ChemCatChem

Research Article
doi.org/10.1002/cctc.202200390

Chemistry Europe
European Chemical Societies Publishing

www.chemcatchem.org

Covalently Immobilized 2-Deoxyribose-5-phosphate Aldolase (DERA) for Biocatalysis in Flow: Utilization of the 3-Hydroxyaldehyde Intermediate in Reaction Cascades

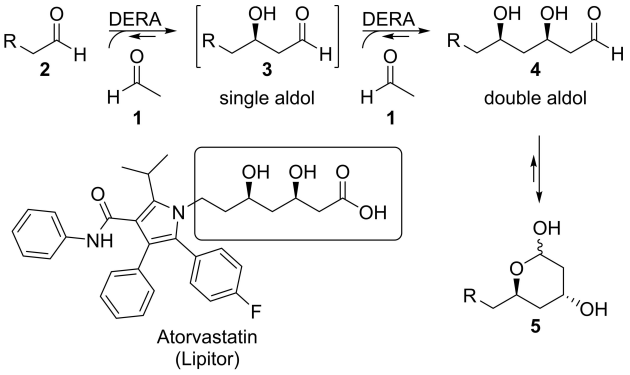
Julia Hindges née Bramski,[a] Johannes Döbber,[b] Marc R. Hayes,[a] Thomas Classen,[b] Martina Pohl,[b] and Jörg Pietruszka*[a, b]

C–C bond forming enzymes, such as 2-deoxyribose-5-phosphate aldolase (DERA), are an important class of enzymes in synthesis. The use of DERA, with its reaction routes to the double aldol product, is an established method for producing statin side chains in industry. Herein, the selective synthesis of 3-hydroxyaldehydes as single aldol products of DERA is presented. Successful covalent immobilization of a suitable *Escherichia coli* (Ec) DERA variants with C47M and C47V exchanges, respectively, enabled their use in continuous synthesis. Precise reaction control using flow chemistry made the selective formation of 3-hydroxyoctanal from hexanal possible. For optimization, a systematic approach of design of experiment (DoE) was used, resulting in 90% conversion. Furthermore, five two-step reaction systems have been established to scavenge the reactive single aldol product combining biocatalysis in flow mode and a consecutive chemical reactions step either in flow (continuous flow mode) or in batch (semi-continuous flow mode).

Introduction

Due to their convincing catalytic efficiencies, high chemo-, stereo- and regioselectivities, enzymes stand out as attractive and noteworthy alternative to classical chemical catalysts that are widely used. At the same time, there has been an increasing public demand for greener and more environmentally friendly production processes.[1] Enzymes could be one measure to increase the sustainability of chemical syntheses.[1a,2] However, in order to be used efficiently on an industrial scale, long-term stability and reusability of the biocatalyst would be an economic boost. One possible solution to this problem is enzyme immobilization.[3] Furthermore, enzyme immobilization strategies have already been demonstrated to provide positive effects on enzyme stability.[3a] In addition, the use of immobilized biocatalysts enables the application of enzymes in continuous flow chemistry mode. This mode of synthesis control, which has already found its way into the fields of bulk and fine chemical industry,[4] offers many advantages over the classical batch mode. A tight control of reaction parameters allows faster reaction optimization resulting in improved reactivity, shorter reaction times, reduced costs and access to new reaction products.[3b,5,20] Even the use of cofactor-dependent enzymes has already been successfully transferred to synthesis in flow mode, paving the way for a further industrially relevant branch of biocatalysts.[6]

The deoxyribose-5-phosphate aldolase (DERA) catalyzes an aldol reaction addressing one of the most important reactions in organic chemistry the formation of new C–C bonds.[7] The enzyme uses acetaldehyde (**1**) as nucleophile (donor) and an electrophilic aldehyde **2** to form an aldol **3**. Since the aldol is again an electrophilic aldehyde, another acetaldehyde (**1**) can be added. Cyclization of the double aldol adduct **4** to the lactol **5** reduces further aldol additions (Scheme 1).[8]



Scheme 1. Sequential enantioselective aldol reaction catalyzed by DERA to synthesize a lactol, which can be further transformed to the typical statin side chains, e.g., finally resulting in the cholesterol-lowering drug Atorvastatin (Lipitor®).[9]

[a] *J. Hindges née Bramski, Dr. M. R. Hayes, Prof. J. Pietruszka*
Institut für Bioorganische Chemie der Heinrich-Heine-Universität Düsseldorf am Forschungszentrum Jülich
Stetternicher Forst, Geb. 15.8
D-52426 Jülich (Germany)
E-mail: j.pietruszka@fz-juelich.de
Homepage: https://www.iboc.uni-duesseldorf.de/
[b] *Dr. J. Döbber, Dr. T. Classen, Prof. M. Pohl, Prof. J. Pietruszka*
Institut für Bio- und Geowissenschaften (IBG-1: Biotechnologie)
Forschungszentrum Jülich GmbH
D-52426 Jülich (Germany)

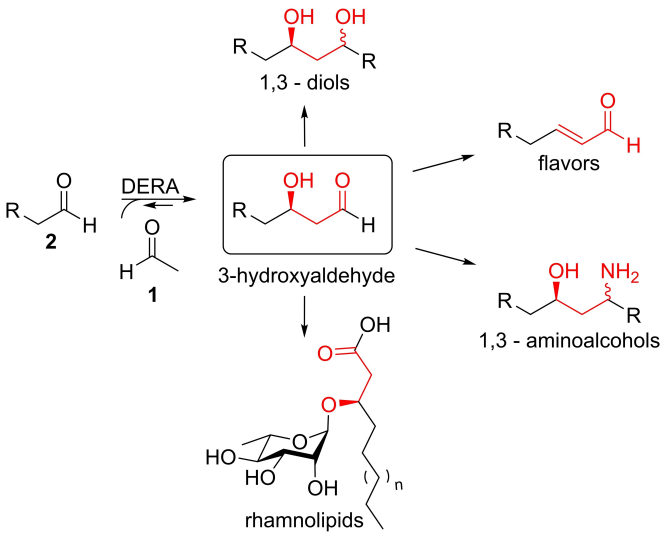
Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.202200390

This DERA-catalyzed reaction towards the double aldol product is already well known and was successfully implemented in the preparation of different types of deoxysugars[8e] and in the industrial synthesis of statin side chains,[9–10] important cholesterol-lowering drugs (Scheme 1). Recently, *Grabner et al.* published a continuous flow process towards the double aldol product as a statin side chain precursor using a DERA variant immobilized in an alginate-Luffa-matrix.[11] In contrast to the double aldol product, the single aldol adduct is highly reactive, which makes its isolation challenging. Yet the single aldol product forms an interesting structural motif and a desirable building block for natural product synthesis such as rhamnolipids,[12] flavors,[13] 1,3-diols[14] or 1,3-aminoalcohols[15] (Scheme 2).

However, the well-known substrate inhibition of DERA severely limits the application of this potentially interesting biocatalyst. Mechanistic studies by *Dick et al.* showed the formation of the irreversible inhibitor crotonaldehyde from two molecules of acetaldehyde, which subsequently blocks the active site. To circumvent this, complete or partial acetaldehyde resistant DERA variants could be obtained by mutating an as crucial identified cysteine (C47 in $DERA_{Ec}$) involved in the process.[16] These are in particular the two DERA variants from *E. coli* DERA-C47M and DERA-C47V,[10b] the latter being characterized by its almost wild-type activity and a half-life of three hours in presence of 300 mM acetaldehyde.

In addition to mechanistic studies leading to mutagenically adapted DERA variants, strategies of immobilization are also being pursued, which can have positive effects on enzyme stability. A number of immobilization strategies for DERA are already known, primarily targeting improvements in substrate tolerance and thus expanding the potential of DERA as a biocatalyst. Adsorptive immobilization methods such as ionic immobilization on multi-walled carbon nanotubes[18] or immobilization on mesoporous silica[19] have been described while maintaining some enzyme activity. However, these methods are known to be characterized by high leaching effects, which severely limits their use in biocatalysis, especially in flow mode.[3a,20] Furthermore, various covalent immobilization strategies have been described for DERA. For example, multipoint attachment to mesocellular silica foam[21] or the immobilization of a DERA to magnetic nanoparticles,[22] which is mainly characterized by its simple separation of the catalyst from the reaction solution. In cooperation with the *Böker* group, we performed the immobilization of *E. coli* DERA in the form of polymer conjugates for the production of biocatalytically active membranes.[23] Here, binding was achieved via lysine or cysteine residues on the enzyme surface. The disadvantage of all known methods for immobilizing aldolase is a (partially drastic) loss of enzyme activity. Further optimized methods are required for the efficient use of DERA as a biocatalyst especially for use in flow mode.

