and (*R*)-1,2-butanediol are used in the industry as moisturizing agents and in synthesizing antiepileptic drug levetiracetam, respectively.[3–5] The synthesis of pure stereoisomers of symmetric diols is challenging. One chemical synthesis strategy is Sharpless' asymmetric hydroxylation starting from the respective (*E*)-alkenes.[6] Several strategies have been developed for the synthesis of chiral diols, complementing previously described methods. Microbial fermentation is one such method, particularly effective for the production of short-chain chiral diols.[7] Another approach utilizes a single-pot system with three solid catalysts: palladium, tungsten, and osmium species.[8] Furthermore, *cis*-diols can be efficiently synthesized through the iron-catalyzed *cis*-dihydroxylation of alkanes.[9] Additionally, chiral 1,2-diols can be obtained *via* asymmetric hydrolysis.[10] In this study, we focus on the synthesis of 2,3-butanediol **3 a**, 3,4-hexanediol **3 b**, 4,5-octanediol **3 c**, and 5,6-decanediol **3 d** (Scheme 1). For the latter three, few literature precedents on properties and synthesis routes are available.[11–15] Due to the symmetric nature of the molecules, three stereoisomers are possible: *RR*, *SS*, and *meso*. An alternative to the chemical synthesis of these diols is employing enzymatic catalysis. This can be achieved using different routes, e. g., by a double reduction of the respective diketone.[16,17] However, most of these enzymatic syntheses have focused on diols with short carbon chains, such as 2,3-butanediol. No synthesis has been shown for bulkier diols. Another enzymatic route is a two-step synthesis based on aldehydes, as used in this work. This strategy has the advantage that the starting aldehydes acetaldehyde **1 a**, propanal **1 b**, butanal **1 c**, and pentanal **1 d** can be derived from microbially produced alcohols[18,19] by oxidation or, in the case of acetaldehyde, microbial production strains directly delivering the starting aldehyde.[20] The enzymatic synthesis approach in this work features the first step of a benzoin-type condensation for the formation of the acyloins, catalyzed by a thiamine diphosphate dependent (ThDP)–lyase forming a C–C bond between two aldehyde molecules. The carboligation introduces the first stereogenic center in the resulting 2-hydroxy ketone. Depending on the stereoselectivity of the lyase, either the (*S*)- or (*R*)-hydroxy ketone is formed. In a second step, the carbonyl function is reduced to a hydroxyl group, forming the second stereogenic center (Scheme 1). Based on the intermediate, oxidoreductases with different substrate- and product stereoselectivity can be employed to produce one of the three possible stereoisomers of the diol. For cofactor recycling, a sacrificial co-substrate is added, depending on the enzyme of choice. Either isopropanol, 2,3-butanediol or 1,2-propanediol were used (Scheme 1). No additional enzyme was added.



Scheme 1. Reaction scheme for the production of vicinal, aliphatic diols from aliphatic aldehydes. R^1 is indicated as (**a** R^1 = Me, **b** R^1 = Et, **c** R^1 = n-Pr, **d** R^1 = n-Bu). Aldehydes are **1 a–d**, 2-hydroxy ketones **2 a-d**, diols **3 a-d**. R^2 of the co-substrate is either a methyl, hydroxymethyl or hydroxymethyl group.

The highest stereoselectivities were achieved when sequential reaction modes were tested, meaning that the second enzyme of the reaction was added after the completion of the first reaction step.

The synthesis of the first three diols (**3 a**, **3 b** and **3 c**) took place in an aqueous buffer, as most enzymes occur in the cytosol and are therefore evolved to function best in the presence of water. However, by increasing the length of the carbon chain of the aldehyde (**3 d**) the hydrophobicity also increases.[21] Thus, an aqueous buffer is not suitable for higher substrate concentrations. To overcome this problem, a micro-aqueous reaction system (MARS) was used as an alternative solvent system in which organic solvents are the main component. The MARS does not only counter the solubility problem but also leads to higher product concentrations and easier downstream processing.[22] In addition, formulating the cells as freeze-dried whole cells protects the enzymes inside from the harsh organic conditions.[22] The potentially biobased organic solvent cyclopentyl methyl ether (CPME) was used. It can be synthesized using various feedstocks from biorefineries such as furfural, lignin or sugar.[23] Furthermore, CPME can be easily separated from the product during subsequent purification steps and is regarded as less toxic to enzymes than other solvents.[22,23]

In the following, it will be shown how all possible stereoisomers of **3 a**, **3 b**, **3 c**, and **3 d** can be accessed using a flexible combination of stereoselective ThDP-dependent lyases and oxidoreductases in either aqueous buffer or organic solvents. In addition, scaling to 400 mL scale is exemplarily shown for (4*S*,5*S*)-octanediol **(*S*,*S*)-3 c** and (5*S*,6*S*)-decanediol **(*S*,*S*)-3 d.**

Results and Discussion

Enzyme Selection for Carboligation

Two ThDP-dependent enzymes were screened for each carboligation reaction to identify the enzyme with the

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highest yields for the desired stereoisomer and its stereoselectivity towards the product formation. Typically, the benzaldehyde lyase from *Pseudomonas fluorescens*[24] (*Pf*BAL) was shown to be (*R*)-selective for the synthesis of acetoin **2 a**, butyroin **2 c**, and valeroin **2 d** from **1 a**, **1 c**, and **1 d**, respectively. As also shown in a previous study, the stereoselectivity of the *Pf*BAL switches slightly from (*R*) to (*S*) when **1 b** is used.[25] For (*S*)-hydroxy ketones, a variant of the pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDCE469G) was selected, in which a moiety in the active site, the so-called *S*-pocket, was enlarged by a mutation. This change increases the portfolio of enzymes that produce products with (*S*)-configured stereocenter.[26]

Enzyme Selection for Reduction

The second step of the cascade is catalyzed by an oxidoreductase. Here, five enzymes were screened. The alcohol reductase from *Lactobacillus brevis*[27,28] (*Lb*ADH) and ketoreductase[29] EM-KRED014, which is commercially available from Enzymaster Deutschland GmbH, are both (*R*)-selective concerning the product formed. The *Bl*BDH has an (*R*)-preference concerning the substrate selection[30] whereas the EM-KRED014 does not distinguish between the substrate stereoisomers and can accept both enantiomers. Two more oxidoreductases were also obtained from Enzymaster Deutschland GmbH, EM-KRED026, and EM-KRED027. These have preferences towards an (*R*)-stereocenter in the substrate and the product. As an enzyme-producing (*S*)-configured product, the butanediol dehydrogenase from *Bacillus licheniformis* (*Bl*BDH) was used.[30] It also has a higher affinity to the substrate in (*R*)-configuration as shown in the following and previous studies.[30] Due to its chemoselectivity, the *Bl*BDH only accepts diols, hydroxy ketones, or diketones with neighboring carbonyl or hydroxy groups. Due to that, a vicinal diol must be used for co-factor regeneration such as the bio-based diols 1,2-propanediol[31] or 2,3-butanediol.[30] The latter is applied for the production of **3 d**, **3 c** and **3 b** and the former for the production of **3 a**.