Here, we demonstrate the covalent immobilization of two optimized DERA variants from *E. coli* $DERA_{EC}$-C47M and $DERA_{EC}$-C47V using the Halotag® system, which allows easy immobilization from the crude extract and prevents leaking upon covalent binding to the Halotag® specific matrix.[24] In this way, packed bed-reactors could be used for synthesis in flow mode. By specifically controlling the contact time between substrate **6** and DERA, we expected to shift the product ratio of the DERA reaction from the double aldol product to the single aldol product **7**. Based on these results, we anticipated two-step syntheses utilizing the reactive intermediate **7** for further transformation towards compounds **8**–**12** (Scheme 3).

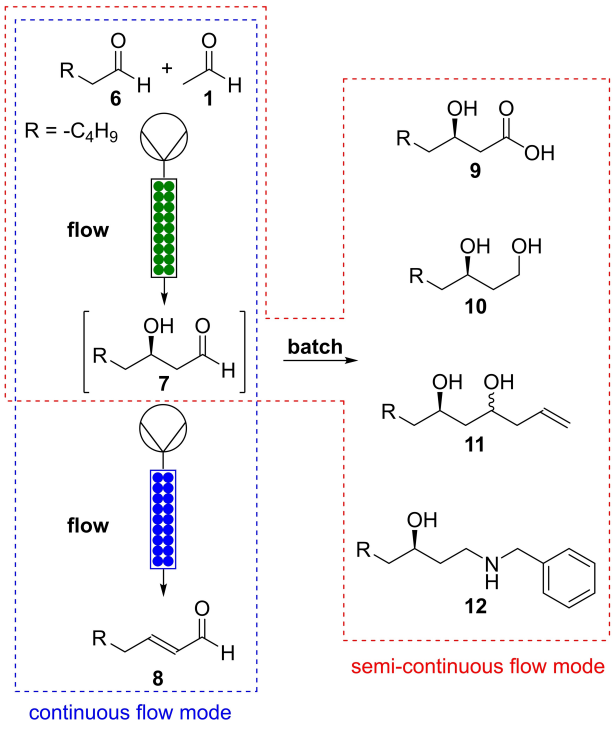


Scheme 2. DERA-catalyzed 3-hydroxyaldehyde as interesting structural motif for unsaturated aldehyde-based flavours, rhamnolipids and various (biologically active) natural products containing 1,3-diols or 1,3-aminoalcohols motifs. The single aldol building block is marked red.

Results and Discussion

DERA in flow – mono aldol product synthesis: Initially a reliable and reproducible method for the formation of the single aldol product (*R*)-3-hydroxyaldehyde **7**, in continuous flow mode was investigated. For this purpose, hexanal (**2**) was selected as model substrate. In order to establish a simple flow-through procedure using immobilized DERA as catalyst in a fed-bed reactor, three crucial parameters must be considered to find the ideal reaction conditions: the concentration of the two substrates acetaldehyde (**1**) and hexanal (**2**) as well as the flowrate, which determines the contact time between catalyst and substrate. The enzyme activity was held constant by the use of a 5 cm *Omnifit*® column (Ø 3 mm, L 50 mm) as reactor.

While a number of by-products (**8, 13**+**14**, see Figure 1) were expected, the primary concern was the ratio of mono- versus double aldol addition products. Furthermore, it was to be expected that considerable batch-to-batch and column-to-column deviation would be observed when quantifying the results. However, for one given batch of Halotag-$DERA_{Ec}$-C47M enzyme packed in a 5 cm Omnifit® glas column reactor a full factorial design was used as shown in Figure 2 and Table 1. Here, the three factors flow rate, concentration of acetaldehyde (**1**) and hexanal (**2**) were examined on two levels resulting in 2^3 experiments each as duplicate, i.e. 16 experiments. In each case



Scheme 3. Anticipated reaction scheme of two-step-syntheses, combining a DERA-catalyzed transformation in flow towards 3-hydroxyaldehyde **7** using hexanal (**6**) and acetaldehyde (**1**) as substrates and a sequential transformation performed in batch (indicated in red) or flow (indicated in blue) mode.

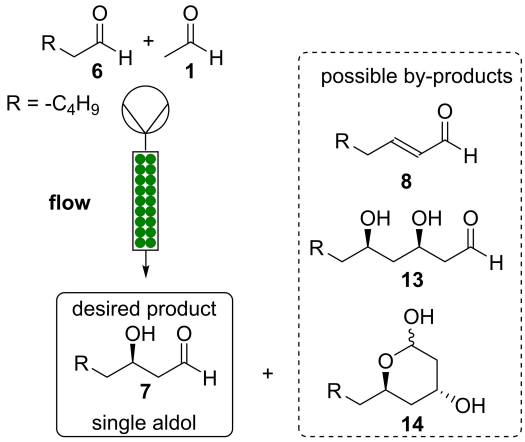


Figure 1. Experimental set-up for the preparation of the single aldol (*R*)-3-hydroxyoctanal (**7**) using the Halotag-DERA$_{Ec}$-C47M in a 5 cm Omnifit® glas column reactor in flow mode. Possible by-products are the double aldol product (**13**) [for clarity, open chain product (**13**) and the actually formed lactol form (**14**) are shown] and the aldol condensation product (*E*)-octenal (**8**). By means of systematic reaction optimization, their formation should be avoided completely or kept as low as possible resulting in the single aldol product (**7**) as major product.

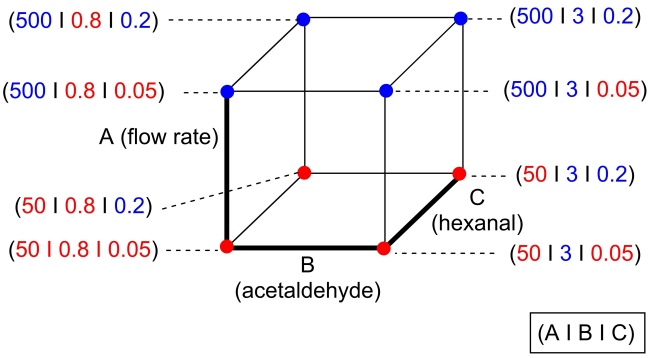


Figure 2. Schematic representation of a two-level factorial design with three factors of the first optimisation round. The cube plot of a factorial design is described, where each factor flow rate [µL/min], acetaldehyde [eq], hexanal [mol/L] represents a dimension. Each factor has two levels, which are indicated here in blue (level 1) and red (level 2).

Table 1. Reaction parameters using a full factorial design. Parameters are calculated by *DesignExpert*® software on basis of three factors A (flowrate), B [eq. acetaldehyde (**1**)], C (hexanal (**2**) concentration). Every experiment was carried out as technical duplicate.

Entry[a]	Flow rate [µL/min]	acetaldehyde [eq]	hexanal [mol/L]	single aldol **7** [%][a]	double aldol **14** [%][b]
1	50	0.8	0.05	32 ± 5.0	0
2	50	3.0	0.05	54 ± 5.0	3 ± 0.0
3	50	0.8	0.20	48 ± 5.0	5 ± 0.4
4	50	3.0	0.20	64 ± 2.0	13 ± 0
5	500	0.8	0.05	23 ± 2.0	0
6	500	3.0	0.05	22 ± 6.0	0
7	500	0.8	0.20	20 ± 1.0	0
8	500	3.0	0.20	23 ± 1.0	0

[a] Relative conversions to 3-hydroxyoctanal (**7**). [b] Relative conversion to double aldol product (**14**).

a minimum and a maximum limit of the respective parameter was set, which differed greatly from each other. This should subsequently provide information on whether a parameter exerts a large or less decisive influence on the reaction. The chosen values were based on the solubilities of both substrates in the aqueous buffer system as well as the concentrations of acetaldehyde (**1**) commonly used in the synthesis in DERA-catalyzed batch reactions.[16a,25] Thus, 0.05 mol/L and 0.2 mol/L were chosen for hexanal (**6**), 0.8 or 3.0 equivalents used for acetaldehyde (**1**), and 500 µL/min or 50 µL/min for the flow rate (s. Table 1). The reaction was carried out in triethanolamine buffer (TEA, pH 7.0, 0.1 M). As catalyst the acetaldehyde-tolerant DERA$_{Ec}$-C47M with HaloTag® was used. On a 5 mL scale, the respective solution was prepared in TEA buffer, filled into a syringe and shaken vigorously. Samples were collected and analyzed via gas chromatography after extraction. Since the desired product (**7**) is not stable enough to be isolated and analyzed in its pure form, the relative conversion was calculated (*cf* SI for detailed calculation). The collected data were evaluated using *DesignExpert*® and *OriginPro*®. For details on the experimental design and all parameters see supplementary information SI. The results show a clear influence of the flow rate on turnover (Table 1). A lower flow rate of 50 µL/min (entries 1–4) was superior with a conversion about two to three times higher compared to the reactions at 500 µL/min (entries 4–8). The influence of the flow rate can be shown

particularly clearly in entries 4 and 8 [3 equivalents of acetaldehyde (**1**) and 0.2 mol/L hexanal (**6**)]: experiments with the highest input of acetaldehyde (**1**) (entries 2 and 4, Table 1) show the highest conversions of 54% and 64%, respectively. These trends can also be seen in Figure 3. The higher the concentration of the substrate, the higher the turnover. The best results in this survey experiments were obtained at a flow rate of 50 µL/min, 200 mM hexanal (**6**) and 600 mM acetaldehyde (**1**), i.e. 3 equivalents yielding a relative conversion of 65% to the single aldol product **7** and 13% to the unwanted double aldol product **14** (Table 1, entry 4).