Determination of Substrate and Product Preference

To design an optimal two-step enzymatic synthesis for the production of the diol of choice with a focus on high stereoselectivity, the kinetics of the mentioned enzymes were tested in regards to affinity to the substrate stereoisomers in the case of the oxidoreductases and in regards to the stereoselectivity of formed product for all enzymes over time. It is important to note that "preference" indicates that the respective enzyme exhibits a higher affinity for one specific enantiomer when both enantiomers are present. However, the enzyme may still be capable of catalyzing the reaction with the alternative enantiomer in certain cases, particularly when the preferred enantiomer is not available in the mixture. Substrate depletion and product formation were tracked by chiral gas chromatography (GC). For the screening of the reduction reaction, racemic mixtures of the respective 2-hydroxy ketone were used as substrates. The stereoisomer with a higher affinity to the enzyme was depleted first, and the respective diol was formed. By tracking the product formation rate, the stereoselectivity towards substrate and product for the oxidoreductases in the second reduction step could be identified (see Table 1).

Based on the stereoselectivities of the chosen enzymes, in principle, every diol should be obtainable by a modular combination of the enzymes. In Figures 1, 3, and 5, this is shown in detail for each diol separately.

2,3-Butanediol Synthesis

To produce **3 a**, two **1 a** molecules are first ligated to form **(*R*)-2 a** using *Pf*BAL, or **(*S*)-2 a** using *Ap*PDCE469G. Achieving high stereoselectivities is difficult with a small molecule such as **1 a**, which is why only a limited stereoselectivity was observed for the intermediate with an *ee* value of about 26% for **(*R*)-2 a**. However, this low selectivity was compensated by adding the oxidoreductase, which predominately reduces the 2-hydroxy ketone in surplus and exploiting its preference to one stereoisomer of the 2-hydroxy ketone, forming the desired diol. Two routes are possible to access ***meso*-3 a**, either **(*R*)-2 a** is formed

Table 1. Stereo-preferences of carboligases towards product and of oxidoreductases towards substrate and product. The substrate taken with higher affinity and the product formed in surplus are given. n.a.: not applicable.

Lyases[a]	Stereo-preference towards Substrate	*ee*% product
*Pf*BAL	n.a.	(*R*): 61% **2 a**, 50% **2 c**, 40% **2 d** (*S*): 42% **2 b**
*Ap*PDCE469G	n.a.	(*S*): 95% **2 a**, **2 b**, 99%**2 c**, **2 d**
Oxidoreductases[a]	**substrate**	***ee*% product**
*Lb*ADH	(*R*)	(*R*): 63% **3 a**, 91% **3 b**
*Bl*BDH	(*R*)	(*S*): 43% **3 a**, 71% **3 b**, 79% **3 c**, 60%**3 d**
EM-KRED014	(*S*), (*R*)	(*R*): 50% **3 a**, 54% **3 b**
EM-KRED026[b]	(*R*)	(*R*): 99% **3 c**
EM-KRED027[b]	(*R*)	(*R*): 99% **3 c**

[a] Reaction conditions can be found in the experimental section.

[b] One-step reaction was carried out with **1 c** as substrate only.

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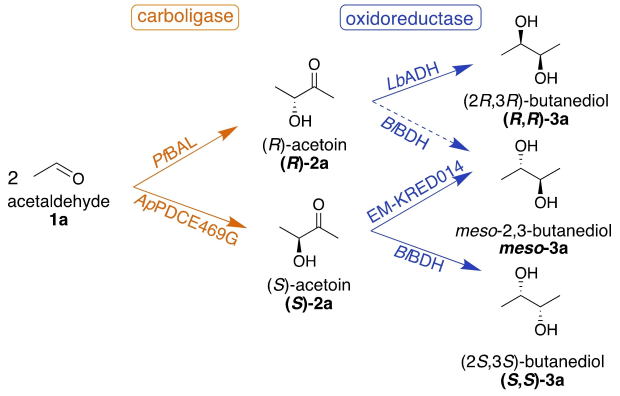

Figure 1. Two-step synthesis of 2,3-butanediol **3 a**. The carboligases, *Pf*BAL and *Ap*PDCE469G were used in the first step (orange) to catalyze the C–C bond formation between two **1 a** molecules to form **(*R*)-2 a** or **(*S*)-2 a**, respectively. For the second step, *Lb*ADH was used to form **(*R*,*R*)-3 a** starting from **(*R*)-2 a** through a reduction step (blue). EM-KRED014 was used for the reduction of the carbonyl group in **(*S*)-2 a** to form **(*S*,*S*)-3 a**. For ***meso*-3 a** production, either *Bl*BDH or EM-KRED014 were possible catalysts starting from either **(*R*)-2 a** or **(*S*)-2 a**, respectively. Because of higher isomeric content (*ic* value), EM-KRED014 was chosen as the preferred catalyst.

first, and the second carbonyl group is reduced by *Bl*BDH to a 2-hydroxy group with an (*S*)–configuration. Alternatively, the first hydroxy group is formed in an (*S*)-configuration and the second in an (*R*)-configuration (Figure 1 and 2). As *Ap*PDCE469G showed higher selectivity than *Pf*BAL, the second route was chosen.

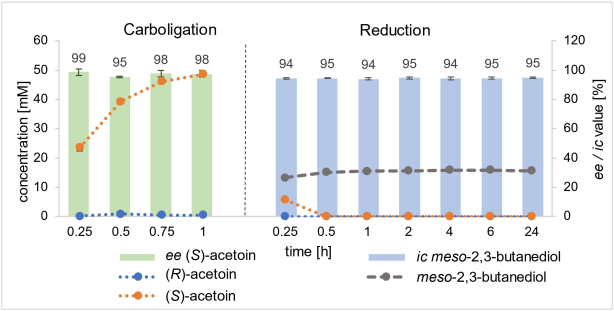

Figure 2. Two-pots two-step synthesis of *meso*-2,3-butanediol ***meso*-3 a** with *Ap*PDCE469G for carboligation and EM-KRED014 for reduction in aqueous buffer (50 mM TEA pH 9). 200 mM acetaldehyde **1 a** were used as substrate. 15 mg of each catalyst was used in a lyophilized whole cell formulation in addition to 1 M of co-substrate. The reaction was performed in technical repeats n = 3. The time courses for the enzymatic production of (2*S*,3*S*)-butanediol **(*S*,*S*)-3 a** and (2*R*,3*R*)-butanediol **(*R*,*R*)-3 a** are given in the supplementary information (SI) in Figures S2 and S4, respectively. GC chromatograms of the three stereoisomers can be found in the SI in Figures S1, S3, and S5.