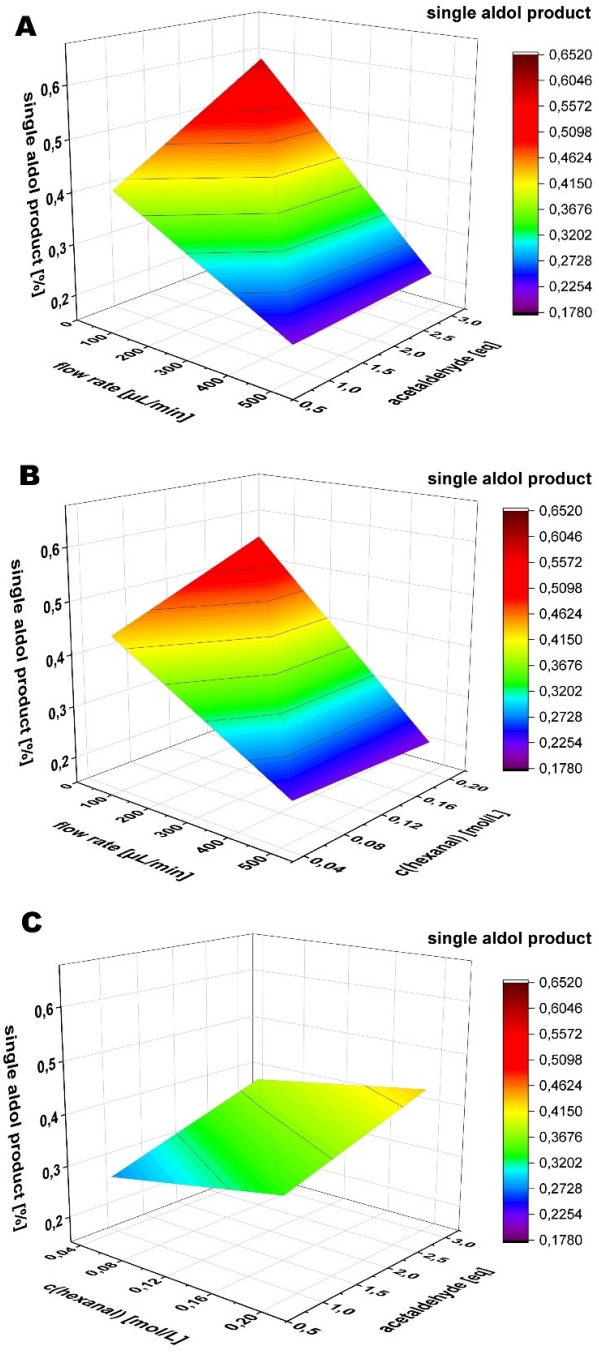


Figure 3. 3D illustration of the Factorial Design for reaction optimization towards the best conditions for the synthesis of the single aldol product 3-hydroxyoctanal (**7**) regarding following parameters: **A**) Flowrate in µL/min and amount of acetaldehyde (**1**); **B**) flowrate in µL/min and hexanal (**6**) concentration in mol/L; **C**) concentration of acetaldehyde (**1**) in equivalents and hexanal (**6**) concentration in mol/L. The darker the shade of red in the illustration, the higher the measured amount of single aldol product (**7**) in the reaction solution (for full data see Supporting Information).

With these findings in hand, the second task was to increase reproducibility of the set-up. Two observations must be addressed: Over time, a) a drastic decrease in conversion up to 20 to 30% was recorded (for details see Supporting Information: during the first 200 min, the conversion remained between 90–100%) and b) a noticeable increase of the by-product (*E*)-octenal (**8**) was observed.

Since the condensation reaction is triggered by acidic conditions, an attempt was made to remove any acidic source in the experimental set-up. For this purpose, the glass column used as reactor was previously inactivated by silyl protection before immobilization. This increased the relative conversion up to 38% of 3-hydroxyoctanal (**7**) as the major product without any traces of unwanted double aldol product (**5**) using the best conditions determined in the initial factorial design. Up to 6% relative conversion towards the unwanted side product (*E*)-octenal (**8**) were still detectable. Based on these findings, a second optimization round using a response surface was performed. The optimization did not further improve the conversion to the desired product **7** (see SI for detailed data). Thus, the next step was to increase the enzyme activity in the reactor. This could principally be achieved by two options: a) By increasing the amount of enzyme (DERA variant C47M), i.e. by using a larger reactor. However, this would generate a larger enzyme consumption. b) Alternatively, a more active, but still sufficiently stable enzyme variant can be used for the synthesis. We decided to test the $DERA_{EC}$ variant C47V. Relatively to C47M, C47V is an improved variant in position 47[16] showing doubled specific activity compared to the C47M variant and a half-life of about 3 h within 300 mM acetaldehyde (**1**) (Table 2).

The higher activity of the C47V variant compared to the previously used C47M variant required adaption of the flow rate. Optimization of the flowrate was carried out by keeping other conditions constant [c(hexanal) = 200 mM, n-(acetaldehyde) = 3 eq, reactor (Ø 3 mm, L 50 mm)]. The results

Table 2. Theoretically immobilized enzyme activity in respective Omnifit columns comparative for HaloTag-$DERA_{EC}$-C47M and C47V.

Halotag $DERA_{EC}$ variant – reactor diameter and length	enzymatic activity [U] before immobilization	after immobilization	theoretically immobilized[a]
C47M – Ø 3, 50 mm	137	99	38
C47V – Ø 3, 50 mm	209	133	75
C47V – Ø 3, 100 mm	194	74	118

[a] Calculated theoretically immobilized enzyme activity: (total enzyme activity in the crude extract measured before immobilization) – (total enzyme activity in the crude extract flow through measured after immobilization).

are shown in Figure 4 (for details see SI). The best conversion towards the desired product **7** was found at a flowrate of 50 μL/min, where up to 56% of 3-hydroxyoctanal (**7**) was obtained. An almost negligible proportion of assignable by-products was produced [under 5% of double aldol (**14**) and 2% (*E*)-octenal (**8**)].

Although the Halotag-DERA$_{Ec}$ variant C47V already clearly increased the conversion and reduced by-product formation, there was some room for improvement in terms of conversion, as up to 35% of substrate hexanal (**6**) still remained in the product mixture. For this, a larger Omnifit® reactor, a 10 cm column (Ø 3 mm, 100 mm) was packed with immobilized DERA$_{Ec}$-C47V. Since the contact time between catalyst and substrate increases with the longer column, the flow rate must also be increased. For this purpose, the screening was repeated as a final optimization step (Figure 5). The substrate concentrations of 0.2 M hexanal (**6**) and 3 equivalents of acetaldehyde (**1**) were kept constant. With a flow rate of 70 μL/min, a relative conversion of up to 90% could be achieved, 73% of which to the desired single aldol product (*R*)-3-hydroxyoctanal (**7**), 10% to the double aldol product **14** and only 6% to the by-product (*E*)-octenal (**8**).

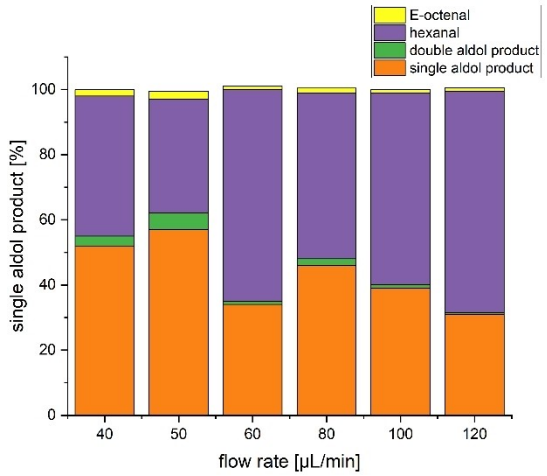


Figure 4. Relative conversions towards the desired single aldol product **7** (orange), the side-products (*E*)-octenal (**8**) (yellow) and the double aldol product **14** (green) as well as remaining substrate hexanal (**6**) (purple), which are determined in the screening of the flow rate using Halotag-DERA$_{Ec}$-C47V (Ø 3 mm, 50 mm).

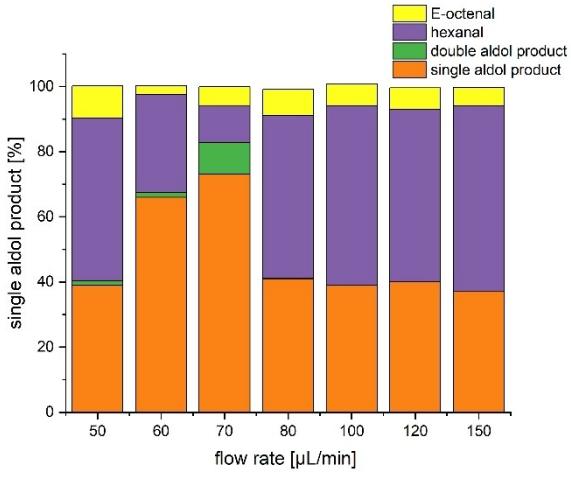


Figure 5. Relative conversions towards the desired single aldol product **7** (orange), the side-products (*E*)-octenal (**8**) (yellow) and the double aldol product **14** (green) as well as remaining substrate hexanal (**6**) (purple), which are determined in the screening of the flow rate using Halotag-DERA$_{Ec}$-C47V (3 mm × 100 mm).

Consecutive reactions utilizing the reactive intermediate 7: In terms of applicability, sequential transformations in continuous flow and in semi-continuous flow mode (combining flow and batch mode) were carried out. For this purpose, five reactions starting from the DERA-produced intermediate (*R*)-3-hydroxyoctanal (**7**) were investigated to demonstrate the feasibility.

The DERA$_{Ec}$ catalyzed reaction step in flow mode was carried out for all semi-continuous flow syntheses under the same optimized conditions as described above. First, we investigated the utilization of the obtained crude (*R*)-3-hydroxyoctanal (**7**) in batch. In order to obtain sufficient amounts of it for further reaction, two 10 cm Omnifit columns (Ø 3 mm, 100 mm, 10 mL reaction volume; Figure 6) were used simultaneously. After the reaction solutions had passed through the enzyme columns, they were combined via a T-piece, extracted using the FLLEX system and the phases separated; the organic phase was collected in a flask on ice.

Combining the DERA-catalyzed (*R*)-3-hydroxyoctanal (**7**) with a sequential *Pinnick* oxidation[26] in batch resulted in (*R*)-β-hydroxyoctanoic acid (**9**). After stirring the reaction mixture for 4 h and column chromatography 43% of pure product **9** was isolated. For the synthesis of the (*R*)-octan-1,3-diol (**10**) a simple reduction with sodium borohydride was performed. After addition of 2.5 equivalents of sodium borohydride the reaction was stirred vigorously for 4 h. Over two steps, 56% of enantiomerically pure product **10** could be isolated. Next, for a reductive amination of the (*R*)-3-hydroxyoctanal (**7**), 1 equivalent of benzylamine was added for the formation of an intermediate imine that was reduced with sodium borohydride. The desired product **12** was isolated in 58% yield. Finally, an allyl addition was performed, which is known to be sensitive to traces of water. Hence, the in-line extracted organic phase containing the DERA-catalyzed product **7** was additionally pumped through a column filled with molecular sieve (4 Å) for drying. The (*R*)-3-hydroxyoctanal (**7**) was then collected in a flask and the allylboronate was added. Again, the traces of by-products of the DERA reaction, (*E*)-octenal (**8**) and double aldol product **14**, led to a product mixture. After column chromatography 58% of desired product (6*R*)-undec-1-ene-4,6-diol (**11**) was isolated as an 80:20 mixture of the two diastereomers.

Besides the presented semi-continuous two-step syntheses, we were able to establish a protocol for a continuous flow synthesis towards the α,β-unsaturated octenal (**8**), which is a known flavor component found in fruits and fungi.[13,27] For this purpose, the DERA$_{Ec}$-C47M variant was used as the biocatalyst instead of the DERA$_{Ec}$-C47V, since the product ratio achieved with this DERA variant is already favoring the (*E*)-octenal (**8**)

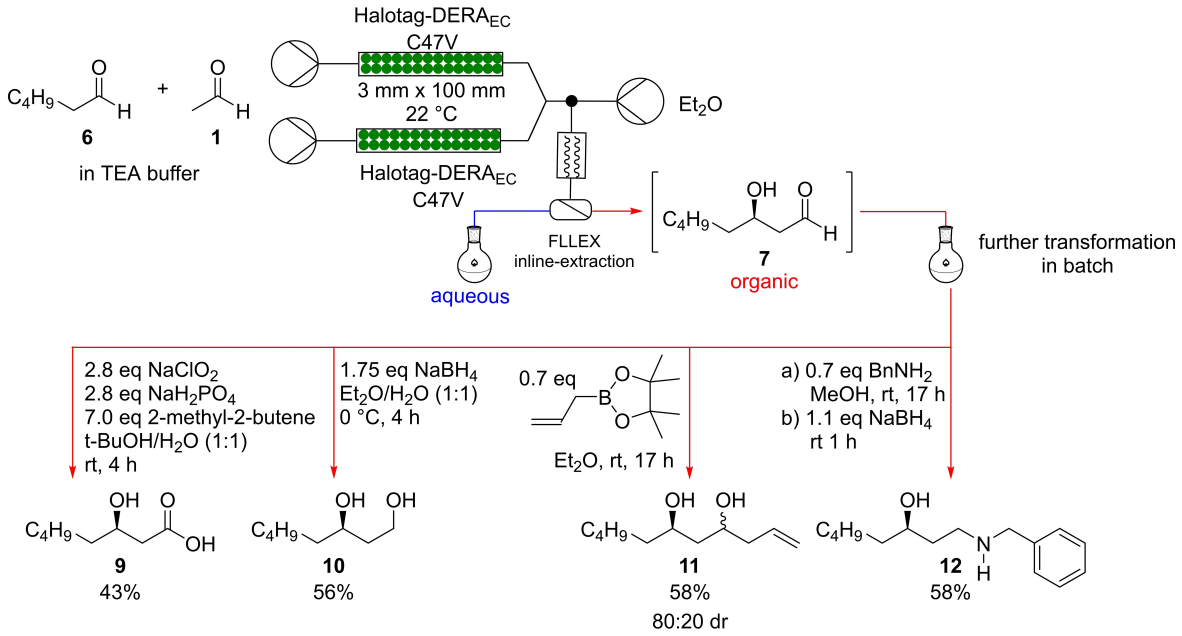


Figure 6. Experimental set-up for the semi-continuous flow mode two-step syntheses. For the first step in flow mode two enzyme reactors (Ø 3 mm, 100 mm each, green indicated) containing Halotag-DERA$_{Ec}$-C47V are used. The reaction mixture including the substrate acetaldehyde (**1**) and hexanal (**6**) in TEA buffer (0.1 M, pH 7.0) is pumped through the enzyme reactors and combined *via* a T-pieces to pass the FLLEX system for inline-extraction and phase separation. The organic phase (indicated red), which contain the desired product **7** is collected in a flask for sequential transformations in batch mode: Four sequential reactions were carried out, more precisely a $NaBH_4$-reduction towards the 1,3-diol **10**, a Pinnick oxidation towards a β-hydroxy acid **9**, an allyl addition resulting in homoallylalcohol **11** and a reductive amination towards product **12**. Isolated yields after purification. The configuration was confirmed by optical rotation measurements.

formation. The first DERA catalyzed reaction step in flow was carried out for the continuous flow synthesis under the optimized conditions described above for the DERA$_{EC}$ variant C47M, i.e. in TEA buffer with 0.2 mol/L hexanal (**6**), 3 equivalents acetaldehyde (**1**) and a flow rate of 50 μL/min. The experimental set-up is illustrated in Figure 7. As with the semi-continuous flow syntheses described above, two 5 cm Omnifit columns (Ø 3 mm, 50 mm) were used simultaneously, each with a reaction volume of 10 mL. After the enzyme reaction in flow, the system was connected to a series of two packed-bed reactors (3 mm × 150 mm) containing basic Amberlyst A-21 to catalyze the elimination of water. Full conversion was observed and resulted in 60% of the desired final product **8**.