Since the *Ap*PDCE469G showed higher activity, the second route was chosen to form ***meso*-3 a** with EM-KRED014 for reduction. Due to the high selectivity of the carboligase *Ap*PDCE469G, **(*S*)-2 a** was produced with an excellent *ic*-value of 98%. Consequently, in the presence of only **(*S*)-2 a, *meso*-3 a** could be formed with high *ic* value of 95% in the second step, using EMKRED-014 (Figure 2).

Also, **(*S*,*S*)-3 a** could be formed in a similar manner. Here, *Bl*BDH typically prefers the (*R*)-enantiomer when both enantiomers are available. However, in our enzyme selection process, by producing only the (*S*)-enantiomer in high selectivity using *Ap*PDCE469G, *Bl*BDH can easily react with it to produce **(*S*,*S*)-3 a** with an *ic* value of 98% (Figure S2 in the SI and Table 2).

(*R*,*R*)-3 a was formed by combining *Pf*BAL and *Lb*ADH. Here, *Pf*BAL produced **(*R*)-2 a** with a low stereoselectivity of 26%, however, *Lb*ADH was able to reduce the available **(*R*)-2 a** and form 13.7 mM **(*R*,*R*)-3 a** with a good *ic* value of 71% (Figure S4 in the SI and Table 2).

As two aldehydes are required to form 2-hydroxy ketone, the maximum concentration of the latter may be half the substrate concentration. If full conversion is achieved in the second step, the diol concentration will be equal to the 2-hydroxy ketone concentration. This means that the conversion of the reactions is equal to the product concentration and is calculated using the following equation:

$$conversion\ [\%] = \frac{product\ c\ [mM]}{\frac{1}{2} substrate\ c [mM]} * 100$$

3,4-Hexanediol Synthesis

The stereoisomers of **3 b** were accessed similarly to the ones of **3 a**. When **1 b** was used as a substrate, the ThDP-dependent carboligase *Pf*BAL switched its stereoselectivity from (*R*) to (*S*) from an *ee* of 61% (*R*) with **1 a** to 42% (*S*) with **1 b**, as expected from literature.[15,25] However, *Pf*BAL was still used to produce **(*R*)-2 b**, even if it was not in surplus, as no other (*R*)-selective lyase for propioin was available. As both *Pf*BAL and *Ap*PDCE469G produced **(*S*)-2 b** in

Table 2. Isomeric content and product concentration of all stereoisomers of 2,3-butanediol **3 a**. [complete conversion corresponds to product concentration of 100 mM, (= 100% yield, not isolated)].

Product	*ic* [%]	c [mM]	Yield (not isolated)
(*S*,*S*)-3a	98	22.8	22.8%
meso-3a	95	16	16%
(*R*,*R*)-3a	71	13.7	13.7%

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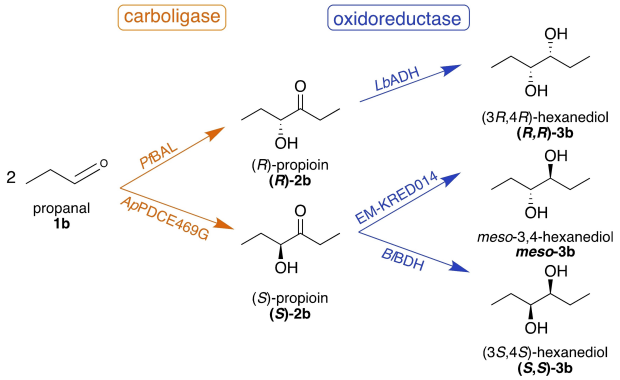

Figure 3. Two-step synthesis of 3,4-hexanediol **3 b**. In the first step, *Ap*PDCE469G was used as carboligase (orange) to build the C–C bond from two **1 b** molecules to form **(*S*)-2 b**. Despite its selectivity shift, *Pf*BAL was used for **(*R*)-2 b** formation, due to the lack of an (*R*)-selective lyase. For the second step, *Lb*ADH was used as an oxidoreductase to access **(*R,R*)-3 b** starting from **(*R*)-2 b** through a reduction step (blue). EM-KRED014 and *Bl*BDH reduced the carbonyl-group in **(*S*)-2 b** to form ***meso*-3 b** and **(*S,S*)-3 b**, respectively.

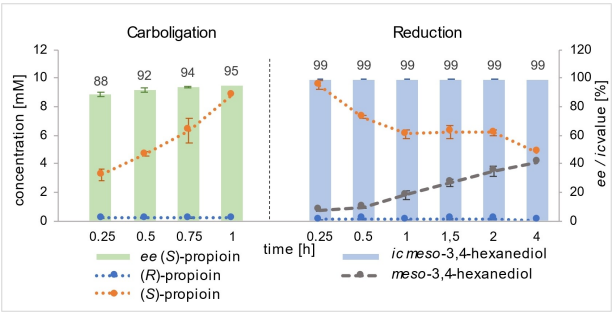

Figure 4. Two-step synthesis of *meso*-3,4-hexanediol ***meso*-3 b** with *Ap*PDCE469G as carboligase and EM-KRED014 as oxidoreductase in aqueous buffer (50 mM TEA pH 9). 200 mM propanal **1 b** was the initial substrate concentration. 15 mg of each catalyst was used in a lyophilized whole cell formulation in addition to 1 M co-substrate. The reaction was performed in 3 technical repeats. The time courses for the enzymatic production of (3*S*,4*S*)-hexanediol **(*S,S*)-2 b** and (3*R*,4*R*)-hexanediol **(*R,R*)-2 b** are given in the SI in Figure S7 and S9, respectively. GC chromatograms of the three stereoisomers can be found in the SI in Figures S6, S8 and S10.

Table 3. Isomeric content and product concentration of all stereoisomers of 3,4-hexanediol **3 b**. [complete conversion corresponds to product concentration of 100 mM, (= 100% yield, not isolated)].

Product	*ic* [%]	c [mM]	Yield (not isolated)
(*S,S*)-3b	>99	5.7	5.7%
meso-3b	>99	4.1	4.1%
(*R,R*)-3b	75	7.4	7.4%

excess, the latter was chosen for the production of ***meso*-3 b** as an excellent *ee* value of >99% was achieved compared to 42% with *Pf*BAL. This allowed the synthesis of ***meso*-3 b** with an *ic* >99% (Figure 3 and 4 and Table 3), similar to **(*S,S*)-3 b** (Figure S7 in the SI and Table 3), for which also an excellent *ic* value of >99% was obtained. To produce **(*R,R*)-3 b**, *Lb*ADH was used in the reduction step. Because *Lb*ADH shows high selectivity towards forming (*R*)-configurated center, 7.4 mM of **(*R,R*)-3 b** was gained with an *ic* value of 75% (Figure S9 and Table 3).

(*R,R*)-3 b was more difficult to access due to the lack of a sufficiently (*R*)-selective lyase for the formation of propioin **2 b**. An *ic* of 75% was reached by taking advantage of the higher affinity to **(*R*)-2 b** by the *Lb*ADH and thus increasing the selectivity from the first step to the second step (Figure S9 in the SI).

4,5-Octanediol Synthesis

The synthesis of both **2 c** enantiomers was possible with all lyases. However, the product is sterically challenging for the oxidoreductase in the second reduction step. Both *Lb*ADH and EM-KRED014 were not able to reduce the carbonyl group of the **2 c** to form **3 c**. The *Bl*BDH, however, can access this group to form both, ***meso*-3 c** (Figure S11 in the SI) and **(*S,S*)-3 c** (Figure 6 and Figure 5). Additionally, EM-KRED026 and EM-KRED027 were tested. Both showed a selectivity towards accepting and forming an (*R*)-configurated stereogenic center. Here, EM-KRED026 was used as an alternative oxidoreductase to synthesize ***meso*-3 c** in combination with *Ap*PDCE469G. From this combination, an increase of both, concentration and *ic* values, was observed from

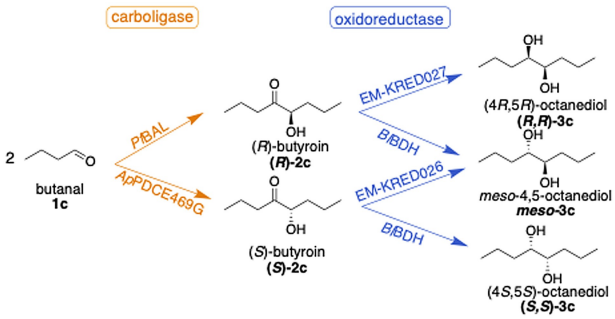

Figure 5. Two-step synthesis of 4,5-octanediol **3 c**. *Pf*BAL and *Ap*PDCE469G were used as carboligases in the first step (orange) to catalyze the C–C bond formation between two **1 c** molecules and form **(*R*)-2 c** and **(*S*)-2 c**, respectively. For the second step, EM-KRED027 was used as the oxidoreductase to form **(*R,R*)-3 c** starting from **(*R*)-2 c** through a reduction step. *Bl*BDH and EM-KRED026 were used to produce ***meso*-3 c** either from **(*R*)-2 c** or **(*S*)-2 c**, respectively (blue). *Bl*BDH reduced the carbonyl group in **(*S*)-2 c** to form **(*S,S*)-3 c.**

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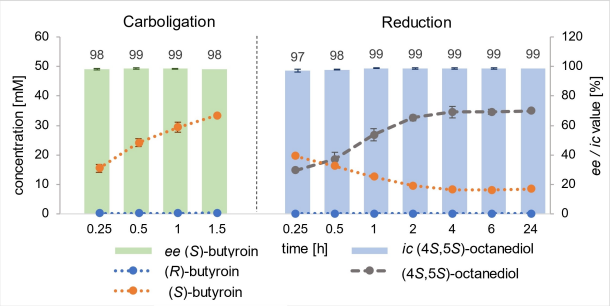

Figure 6. Two-step synthesis of (4*S*,5*S*)-octanediol **(*S,S*)-3 c** with *Ap*PDCE469G as lyase and *Bl*BDH as oxidoreductase in aqueous buffer (50 mM TEA pH 9). As substrate, 200 mM butanal **1 c** was used. In addition to 1 M of co-substrate, 15 mg of each catalyst was used in a lyophilized whole cells formulation. The reaction was performed in technical repeats n = 3. The time courses for the enzymatic production of (4*R*,5*R*)-octanediol **(*R,R*)-3 c** and *meso*-4,5-octanediol ***meso*-3 c** are given in the SI in Figures S16 and S11/S13, respectively. GC chromatograms of the three stereoisomers can be found in the SI in Figures S12, S14, S15, and S17.

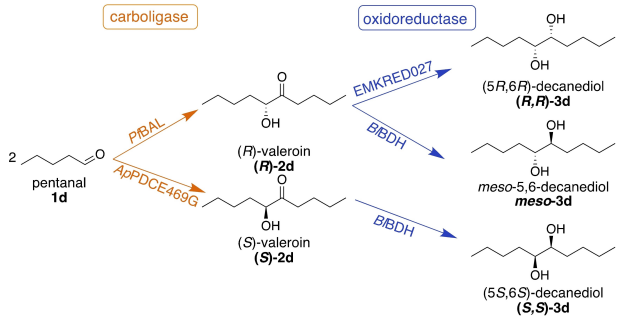

Figure 7. Two-step synthesis of 5,6-decanediol **3 d**. In the first step, *Pf*BAL and *Ap*PDCE469G were used as carboligases (orange) to catalyze the C–C bond formation between two **1 d** molecules forming **(*R*)-2 d** and **(*S*)-2 d**, respectively. For the second step, EM-KRED027 was used as oxidoreductase, forming **(*R,R*)-3 d** from **(*R*)-2 d**. *Bl*BDH was used to produce ***meso*-3 d** from **(*R*)-2 d** (blue). *Bl*BDH reduced the carbonyl-group in **(*S*)-2 d** to form **(*S,S*)-3 d**.

23 mM and 90% to 38.4 mM and 98%, respectively (Figure S13 in the SI). For **(*R,R*)-3 c**, EM-KRED027 showed the best results. Although **(*R*)-2 c** was formed with a low *ee* value of 51% by *Pf*BAL, the highly selective EM-KRED027 produced 60 mM of **(*R,R*)-3 c** after 4 h with a high *ic* value of 93% as the intermediate **2 c** was accepted with much higher affinity (Figure S16 in the SI). All values are presented in Table 4.

5,6-Decanediol Synthesis

For the synthesis of **(*R*)-2 d** and **(*S*)-2 d**, similar enzymes as in the last mentioned carboligation step were used, namely *Pf*BAL and *Ap*PDCE469G (Figure 7). Here, a conversion and *ic* value of 67 mM, 99% (Figure 8) and 66 mM, 41% (Figure S21) were obtained for the (*S*)- and (*R*)-enantiomer, respectively. As oxidoreductase, *Bl*BDH was the best candidate for the **(*S,S*)-3 d** synthesis using **(*S*)-2 d** as a substrate. Around 29 mM was formed with a high *ic* value > 99% (Figure 8, Table 5). For the production of ***meso*-3 d** and

Table 4. Isomeric content and product concentration of all stereoisomers of 4,5-octanediol **3 c**. [complete conversion corresponds to product concentrations of 100 mM, (= 100% yield, not isolated)].