Conclusion

To the best of our knowledge, we here present the first effective protocol for the use of DERA in flow for the targeted synthesis of the single aldol product 3-hydroxyaldehyde **7**. *E. coli* DERA variants C47V and C47M were found to be suitable catalysts that could be applied in flow after covalent immobilization via the Halotag® system. The decisive reaction parameters for the

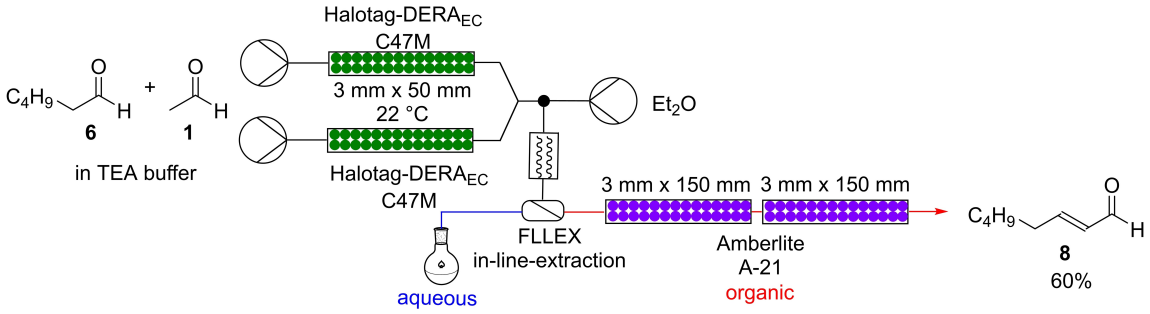


Figure 7. Experimental set-up for continuous flow mode two-step synthesis combining a DERA-C47M-catalyzed reaction step towards the (R)-3-hydroxyoctanal (**7**) and a further in-line elimination catalyzed by a basic Amberlyst resin. Two enzyme columns (3 mm × 50 mm) were used parallel. After extraction in flow and phase separation via FLLEX module, the reaction mixture is pumped through two columns (3 mm × 150 mm each) connected in series containing the basic A-21 resin for in-line elimination towards the desired α,β-unsaturated (E)-octenal (**8**) (60% yield of isolated product).

synthesis of the 3-hydroxyaldehyde **7** were primarily the flow rate and the concentrations of substrate. In the end, up to 90% conversion could be achieved with 73% of the desired 3-hydroxyaldehyde product **7** and only 10% of the otherwise difficult to separate double aldol DERA product **14**.

Finally, the optimized biocatalytic flow step was combined with different consecutive reaction steps, either using the collected crude product for further transformation in batch or directly using the flow stream in a second reaction in a packed-bed reactor. Application of a continuous extraction module allowed the biocatalytic reaction system in buffer to be combined with the organic synthesis environment of the following reaction system for optimized reaction conditions in both steps. Our findings provide a basis for further studies utilizing otherwise difficult to obtain single-aldol intermediates for reaction cascades.

Experimental Section

General information

All chemicals for synthesis were obtained from commercial suppliers and used without further purification unless otherwise stated. Solvents were reagent grade and were dried as well as purified by common methods. Thin-layer chromatography (TLC) was performed using pre-coated silica gel plates (Polygram® SIL G/UV, Macherey-Nagel) and components were visualized via oxidative staining or UV-light. Flash chromatography was performed on silica gel (Merck silica gel 60 (0.063–0.200 μm) and solvents for flash chromatography (petroleum ether/ethyl acetate/dichloromethane/*n*-pentane) were distilled prior to use. NMR spectra were measured on a *Bruker 600 Ultra Shield*™ with a frequency of 600 MHz for ^{1}H- and 151 MHz for ^{13}C-NMR spectra. Chemical shifts were determined by using tetramethyl silane as an internal standard and is given in ppm. Only deuterated solvents were used. Coupling constants *J are* given in Hz. The signals are described as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet) and m (multiplet). Besides, DEPT-(135°-pulse)-, ^{1}H-, ^{1}H- COSY, ^{1}H- ^{13}C- HSQC and ^{1}H- ^{13}C-HMBC-spectra were measured for evaluation. Infrared spectra were recorded on a *PerkinElmer FT-IR SpectrumTwo* spectrometer in the ATR mode. Absorption bands were given in $\tilde{\nu}$ [cm^{-1}]. The rotary power measurements were performed on a *KRÜSS P-8000-TF* in a 5.00 cm cuvette at 589 nm (sodium-D-line). As a solvent $CHCl_3$ was chosen. The specific rotation was calculated with *Biot* equation. Conversions were determined by using gas-liquid chromatography. GC-Chromatograms were measured on a *Thermo Scientific Trace® GC Ultra.* The probes were solved in ethyl acetate. Carrier gas was H_2 at 0.06 bar. For detection a FID detector was used. For exact GC methods, see SI.

Flow devices

Syringe pump and syringes: For experiments in flow mode a LegatoTM 100 Syringe pump (single syringe infusion pump) was used. To enable use with several syringes at the same time, a corresponding attachment was produced using a 3D printer. Luer-lock syringes from *B. Braun* Injekt® (5 mL; 10 mL 20 mL) were used.

FLLEX: For in-flow extraction the FLLEX module from *Syrris Asia* was used. The module was equipped with original *FLLEX* PTFE or *Merck* FHLP02500 PTFE membranes. For the latter, the polyethylene support was removed before use. The total system pressure was set to 3.00 bar. Depending on the solvent, the cross-membrane pressure (CMP) was chosen.

Silylation of glassware: To avoid any acidic source, the *Omnifit* glass columns used for the flow experiments with DERA were beforehand inactivated. For this purpose, the columns were immersed in a 10% Me_3SiCl solution in dichloromethane for 30 minutes. Afterwards they were washed thoroughly with dichloromethane and, in a second cleaning step, with acetone. The columns were then stored in the heating cabinet until use.

Enzyme activity measurement of 2-deoxyribose-5-phosphate aldolase

Enzyme activity was monitored using the natural substrate 2-deoxyribose-5-phosphate as described before.[28]

Enzyme production and immobilization procedure

2.00 g wet cell pellet of Halotag-$DERA_{EC}$ expressed in *E. coli* BL21 (DE3) was suspended in 10 mL triethanolamine buffer (0.1 M; pH 7.0). After ultrasonication using a *Bandelin SONOPLUS* with a *SONOPLUS KE76* cone tip for cell lysis, the crude extract has been centrifuged at 7000×g. Cell lysis was performed 2×10 min, 5×10% cycle at 35% power. The obtained supernatant was frozen in liquid nitrogen and lyophilized overnight. It was used without any further purification for the following immobilization. For immobilization in flow, an *Omnifit* column (Ø 3 mm, 50 mm or Ø 3 mm, 100 mm) was filled with HaloLink™ resin (*Promega*; stored in 20 vol% ethanol). The column was connected to the syringe pump. First, the freshly packed column was washed with deionized water to remove residual ethanol and afterwards equilibrated with five column volumes of triethanolamine buffer (0.1 M; pH 7.0). 25 mg of lyophilized crude extract per mL was dissolved in triethanolamine buffer and stored on ice for 20 min. Depending on the column size, 1 mL of the crude cell extract per cm of the column was used for immobilization. The immobilization step was performed at a flowrate of 30 μL/min. This was followed by another washing step with five column volumes of the corresponding buffer.[29] The theoretically immobilized enzyme activity on the column was determined by measuring the enzyme activity of the cell extract before the immobilization and in the flow through after the immobilization (Table 2).

Response factor analysis

Due to the instability of the single aldol product gas chromatography was chosen to investigate the reaction conversions. In order to be able to make quantitative statements about the investigated substances, standard series were measured to calculate response factors. (s. SI)

(*R*)-3-Hydroxyoctanal (7): isolation of the single aldol product and optimization using Design of Experiment

General Procedure: For the first experiments towards the single aldol product, hexanal was chosen as a model substrate. For the proof-of-concept, a 5 cm Omnifit® bed reactor (Ø 3 mm, 50 mm) containing the biocatalyst $DERA_{EC}$-C47M or $DERA_{EC}$-C47V was used. The two substrates hexanal and acetaldehyde were added in appropriate concentrations to triethanolamine buffer (0.1 M, pH 7.0) with a total volume of 5 mL in a beaker, taken up with a syringe and the syringe was shaken well. The reaction solution was then pumped through the catalyst column at varying flow rates using a syringe pump. The first milliliter of the product mixture was discarded to

ensure an equilibrium within the reactor. The product was collected on ice in vials. The conversion was determined by GC analysis. For preparation of the GC probes, 500 μL of product solution was extracted three times with ethyl acetate (HPLC grade) containing 1 mM 2-phenylethanol as internal standard. The organic phase was dried with $MgSO_4$ and transferred to GC vials for measuring.

Optimization using Design of Experiments: A systematic approach by means of design of experiments was chosen to optimize the turnover. This allows a systematic approach to the variation of the reaction parameter. The program Design Expert® (Version 7) and OriginPro® 2021was used. First, the DERA variant $DERA_{EC}$-C47M was chosen for the optimization experiments. A factorial design was used as the first optimization round. This was followed by another optimization round with a response-surface design. For exact data see SI. All experiments were carried out in an air-conditioned laboratory, so that a constant room temperature of 22 degrees was guaranteed.