Product	Enzyme	*ic* [%]	c [mM]	Yield (not isolated)
(*S,S*)-3c	*Bl*BDH	> 99	35	35%
meso-3c	*Bl*BDH	90	23	23%
	EM-KRED026	98	38.4	38.4%
(*R,R*)-3c	EM-KRED027	93	60	60%

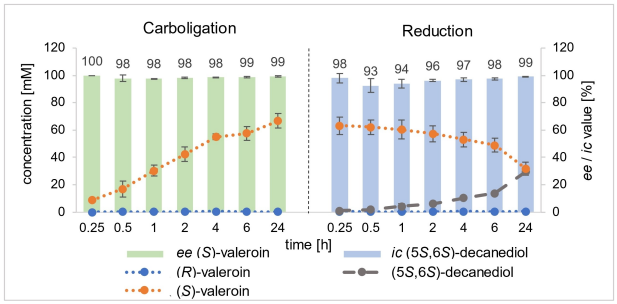

Figure 8. Two-step synthesis of (5*S*,6*S*)-decanediol **(*S,S*)-3 d** with *Ap*PDCE469G as carboligase and *Bl*BDH as oxidoreductase in organic solvent (CPME). 200 mM pentanal **1 d** was the initial substrate concentration. 30 mg of each catalyst was used in a lyophilized whole cells formulation in addition to 1 M of co-substrate. The reaction was performed in 3 technical repeats. The time courses for the enzymatic production of (5*R*,6*R*)-decanediol **(*R,R*)-3 d** and *meso*-5,6-decanediol ***meso*-3 d** are shown in the SI in Figures S18 and S21, respectively. GC chromatograms of the three stereoisomers can be found in the SI in Figures S19, S22 and S23.

Table 5. Isomeric content and product concentration of all stereoisomers of 5,6-decanediol. [complete conversion corresponds to product concentration of 100 mM, (= 100% yield, not isolated)].

Product	*ic* [%]	c [mM]	Yield (not isolated)
(*S,S*)-3d	> 99	29	29%
meso-3d	72	35	35%
(*R,R*)-3d	78	7	7%

(*R,R*)-3 d, **(*R*)-2 d** was used as an initial substrate. *Bl*BDH was again chosen along with *Pf*BAL to form 35 mM ***meso*-3 d** with a purity of 72% (Figure S21,

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Table 5). Among the three stereoisomers, **(*R*,*R*)-3 d** was the most challenging one. Due to its long carbon chain, *Lb*ADH and EM-KRED014 did not accept valeroin as a substrate. In addition, the oxidoreductase used here has a higher affinity for the oxidization of **1 d** than the reduction of 2-hydroxy ketone under the reaction conditions applied (Figure S20). It was therefore challenging to achieve high concentrations. However, EM-KRED027 led to the production of **(*R*,*R*)-3 d** with a concentration of 7 mM and *ic* value of 78% (Figure S18, Table 5).

Scale-Up of the (4*S*,5*S*)-Octanediol Synthesis (400 mL)

To bridge between proof-of-concept and applicability, a scale-up and process optimization of the production of these diols is important. Here, we chose **(*S*,*S*)-3 c** for scaling up from 1 mL to 400 mL with *Ap*PDCE469G and *Bl*BDH as a suitable combination. To achieve higher concentrations, some parameters were optimized (Table 6 in experimental section). Instead of two sequential steps, a simultaneous two-step synthesis in one pot was performed with a starting concentration of **1 c** of 400 mM. The results are shown in Figure 9.

Here, **1 c** was almost fully consumed after 2 h, forming **(*S*)-2 c**, which reached its maximum concentration of 96.4 mM after 22.5 min. **(*S*)-2 c** was further reduced to the product by *Bl*BDH, yielding 115.2 mM of **(*S*,*S*)-3 c** after 24 h, resulting in a conversion of 57.6%, percentage yield of 57% and an excellent *ic*-value >99%. After purification, 3.76 g (50.4%) of **(*S*,*S*)-3 c** were obtained. The specific rotation of the purified product yielded a value of $[\alpha]D^{22} = -42°$. This value is nearly double the value reported in the literature (−23.9°).[32] However, the literature measurement was conducted in $CHCl_3$, whereas ethanol was used as the solvent in our analysis. Solvent choice significantly affects the specific rotation, as evidenced by previous studies showing that the specific rotation of vitamin D doubled when the solvent was changed from $CHCl_3$ to ethanol.[33] Overall, by performing the scale-up, higher product concentrations and conversion were achieved compared to the 1 mL scale.

Table 6. Optimized parameters for the scale-up approach towards (4*S*,5*S*)-octanediol **(*S*,*S*)-3 c.**

Parameter	Initial	Optimized
reaction modus	sequential	simultaneous
co-substrate	1 M	2.2 M
substrate concentration	200 mM	400 mM
buffer pH	9	10
concentration	35 mM	115.2 mM
ic value	> 99%	> 99%
yield (not isolated)	35%	57.6%

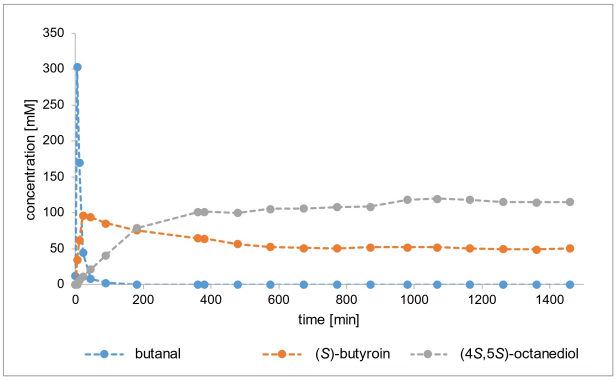

Figure 9. In 400 mL scale, one-pot two-step synthesis of (4*S*,5*S*)-octanediol **(*S*,*S*)-3 c** in an aqueous buffer pH 9. *Ap*PDCE469G was used as lyase and *Bl*BDH was applied as reductase. 400 mM butanal **1 c** was used as the initial substrate concentration. 30 mg mL^{-1} per enzyme was used in addition to 2,2 M racemic 2,3-butanediol as co-substrate. n = 1. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra of the purified product in addition to its GC chromatogram can be found in the SI Figure S29, Figure S30 and Figure S31, respectively.