(*R*)-Hydroxyoctanoic acid (9)

Two *Omnifit*® columns (3 mm × 100 mm) containing immobilized Halotag-$DERA_{EC}$-C47V parallel were used for the reaction in flow mode. 10 mL reaction volume was performed per column [in total: 20 mL solution containing 4.0 mmol hexanal (**6**)]. 10 mL substrate solution containing 0.2 M hexanal (**6**), 0.6 M acetaldehyde (**1**), and 0.1 M triethanolamine buffer (pH 7.0) was mixed in a 10 mL syringe and pumped with 70 μL/min through the packed-bed reactor containing the biocatalyst. For easier handling of the volatile acetaldehyde (**1**), a 1 M stock solution was used. The efflux of both columns was combined via a T-piece and passed through the FFLEX module for in-line-extraction. As extraction solvent diethyl ether was used, which was pumped with 70 μL/min into the FFLEX module. The extracted organic phase was pumped into a flask and the solvent was removed carefully under reduced pressure (not until completely dry). The intermediate was solved in 20 mL *t*-butanol. After addition of 7.0 eq 2-methyl-2-butene (28 mmol, 2.9 mL) and 10 mL of a solution of $NaClO_2$ (2.8 eq, 11.2 mmol, 1.00 g) and NaH_2PO_4*H_2O (2.8 eq, 11.2 mmol, 1.35 g) in water dropwise, the reaction was stirred for 4 hours at rt. After completed reaction, the reaction was diluted with 30 mL water and 1 M HCl was added until pH 1–3. The reaction mixture was three times extracted with diethyl ether and the organic phase washed with a saturated solution of NaCl. After drying with $MgSO_4$, the solvent was removed under reduced pressure. For product purification flash chromatography (CH_2Cl_2:MeOH:AcOH 98:2:0.1; R_f = 0.16) was performed. 270 mg (1.7 mmol, 43 %) of pure product **9** were isolated ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.04 (s, 1H, 3-OH), 2.57 (dd, $^2J_{2b,2a}$ = 16.56 Hz, $^3J_{2b,3}$ = 2.97 Hz, 1H, 2-H_b), 2,48 (dd, $^2J_{2a,2b}$ = 16.53 Hz, $^3J_{2a,3}$ = 9.02 Hz, 1H, 2-H_a), 1.57–1.54 (m, 1H, 3-H), 1.47–1.39 (m, 2H, 4-H), 1.36–1.24 (m, 5H, 5-H, 6-H, 7-H), 0.89 (t, 3H, 8-H). ^{13}C (151 MHz, $CDCl_3$): δ 176.58 (C-1), 67.01 (C-3), 40.06 (C-2), 35.45 (C-4), 30.63 (C-5), 24.10 (C-6), 21.55 (C-7), 12.98 (C-8). IR (ATR): 3387, 2930, 2864, 2657, 1711, 1405, 1262, 173, 1079, 941, 882, 805, 621. $[\alpha]^{25}_D$ = −10 (c = 0.1, $CHCl_3$). HRMS (ESI, positive-ion): *m/z* (%) calcd for $C_8H_{16}NaO_3$ $[M+Na]^+$: 183.0992; found: 183.0992. The analytic data are consistent with previously published data in literature.[31]

(*R*)-Octane-1,3-diol (10)

Two *Omnifit*® columns (3 mm × 100 mm) containing immobilized Halotag-$DERA_{EC}$-C47V parallel were used for the reaction in flow mode. 10 mL reaction volume was performed per column [in total: 20 mL solution containing 4.00 mmol hexanal (**6**)]. 10 mL substrate solution containing 0.2 M hexanal (**6**), 0.6 M acetaldehyde (**1**), and 0.1 M triethanolamine buffer (pH 7.0) was mixed in a 10 mL syringe and pumped with 70 μL/min through the packed-bed reactor containing the biocatalyst. For easier handling of the volatile acetaldehyde (**1**), a 1 M stock solution was used. The efflux of both columns was combined via a T-piece and passed through the FFLEX module for in-line-extraction. As extraction solvent diethyl ether was used, which was pumped with 70 μL/min into the FFLEX module. The extracted organic phase was pumped into a flask placed on ice. After addition of 1.75 eq $NaBH_4$ (265 mg, 7.00 mmol), the reaction was stirred for 4 hours at 0 °C. After completed reaction, the reaction was quenched with saturated NH_4Cl and three times extracted with CH_2Cl_2. The combined organic phase was washed with distilled water and dried with $MgSO_4$. The solvent was removed under reduced pressure. For product purification flash chromatography (PE:EE 50:50; R_f = 0.23) was performed. 326 mg (2.24 mmol, 56 %) of pure product **10** were isolated. ^{1}H NMR (600 MHz, $CDCl_3$): δ .4.12 (q, 1H, 6-H), 3.88–3.86 (m, 2H, 9-H), 2.75 (bs, 1H, 6-OH), 2.17 (s, 1H, 9-OH), 1.75–1.63 (m, 2H, 5-H), 1.58–1.37 (m, 2H, 7-H), 1.31–1.25 (m, 6H, 4-H, 3-H, 2-H), 0.90 (t, 3H, 1-H). ^{13}C (151 MHz, $CDCl_3$): δ 72.49 (C-1), 62.00 (C-3), 38.31 (C-2), 37.86 (C-4), 31.81 (C-5), 25.20 (C-7), 22.63 (C-6), 14.03 (C-8). IR (ATR): 3338, 2930, 2859, 1741, 1458, 1373, 1212, 1128, 1055, 715, 637. $[\alpha]^{25}_D$ = −6 (c = 0.1, $CHCl_3$). HRMS (ESI, positive-ion): *m/z* (%) calcd for $C_8H_{18}NaO_2$ $[M+Na]^+$: 169.1199; found: 169.1201. The analytic data are consistent with previously published data in literature.[30]

(6*R*)-Undec-1-ene-4,6-diol (11)

Two *Omnifit*® columns (3 mm × 100 mm) containing immobilized Halotag-$DERA_{EC}$-C47V parallel were used for the reaction in flow mode. 10 mL reaction volume was performed per column [in total: 20 mL solution containing 4.0 mmol hexanal (**6**)]. 10 mL substrate solution containing 0.2 M hexanal (**6**), 0.6 M acetaldehyde (**1**) and 0.1 M triethanolamine buffer (pH 7.0) was mixed in a 10 mL syringe and pumped with 70 μL/min through the packed-bed reactor containing the biocatalyst. For easier handling of the volatile acetaldehyde (**1**), a 1 M stock solution was used. The efflux of both columns was combined via a T-piece and passed through the FFLEX module for in-line-extraction. As extraction solvent diethyl ether was used, which was pumped with 70 μL/min into the FFLEX module. The extracted organic phase was pumped through a column containing molecular sieve (4 Å) for drying to avoid any side reactions during allylation and transferred in a flask. After addition of 0.7 eq allylboronic ester (525 μL, 2.80 mmol), the reaction was stirred overnight at rt. After completed reaction, the solvent was concentrated in *vacuo* and the product purified using flash chromatography (PE:EE 80:20; R_f = 0.64). 351 mg (1.88 mmol, 58 %) of product **11** were isolated in a syn:anti mixture of 80:20. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.94–5.72 (m, 2H, 2-H_{syn}, 2-H_{anti}), 5.14–5.05 (m, 2H, 1-$H_{a/syn}$, 1-$H_{b/syn}$), 4.96–4.82 (m, 2H,1-$H_{a/anti}$, 1-$H_{b/anti}$), 4.09–3.85 (m, 2H, 4-H, 6-H), 2.42–2.30 (m, 1H, 3-H_a), 2.27–2.16 (m, 1H, 3-H_b), 1.89 (dt, 1H, $^3J_{syn}$ = 13.85 Hz, 5-H_a), 1.75 (dt, 1H, $^3J_{anti}$ = 4.42 Hz, 5-H_b), 1.64 (d, 2H, 4-OH, 6-OH), 1.43–1.41 (m, 2H, 7-H), 1.34–1.19 (m, 6H, 8-H, 9-H, 10-H), 0.89 (t, 3H, 11-H). ^{13}C (151 MHz, $CDCl_3$): δ 134.91 (C-2syn), 132.91 (C-2anti), 116.60 (C-1syn), 112.48 (C-1anti), 70.37 (C-4), 67.30 (C-6), 40.55 (C-3), 40.06 (C-5), 37.00 (C-7), 30.73 (C-9), 23.66 (C-8), 21.57 (C-10), 13.00 (C-11). IR (ATR): 3064, 2930, 2864, 1712, 1418, 1296, 1186, 1128, 1083, 1050, 934, 876, 611. $[\alpha]^{25}_D$ = +2 (c = 0.1, $CHCl_3$). HRMS (ESI, positive-ion): *m/z* (%) calcd for $C_{11}H_{23}O_2$ $[M+H^+]$.: 187.1693; found: 187.1694. The analytic data are consistent with previously published data in literature.[32]

(*R*)-1-(Benzylamino)octan-3-ol (12)

Two *Omnifit*® columns (3 mm × 100 mm) containing immobilized Halotag-$DERA_{EC}$-C47V parallel were used for the reaction in flow

mode. 10 mL reaction volume was performed per column [in total: 20 mL solution containing 4.0 mmol hexanal (**6**)]. 10 mL substrate solution containing 0.2 M hexanal (**6**), 0.6 M acetaldehyde (**1**) and 0.1 M triethanolamine buffer (pH 7.0) was mixed in a 10 mL syringe and pumped with 70 μL/min through the packed-bed reactor containing the biocatalyst. For easier handling of the volatile acetaldehyde (**1**), a 1 M stock solution was used. The efflux of both columns was combined via a T-piece and passed through the FFLEX module for in-line-extraction. As extraction solvent diethyl ether was used, which was pumped with 70 μL/min into the FFLEX module. The organic phase was collected a flask placed on ice. Under reduced pressure the solvent was carefully removed (not until completely dry) and solved in 15 mL methanol. After addition of 0.7 eq benzylamine (206 μL, 2.8 mmol), the reaction was stirred for 17 h at rt for imine formation. Subsequently 1.1 eq $NaBH_4$ (170 mg, 4.48 mmol) were added to the reaction mixture and stirred for 1 h at rt. After completed reaction, the reaction was three times extracted with diethyl ether. The organic phase was washed with saturated solution of NaCl and dried with $MgSO_4$. The solvent was removed under reduced pressure. For product purification flash chromatography (CH_2Cl_2:MeOH:NH_3 98:2:0.01; $R_f = 0.15$) was performed. 270 mg (1.15 mmol, 58%) of pure product **12** (base form) was isolated. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.34–7.29 (m, 4H, Ar-H_{ortho}, Ar-H_{para}), 7.25–7.21 (m, 1H, Ar-H_{meta}), 3.78 (d, 2H, 9-H), 2.65–2.57 (m, 2H, 1-H), 2.14–1.90 (m, 2H, 2-H), 1.55–1.48 (m, 2H, 4-H), 1.40–1.17 (m, 6H, 5-H, 6-H, 7-H), 0.88 (t, 3H, 8-H). ^{13}C (151 MHz, $CDCl_3$): δ 140.26 (C-10), 128.31 (C-11, C-12, C-13, C-14, C15), 54.00 (C-9), 49.45 (C-1), 31.61 (C-4), 29.95 (C-2), 29.02 (C-5), 27.05 (C-6), 22. (C-7), 14.07 (C-8). IR (ATR): 3032, 2926, 2858, 2812, 1747, 1496, 1454, 1354, 1218, 121, 1031, 966, 728, 698. $[\alpha]^{25}_D = -6$ (c = 0.1, $CHCl_3$). HRMS (ESI, positive-ion): *m/z* (%) calcd for $C_{15}H_{26}NO$ $[M+H^+]$: 236.009; found: 236.2008.

(*E*)-Oct-3-enal (8)

Two *Omnifit*® columns (3 mm × 100 mm) containing immobilized Halotag-$DERA_{EC}$-C47M parallel were used for the reaction in flow mode. 10 mL reaction volume was performed per column [in total: 20 mL solution containing 4.0 mmol hexanal (**6**)]. 10 mL substrate solution containing 0.2 M hexanal (**6**), 0.6 M acetaldehyde (**1**) and 0.1 M triethanolamine buffer (pH 7.0) was mixed in a 10 mL syringe and pumped with 50 μL/min through the packed-bed reactor containing the biocatalyst. For easier handling of the volatile acetaldehyde (**1**), a 1 M stock solution was used. The efflux of both columns was combined via a T-piece and passed through the FFLEX module for in-line-extraction. As extraction solvent ethyl acetate was used, which was pumped with 50 μL/min into the FFLEX module. The extracted organic phase was then pumped through a column containing molecular sieve (4 Å) for drying. The reaction solution was then passed through two *Omnifit*® columns (3 mm × 150 mm) with 10 μL/min connected in series containing an alkaline resin (Amberlyst A-21) and collected in a flask on ice. The solvent was removed and the final product was isolated without further work up. 304 mg (2.40 mmol, 60%) of pure product **8** were isolated. ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.51 (d, 1H, $^3J = 1.1$ Hz, 1-H), 6.89–6.81 (m, 1H, 3-H), 6.12 (ddt, 1H, $^3J = 15.62$ Hz, $^3J = 7.89$ Hz, $^4J = 1.58$ Hz), 2.34 (td, 2H, $^3J = 8.27$ Hz, $^3J = 7.69$ Hz, $^3J = 6.18$ Hz, 4-H), 1.52 (p, 2H, 5-H), 1.38–1.34 (m, 4H, 7-H, 8-H), 0.91 (t, 3H, 8-H). ^{13}C (151 MHz, $CDCl_3$): δ 191.74 (C-1), 157.62 (C-3), 133.14 (C-2), 33.08 (C-4), 311.67 (C-6), 29.14 (C-5), 22.66 (C-7), 14.06 (C-8). IR (ATR): 2955, 2930, 2860, 1688, 1635, 1467, 1381, 1146, 1095, 974, 900, 632. HRMS (ESI, positive-ion): *m/z* (%) calcd for $C_8H_{14}O$ $[M+H^+]$.: 128.1196; found: 128.1194.

Acknowledgements

We gratefully acknowledge the state of North Rhine Westphalia (NRW) and the European Regional Development Fund (EFRE) for funding the project within the "CLIB-Kompetenzzentrum Biotechnologie", grant numbers 34-EFRE-0300096 and 34-EFRE-0300097, as well as the Heinrich Heine University Düsseldorf and the Forschungszentrum Jülich GmbH for their ongoing support. Part of the work was further supported by the AiF ("IGF-Vorhaben" 20341 BG). We thank Beatrix Paschold for provision of enzymes, Birgit Henßen for analytical support, Benedikt Baumer and Martin Wäscher for their scientific consultation. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

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

Keywords: biocatalysis · design of experiment · flow chemistry · stereoselective synthesis

[1] a) J. M. Woodley, *Trends Biotechnol.* **2008**, *26*, 321–327; b) G. Hughes, J. C. Lewis, *Chem. Rev.* **2018**, *118*, 1–3.
[2] a) J.-M. Choi, S.-S. Han, H.-S. Kim, *Biotechnol. Adv.* **2015**, *33*, 1443–1454; b) S. Panke, M. Held, M. Wubbolts, *Curr. Opin. Biotechnol.* **2004**, *15*, 272–279; c) M. D. Truppo, *ACS Med. Chem. Lett.* **2017**, *8*, 476–480; d) G. Qu, A. Li, C. G. Acevedo-Rocha, Z. Sun, M. T. Reetz, *Angew. Chem. Int. Ed.* **2020**, *59*, 13204–13231; *Angew. Chem.* **2020**, 132, 13304–13333.
[3] a) R. A. Sheldon, S. van Pelt, *Chem. Soc. Rev.* **2013**, *42*, 6223–6235; b) M. P. Thompson, I. Peñafiel, S. C. Cosgrove, N. J. Turner, *Org. Process Res. Dev.* **2018**, *23*, 9–18.
[4] a) R. Porta, M. Benaglia, A. Puglisi, *Org. Process Res. Dev.* **2016**, *20*, 2–25; b) M. Baumann, T. S. Moody, M. Smyth, S. Wharry, *Org. Process Res. Dev.* **2020**, *24*, 1802–1813.
[5] a) B. Gutmann, D. Cantillo, C. O. Kappe, *Angew. Chem. Int. Ed.* **2015**, *54*, 6688–6728; *Angew. Chem.* **2015**, *127*, 6788–6832; b) M. Baumann, I. R. Baxendale, S. V. Ley, *Mol. Diversity* **2011**, *15*, 613–630; c) J. Britton, S. Majumdar, G. A. Weiss, *Chem. Soc. Rev.* **2018**, *47*, 5891–5918.
[6] B. Baumer, T. Classen, M. Pohl, J. Pietruszka, *Adv. Synth. Catal.* **2020**, *362*, 2894–2901.
[7] a) M. Brovetto, D. Gamenara, P. Saenz Mendez, G. A. Seoane, *Chem. Rev.* **2011**, *111*, 4346–4403; b) B. Alcaide, P. Almendros, *Angew. Chem. Int. Ed.* **2003**, *42*, 858–860; *Angew. Chem.* **2003**, *115*, 884–886.
[8] a) A. Heine, G. DeSantis, J. G. Luz, M. Mitchell, C.-H. Wong, I. A. Wilson, *Science* **2001**, *294*, 369–374; b) A. Heine, J. G. Luz, C.-H. Wong, I. A. Wilson, *J. Mol. Biol.* **2004**, *343*, 1019–1034; c) T. Gefflaut, C. Blonski, J. Perie, M. Willson, *Prog. Biophys. Mol. Biol.* **1995**, *63*, 301–340; d) M. Haridas, E. M. Abdelraheem, U. Hanefeld, *Appl. Microbiol. Biotechnol.* **2018**, *102*, 9959–9971; e) H. J. M. Gijsen, C.-H. Wong, *J. Am. Chem. Soc.* **1994**, *116*, 8422–8423; f) P. Clapés, W.-D. Fessner, G. A. Sprenger, A. K. Samland, *Curr. Opin. Chem. Biol.* **2010**, *14*, 154–167.
[9] W. A. Greenberg, A. Varvak, S. R. Hanson, K. Wong, H. Huang, P. Chen, M. J. Burk, *PNAS* **2004**, *101*, 5788–5793.
[10] a) S. Jennewein, M. Schürmann, M. Wolberg, I. Hilker, R. Luiten, M. Wubbolts, D. Mink, *Biotechnol. J.* **2006**, *1*, 537–548; b) M. A. Huffman, A. Fryszkowska, O. Alvizo, M. Borra-Garske, K. R. Campos, P. N. Devine, D. Duan, J. H. Forstater, S. T. Grosser, H. M. J. S. Halsey, G. J. Hughes, J. Jo,