Scale-Up of the (5*S*,6*S*)-Decanediol Synthesis (200 mL)

A second scale-up was carried out, however, by using MARS as a solvent system. Of the three **3 d** stereoisomers, **(*S*,*S*)-3 d** showed the best results in terms of concentration and stereoselectivity. Thus, an optimization process (Table 7 in experimental section) with subsequent final synthesis in 200 mL was performed. Here, 151 mM **(*S*)-2 d** was reached after 24 h with an *ic* value >99%. For that, another 200 mM were added after 2 h to the initial substrate concentration (200 mM). This was then followed by a reduction of the keto group in **(*S*)-2 d** by *Bl*BDH to form the **(*S*,*S*)-3 d**. The latter was synthesized in a final concentration of 88 mM, a percentage yield of 44%, and an excellent *ic* value >99% (Figure 10). After product purification, 1.1 g of **(*S*,*S*)-3 d** (40%) was obtained. The specific rotation of the purified **(*S*,*S*)-3 d** was also measured, yielding a value of $[\alpha]D^{22} = -40.2°$. This aligns closely

Table 7. Optimized parameters for the scale-up approach towards (5*S*,6*S*)-decanediol **(*S*,*S*)-3 d.**

Parameter	Initial	Optimized
co-substrate	1 M	2 M
substrate concentration	200 mM	200 mM + 200 mM
reaction time	24 h	48 h
buffer pH	9	10
concentration	29 mM	88 mM
ic value	> 99%	> 99%
yield (not isolated)	29%	44%

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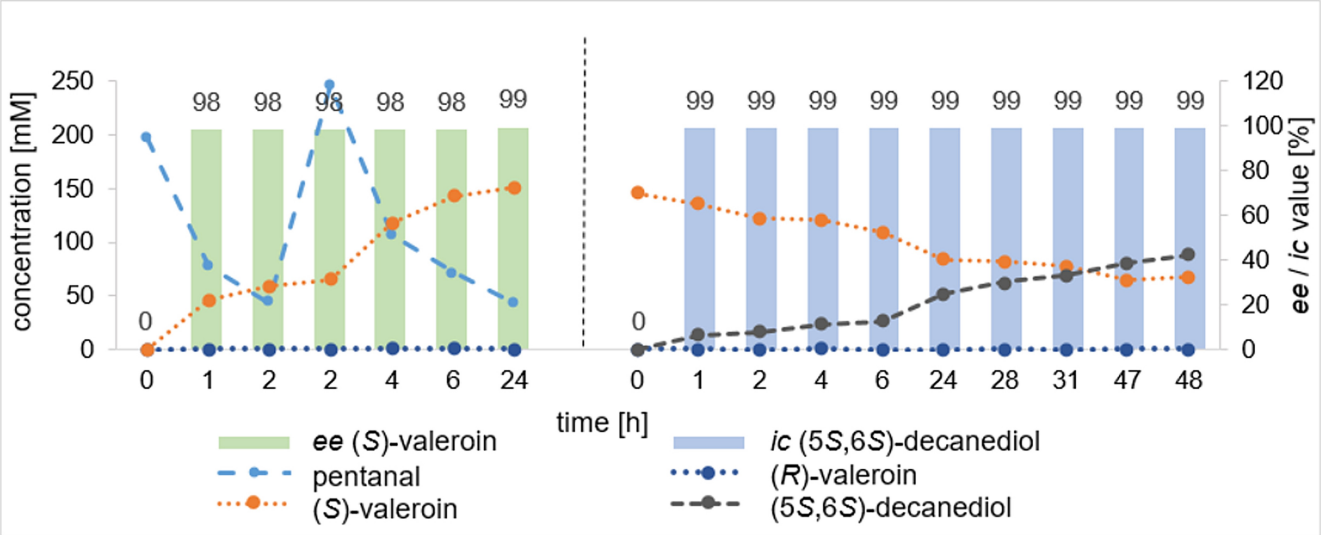

Figure 10. In 200 mL scale, one-pot two-step synthesis of (5*S*,6*S*)-decanediol **(*S*,*S*)-3 d** in MARS with CPME as an organic solvent. *Ap*PDCE469G was used as lyase and *Bl*BDH as reductase. 200 mM pentanal **1 d** was used as the initial substrate concentration and an additional 200 mM were added after 2 h. 30 mg mL^{-1} LWC for each enzyme was used in addition to 2 M racemic 2,3-butanediol as co-substrate. n = 1. ^{1}H and ^{13}C{^{1}H} NMR spectra of the purified product in addition to its GC chromatogram can be found in the SI (Figure S32, Figure S33 and Figure S34, respectively).

with the literature value of −36.6°,[34] considering that the literature product had an *ic* value of 93%, while our sample had an *ic* > 99%. Both values were determined in ethanol. The corrected rotation value of −38° is therefore consistent with the observed −40.2°.

Conclusion

Over the past decades, various approaches have been developed for the synthesis of chiral diols. However, many of these efforts focused primarily on short-chain chiral diols or failed to achieve the high selectivity required for industrial applications. In this study, we show the biocatalytic synthesis of all possible stereoisomers of **3 a**, **3 b**, **3 c**, and **3 d** starting from **1 a**, **1 b**, **1 c** and **1 d**, encompassing a diverse range of carbon chain lengths from C4 to C10. The latter aldehydes can be accessed from the corresponding bio-based alcohol by oxidation, rendering this process more environmentally friendly. The enzymatic route is comprised of a carboligation step using ThDP-dependent lyases, which shows either (*R*)- or (*S*)-selectivity, depending on the chosen enzyme. Although high stereoselectivity is limited to small molecules such as **1 a**, the overall selectivity of the cascade can be increased by the second step. Here, very stereoselective oxidoreductases were used by exploiting both, substrate specificity and product selectivity. The reaction time for each step is adjusted to achieve maximal stereoselectivity with acceptable product concentrations. Hence, by optimizing the reaction parameters and exploiting the kinetics of the enzymes, all possible molecules can be accessed with good to excellent *ic* values between 93–>99%. Only the **(*R*,*R*)-3 a**, **(*R*,*R*)-3 b**, **(*R*,*R*)-3 d**, and ***meso*-3 d** remained challenging with *ic* values of 78%, 75%, 86% and 72%, respectively. Compared to the 1 mL scale, conversions and concentrations could be increased in the 400 mL scale by 1.6- and 3.3-fold, respectively. Furthermore, the scale-up in 200 mL for **(*S*,*S*)-3 d** gave 88 mM product with an excellent *ic* value >99%, making MARS a suitable alternative solvent system for aqueous buffer. For further optimization, a more (*R*)-selective lyase is needed to enable the production of the (*R*)-enantiomer with high purity in the first step which will increase eventually the *ic*-values in the reduction step. When high concentration should be gained in combination with excellent stereoselectivities, further reaction optimization seems to be possible.

Experimental Section

Chemicals

Acetaldehyde **1 a** ≥99.5% and *meso*-2,3-butanediol ***meso*-3 a** >99% were purchased from Fluka. Acetoin >98.0%, 2,3-butanediol 98%, (2*R*,3*R*)-butanediol **(*R*,*R*)-3 a** 97%, (2*S*,3*S*)-butanediol **(*S*,*S*)-3 a**, 1,2-propanediol >99% and pentanal **1 d** 97% were obtained from Sigma-Aldrich. Butanal **1 c** 99%, butyroin **2 c** 97%, and propanal **1 b** 99% were bought from Acros Organics. Propioin **2 b** >95.0% was purchased from TCI. The stereoisomers (3*R*,4*R*)-hexanediol **(*R*,*R*)-3 b**, (3*S*,4*S*)-hexanediol **(*S*,*S*)-3 b**, (4*R*,5*R*)-octanediol **(*R*,*R*)-3 c** and (4*S*,5*S*)-octanediol **(*S*,*S*)-3 c** were chemically synthesized by Sharpless' asymmetric dihydroxylation[11,12,14,32] as previously published.[15]

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Meso-3,4-hexanediol ***meso*-3 b** and *meso*-4,5-octanediol ***meso*-3 c** were supplied by enzymatic synthesis as published.[15]

Chemical Synthesis of Stereoisomers of 5,6-Decanediol

(5*R*,6*R*)-decanediol (***R,R*)-3 d**, and (5*S*,6*S*)-decanediol **(*S,S*)-3 d** were synthesized using the protocol of Denmark and Vogler.[12] *meso*-5,6-decanediol ***meso*-3 d** was on the other hand enzymatically synthesized following the below described protocol under "Setup of two-step biotransformation" and subsequently purified. Further information on the synthesis process, purification methods, and properties can be found in the Supporting Information.

Biocatalyst Preparation

Enzymes formulated as lyophilized whole cells (LWC) were prepared as follows: *Pf*BAL (GenBank AY007242.1), *Ap*PDC varE469G (PDB 2VBI), *Lb*ADH (GenBank CAD66648.1), EM-KRED14, EM-KRED026 and EM-KRED027 (commercially available from Enzymaster Deutschland GmbH), and *Bl*BDH (PDB 3WYE) were produced in *Escherichia coli* (*E. coli*) BL21(DE3) in an autoinduction medium in 5 L shaking flasks[35] at 37 °C and 75 rpm for 2 hours followed by a temperature reduction to 20 °C for 2 days. To harvest the cells, the broth was centrifuged for 40 min at 7,000 rpm and 10 °C. Pellets were stored at −20 °C and subsequently lyophilized at −52 °C for 72 h with a pressure of 1.0 mbar. After lyophilization, cells were ground with a spatula or spoon into fine powder.

Setup of One-Step Biotransformation Towards 2-Hydroxy Ketone (2 a, 2 b, 2 c)

The reaction was carried out in a thermal shaker at 30 °C and 1,000 rpm. In a 1.5 mL glass vial, 50 mM triethanolamine (TEA) buffer (800 µL, 50 mM, pH 9) was added to the catalyst (15 mg mL^{-1}, carboligases formulated as lyophilized whole cells) and aldehyde stock solution (200 µL of a 1 M solution in 50 TEA buffer pH 9) was added to start the reaction. To stop the carboligation step, the mix was centrifuged at 14,000 rpm for 3 min. All reactions were performed in technical repeats n = 3.[36]

Setup of One-Step Biotransformation Towards Diols Stereoisomers (3 a, 3 b, and 3 c)

The reaction was carried out in a thermal shaker at 30 °C and 1,000 rpm. In a 1.5 mL glass vial, 50 mM triethanolamine (TEA) buffer (950 µL, 50 mM, pH 9) was added to the catalyst (15 mg mL^{-1}, carboligases formulated as lyophilized whole cells) and aldehyde stock solution (50 µL of a 1 M solution in 50 TEA buffer pH 9) was added. To this, co-substrate (see 'cofactor regeneration') was added to start the reduction reaction. To stop the reaction, the mix was centrifuged at 14,000 rpm for 3 min. All reactions were performed in technical repeats n = 3.[36]

Setup of One-Step Biotransformation Towards Valeroin and 3 d Stereoisomers in MARS

The same protocol as described above was used, but instead of TEA (50 mM, pH 9), cyclopentyl methyl ether (CPME) was added as the organic solvent. In addition, LWC (30 mg mL^{-1})* was used and TEA (1 µL per 1 mg LWC, 1 M, pH 9) was added to activate the enzymes and start the reaction.

Setup of Two-Step Biotransformation Towards 3 a, 3 b, and 3 c in Aqueous Buffer

Carboligation and oxidoreduction steps were carried out in a thermal shaker at 30 °C and 1,000 rpm. In a 1.5 mL glass vial, 50 mM triethanolamine (TEA) buffer (800 µL, 50 mM, pH 9) was added to the catalyst (15 mg mL^{-1}, carboligases formulated as LWC) and aldehyde stock solution (200 µL of a 1 M solution in 50 TEA buffer pH 9) was added to start the reaction. To stop the carboligation step, the mix was centrifuged at 14,000 rpm for 3 min and the supernatant was transferred to a new glass vial with cells formulated as LWC of oxidoreductase (15 mg mL^{-1}). To this, co-substrate (see 'cofactor regeneration') was added to start the reduction reaction. All reactions were performed in technical repeats n = 3.[36]

Setup of Two-Step Biotransformation Towards 3 d Stereoisomers in MARS

Due to the hydrophobicity of **1 d**, the reaction was carried out in organic solvents. Therefore, the same protocol as described above was used, but instead of TEA (50 mM, pH 9), cyclopentyl methyl ether (CPME) was added as the organic solvent. In addition, LWC (30 mg mL^{-1})* was used and TEA (1 µL per 1 mg LWC, 1 M, pH 9) was added to activate the enzymes and start the reaction.

*For the production of ***meso*-3 d**, new enzyme batch was prepared. However, the activity of *Bl*BDH was lower compared to the one used in the previous experiments. Hence, after 24 h, another set of LWC (30 mg mL^{-1}) were added in the second step.

Cofactor regeneration: 2,3-Butanediol (98%, 1 M) was used for cofactor recycling with reactions starting from **1 b**, **1 c** and **1 d**. 1,2-Propanediol (1 M) was used with **1 a** as substrate. Isopropanol (1 M) was applied with EM-KRED026 and EM-KRED017.

Sample preparation: For the preparation of samples with **1 a**, **1 c**, and **1 d**, 40 µL of reaction solution were mixed with acetonitrile and *n*-decane (360 µL, 0.16% v/v) as an internal standard. When **1 b** was the substrate, acetonitrile was substituted with ethyl acetate to stop the reaction. Samples in Eppendorf tubes were centrifuged for 3 min at 14,000 rpm, and 380 µL were used for instrumental analysis (gas chromatography, GC).

Scale up towards (4*S*,5*S*)-octanediol: The synthesis of **(*S,S*)-3 c** was scaled to 400 mL. Here, carboligation and oxidoreduction were performed simultaneously in one pot. The reaction was carried out in an EasyMax® device from Mettler Toledo at 30 °C and 250 rpm.

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For the scale-up approach, some parameters were optimized to increase the concentration of **(*S*,*S*)-3 c** (Table 6).