L. A. Joyce, J. N. Kolev, J. Liang, K. M. Maloney, B. F. Mann, N. M. Marshall, M. McLaughlin, J. C. Moore, G. S. Murphy, C. C. Nawrat, J. Nazor, S. Novick, N. R. Patel, A. Rodriguez-Granillo, S. A. Robaire, E. C. Sherer, M. D. Truppo, A. M. Whittaker, D. Verma, L. Xiao, Y. Xu, H. Yang, *Science* **2019**, *366*, 1255–1259; c) M. Müller, *Angew. Chem. Int. Ed.* **2005**, *44*, 362–365; *Angew. Chem.* **2005**, *117*, 366–369.

[11] B. Grabner, Y. Pokhilchuk, H. J. C. Gruber-Woelfler, *Catalysts* **2020**, *10*, 137.

[12] A. M. Abdel-Mawgoud, F. Lépine, E. Déziel, *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1323–1336.

[13] a) T. Adams, C. L. Gavin, S. Taylor, W. Waddell, S. M. Cohen, V. Feron, J. Goodman, I. Rietjens, L. Marnett, P. Portoghese, *Food Chem. Toxicol.* **2008**, *46*, 2935–2967; b) D. Rowe, *Chem.Technol. Flav. Fragr.*, John Wiley & Sons, **2009**.

[14] a) K. Chen, J. M. Richter, P. S. Baran, *J. Am. Chem. Soc.* **2008**, *130*, 7247–7249; b) M. Fresia, M. Kock, T. Lindel, *Chemistry* **2020**, *26*, 12733; c) J. Staunton, K. J. Weissman, *Nat. Prod. Rep.* **2001**, *18*, 380–416; d) D. Holt, M. J. Gaunt, *Angew. Chem.* **2015**, *127*, 7968–7972; *Angew. Chem. Int. Ed.* **2015**, *127*, 7968–7972; e) C. Hertweck, *Angew. Chem. Int. Ed.* **2009**, *48*, 4688–4716; *Angew. Chem.* **2009**, *121*, 4782–4811; f) M. Saifuddin, C. Guo, L. Biewenga, T. Saravanan, S. J. Charnock, G. J. Poelarends, *ACS Catal.* **2020**, *10*, 2522–2527.

[15] a) T. Kochi, T. P. Tang, J. A. Ellman, *J. Am. Chem. Soc.* **2003**, *125*, 11276–11282; b) S. Shibahara, S. Kondo, K. Maeda, H. Umezawa, M. Ohno, *J. Am. Chem. Soc.* **1972**, *94*, 4353–4354; c) S. Knapp, *Chem. Rev.* **1995**, *95*, 1859–1876; d) H. L. Sham, C. Zhao, L. Li, D. A. Betebenner, A. Saldivar, S. Vasavanonda, D. J. Kempf, J. J. Plattner, D. W. Norbeck, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3101–3103; e) L. Schreyer, P. S. J. Kaib, V. n. Wakchaure, C. Obradors, R. Properzi, S. Lee, B. List, *Science* **2018**, *362*, 216–219.

[16] a) M. Dick, R. Hartmann, O. H. Weiergraber, C. Bisterfeld, T. Classen, M. Schwarten, P. Neudecker, D. Willbold, J. Pietruszka, *Chem. Sci.* **2016**, *7*, 4492–4502; b) J. Bramski, M. Dick, J. Pietruszka, T. Classen, *J. Biotechnol.* **2017**, *258*, 56-58. For further investigations on the structural dynamics, see: c) M. Schulte, D. Petrovic, P. Neudecker, R. Hartmann, J. Pietruszka, S. Willbold, D. Willbold, V. Panwalkar, *ACS Catal.* **2018**, *8*, 3971–3984.

[17] R. C. Rodrigues, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. Fernández-Lafuente, *Chem. Soc. Rev.* **2013**, *42*, 6290–6307.

[18] F. Subrizi, M. Crucianelli, V. Grossi, M. Passacantando, G. Botta, R. Antiochia, R. Saladino, *ACS Catal.* **2014**, *4*, 3059–3068.

[19] T. Y. Nara, H. Togashi, S. Ono, M. Egami, C. Sekikawa, Y.-h. Suzuki, I. Masuda, J. Ogawa, N. Horinouchi, S. Shimizu, F. Mizukami, T. Tsunoda, *J. Mol. Catal. B* **2011**, *68*, 181–186.

[20] P. De Santis, L.-E. Meyer, S. Kara, *React. Chem. Eng.* **2020**, *5*, 2155–2184.

[21] A. Wang, W. Gao, F. Zhang, F. Chen, F. Du, X. Yin, *Bioprocess Biosyst. Eng.* **2012**, *35*, 857–863.

[22] H. Fei, G. Xu, J.-P. Wu, L.-R. Yang, *J. Mol. Catal. B* **2014**, *101*, 87–91.

[23] a) S. Reinicke, H. C. Rees, P. Espeel, N. Vanparijs, C. Bisterfeld, M. Dick, R. Rosencrantz, G. Brezesinski, B. G. De Geest, F. E. Du Prez, J. Pietruszka, A. Böker, *ACS Appl. Mater. Interfaces* **2017**, *9*, 8317–8326; b) S. Zhang, C. Bisterfeld, J. Bramski, N. Vanparijs, B. G. De Geest, J. Pietruszka, A. Böker, S. Reinicke, *Bioconjugate Chem.* **2018**, *29*, 104–116; c) S. Zhang, J. Bramski, M. Tutus, J. Pietruszka, A. Böker, S. Reinicke, *ACS Appl. Mater. Interfaces* **2019**, *11*, 34441–34453.

[24] a) L. P. Encell, R. F. Ohana, K. Zimmerman, P. Otto, G. Vidugiris, M. G. Wood, G. V. Los, M. G. McDougall, C. Zimprich, N. Karassina, *Curr. Chem. Genomics* **2012**, *6*, 55; b) G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R. Learish, R. F. Ohana, M. Urh, *ACS Chem. Biol.* **2008**, *3*, 373–382; c) J. Döbber, M. Pohl, *J. Biotechnol.* **2017**, *241*, 170–174.

[25] a) L. Chen, D. P. Dumas, C. H. Wong, *J. Am. Chem. Soc.* **1992**, *114*, 741–748; b) W.-C. Chou, L. Chen, J.-M. Fang, C.-H. Wong, *J. Am. Chem. Soc.* **1994**, *116*, 6191–6194.

[26] R. K. Boeckman Jr, J. R. Miller, *Org. Lett.* **2009**, *11*, 4544–4547.

[27] H. B. Heath, *Source book of flavors:(AVI Sourcebook and Handbook Series), Vol. 2*, Springer Science & Business Media, **1981**.

[28] M. Dick, O. H. Weiergräber, T. Classen, C. Bisterfeld, J. Bramski, H. Gohlke, J. Pietruszka, *Sci. Rep.* **2016**, *6*, 17908.

[29] J. Döbber, M. Pohl, S. Ley, B. Musio, *React. Chem. Eng.* **2018**, *3*, 8–12.

[30] M. Campagna, M. Trzoss, S. Bienz, *Org. Lett.* **2007**, *9*, 3793–3796.

[31] J. L. Motley, B. W. Stamps, C. A. Mitchell, A. T. Thompson, J. Cross, J. You, D. R. Powell, B. S. Stevenson, R. H. Cichewicz, *J. Nat. Prod.* **2017**, *80*, 598–608.

[32] F. Allais, M.-C. Louvel, J. Cossy, *Synlett* **2007**, 451–452.

Manuscript received: March 22, 2022
Revised manuscript received: May 13, 2022
Accepted manuscript online: May 16, 2022
Version of record online: June 23, 2022