In a 400 mL vessel glass reactor, TEA buffer (50 mM, pH 10) was added to the carboligase and oxidoreductase (30 mg mL^{-1} LWC per catalyst). 2,3-butanediol (98%, 2.2 M) was used as a co-substrate. **1 c** (400 mM) was added to start the reaction.

Scale up towards (5*S*,6*S*)-decanediol: The synthesis of **(*S*,*S*)-3 d** was scaled to 200 mL. Here, carboligation and oxidoreduction were performed as described before. The reaction was carried out in an EasyMax® device from Mettler Toledo at 30 °C and 250 rpm.

For the scale-up approach, some parameters were optimized to increase the concentration of **(*S*,*S*)-3 d** (Table 7).

Instrumental Analytics and References

Samples were analyzed on a Thermo TRACE_1300_1310 GC with a chiral column CP-Chirasil-Dex CB (Agilent J&W) column (i.d. 0.25 mm; 25 m x 0.25 mm). To analyze the reaction towards **3 a**, **3 b**, and **3 c**, an atmosphere gradient from 40 to 190 °C (40 °C hold 0.5 min, to 77 °C with 10 °C.min^{-1}, hold 0.75 min, to 190 °C with 40 °C min, hold 5 min) was used. The total run time was 13 min.

Regarding the reaction towards **3 d**, an atmosphere gradient from 77 to 190 °C (77 °C hold 1.25 min, to 190 °C with 10 °C.min^{-1}, hold 3 min) was used with a run time of 16 min. For calibration, all possible stereoisomers of the analyzed diols were used. Retention times for diols were as follows in Table 8, and their chromatogram are presented in SI Figure S25, S26 and S27.

Calibration: Gas chromatography was calibrated using all aldehydes, intermediates and the 12 stereoisomers. For this purpose, a dilution series was prepared for all compounds (0, 1, 5, 10, 25, 50 and 100 mM) and the retention time was measured (Table 8). By plotting the known concentrations against the measured areas of the GC peak, a linear curve was obtained. This equation $x = \frac{y-b}{m}$ was used to determine the concentration of unknow samples (Figure S24).

Table 8. Retention time of the stereoisomeric diols.

Stereoisomer	Retention time	Stereoisomer	Retention time
(2*S*,3*S*)-butanediol **(*S*,*S*)-3 a**	8.453	(4*S*,5*S*)-octanediol **(*S*,*S*)-3 c**	10.425
meso-2,3-butanediol ***meso*-3 a**	8.735	*meso*-4,5-octanediol ***meso*-3 c**	10.575
(2*R*,3*R*)-butanediol **(*R*,*R*)-3 a**	8.502	(4*R*,5*R*)-octanediol **(*R*,*R*)-3 c**	10.473
(3*S*,4*S*)-hexanediol **(*S*,*S*)-3 b**	9.528	(5*S*,6*S*)-decanediol **(*S*,*S*)-3 d**	13.31
meso-3,4-hexanediol ***meso*-3 b**	9.675	*meso*-5,6-decanediol ***meso*-3 d**	13.59
(3*R*,4*R*)- hexanediol **(*R*,*R*)-3 b**	9.577	(5*R*,6*R*)-decanediol **(*R*,*R*)-3 d**	13.36

Purification of (4*S*,5*S*)-octanediol: LWCs were separated from the reaction mixture by centrifuging the mixture at 10,000 rpm for 30 min at 25 °C. To avoid the formation of an interphase, protein precipitation was followed by adding HCl (20%) to lower the pH to 2. After a further centrifugation step (10,000 rpm, 45 min, 25 °C) to remove the precipitated proteins, **(*S*,*S*)-3 c** was extracted from the hydrophilic supernatant into the organic phase by mixing it twice with an equal volume of ethyl acetate in a separatory funnel and left over-night. The organic phase was separated and dried by adding $MgSO_4$, which was then removed by filtration. Using a rotary evaporator, the volume of the solvent was reduced at 100 mbar and 40 °C. The remaining volume (~4 mL) was added to the top of a silica gel column (29 cm length and 50 mm diameter). This was prepared with a mixture of petroleum ether and ethyl ether (5:1). After elution of the intermediate **(*S*)-2 c** with this mixture (300 mL), **(*S*,*S*)-3 c** was eluted with a 1:1 mixture of petroleum ether and diethyl ether. In a final step, the fractions containing the product were pooled and the solvent was removed at 40 °C and 40 mbar to obtain the purified product.

Purification of (5*S*,6*S*)-decanediol: LWCs were separated from the reaction mixture by filtration. To avoid the formation of an interphase, protein precipitation was followed by adding HCl (20%) to lower the pH to 2. After a further filtration step, all the hydrophilic components like the co-substrate were extracted from the hydrophobic supernatant into the aqueous phase by mixing it twice with an equal volume of water in a separatory funnel and left over-night. The organic phase was separated and dried by adding $MgSO_4$, which was then removed by filtration. The solvent volume was reduced to ~5 mL using a rotary evaporator at 40 °C and 100 mbar. The remaining volume was added to the top of a silica gel column (29 cm length and 50 mm diameter). This was prepared with a mixture of petroleum ether and ethyl ether (5:1). After elution of the intermediate **(*S*)-2 d** with this mixture (300 mL), **(*S*,*S*)-3 d** was eluted with a 1:1 mixture of petroleum ether and diethyl ether. In a final step, the fractions containing the product were pooled and the solvent was removed at 40 °C and 40 mbar to obtain the purified product.

Samples preparation for NMR: 10 mg and 50 mg from **(*S*,*S*)-3 c** and **(*S*,*S*)-3 d** were used separately for ^{1}H-, ^{13}C{^{1}H}-NMR measurement, respectively. $CDCl_3$ (0.55 mL) was added as a solvent and filtered with the product into an NMR tube. TMS was provided with $CDCl_3$ and was used as an internal standard.

NMR measurement: ^{1}H-, ^{13}C{^{1}H} NMR spectra were recorded on an Advance/DRX 600 nuclear magnetic resonance spectrometer (Bruker, Billerica, USA) at 600 MHz and 151 MHz with TMS as internal standard. Chemical shifts δ are given in ppm and were referenced to residual chloroform at $\delta = 7.26$ ppm (^{1}H) or $\delta = 77.16$ ppm (^{13}C). Multiplicities are labeled by s (singlet), d (doublet), t (triplet), dd (doublet of doublet), q (quartet) and m (multiplet). Coupling constants are given in Hz.

Isomeric content (*ic*) calculation: The isomeric content (*ic*) indicates the ratio of one stereoisomer over the sum of all possible stereoisomers. It gives the proportion of the stereoisomer of interest when the samples contain both, enantiomers and diastereomers and is calculated as follows:

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$$\% \text{ ic} = \left(\frac{P1}{(P1 + P2 + ... + Pn)}\right)^* 100$$

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