Electronic Supplementary Information

Tailor-made catalytically active inclusion bodies for different applications in biocatalysis

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2 Experimental

2.1 Cloning & sequences

The gene encoding for *Pf*BAL was cloned into a pET28a vector containing the gene fragment encoding for the TDoT-domain, a linker region consisting of 3xGGGS linker and the Factor Xa protease recognition site (L), which was performed based on the earlier described cloning strategy.[1] In brief, the gene coding for *Pf*BAL was amplified by PCR using the below listed oligonucleotide primers (Table S1), and subsequently inserted into the above described pET28a vector by restriction with *BamH*I and *Not*I and ligation, resulting in an N-terminal fusion of the target enzyme to linker and TDoT (pTDoT-Xa-L-*Pf*BAL) (Table S2). To insert the 3HAMP domain into the so generated vector (pTDoT-Xa-L-*Pf*BAL), the 3HAMP gene fragment was codon-optimized, synthesized by Eurofins Genomics (Ebersberg, Germany) and supplied on a plasmid (pEX-A-3HAMP-Linker). The DNA fragment coding for 3HAMP-Linker was subsequently cloned into the above described vector (pTDoT-Xa-L-*Pf*BAL) by restriction with endonucleases *Nde*I and *Spe*I and ligation to obtain a vector consisting of 3HAMP-domain, a linker region consisting of 3xGGGS linker and the Factor Xa protease recognition site (L) and the enzyme *Pf*BAL (p3HAMP-Xa-L-*Pf*BAL). All final constructs were verified by sequencing (LGC genomics, Berlin, Germany). Plasmid amplification was performed in *E. coli* DH5α (Table S3).

Table S1: Primer sequences for amplification of *Pf*BAL gen with *BamH*I and *Not*I cleavage sites (underlined)

name	sequence
BamHI_BAL _fw	5'- ATATATGGATCCATGGCGATGATTACAGGCGGCGAAC -3'
BAL_NotI_rev	5'- ATATAT GCGGCCGCTTATGCGAAGGGGTCCATG -3'

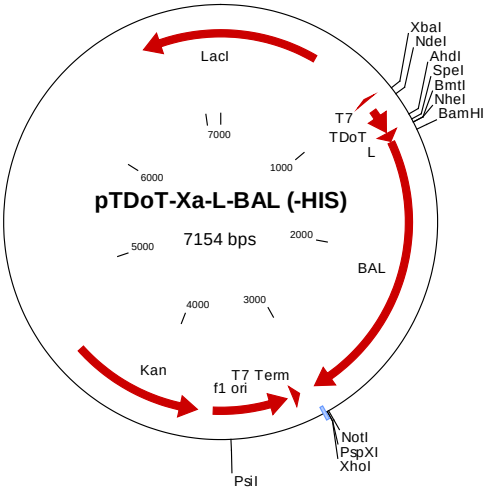
Table S2: The used vectors are given with genotype and cloning description. DNA and amino acid sequences are stated in section 2.3-2.10.

vector	genotype	description
pET28a	*ColE1 lacZ'* Kan^R P_{T7} P_{lac}	Merck (Darmstadt, Germany)
BAL*His*/ pKK233_2	pKK233_2 P_{trc}, gene fusion [*pfbal, His-tag]*	Janzen et al. 2006 [2]
pTDoT-L-*Pf*BAL	pET28a, P_{T7}, gene fusion [*tdot*-factor Xa recognition site-*(GGGS)$_3$ linker- pfbal*]	pTDoT-L-*At*HNL derivative, insertion of 1699 bp PCR-amplified *Bam*HI/*Not*I *pfbal* fragment in pTDoT-L-*At*HNL; without the 784 bp fragment containing *hnl* [1]
p3HAMP-L-*Pf*BAL	pET28a, P_{T7}, gene fusion [*3hamp*-factor Xa recognition site-*(GGGS)$_3$ linker-pfbal*]	pTDoT-L-*Pf*BAL derivative, insertion of a 518 bp *Nde*I/*Spe*I *3hamp*-containing fragment in pTDoT-L-*Pf*BAL; without the 155 bp fragment containing *tdot*

Table S3: The used strains are given with genotype and reference or source.

strains	genotype	reference or source
E. coli BL21 (DE3)	*F⁻ ompT hsdSB(rB⁻ mB⁻) gal dcm (λIts857ind1 Sam7 nin5 lacUV5-T7 gene1)*	Studier & Moffatt, 1986 [3], Invitrogen (Carlsbad, USA)
E. coli DH5α	*supE44 ΔlacU169 (Φ80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen (Carlsbad, USA)
E. coli SG 13009	*F⁻ ompT hsdS$_B$ (rB⁻mB⁻) dcm gal* (DE3)	Qiagen (Hilden Germany)

2.2 Expression vector of TDoT-*Pf*BAL



2.3 DNA-sequence of the pET28a vector containing the gene fusion encoding for TDoT-*Pf*BAL

vector DNA (grey), start and stop codon of the *tdot-pfbal*-ORF (red), *Pf*BAL gen (black), linker (green), TDoT (orange), restrictions sites (blue)

CAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCT
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TGCTATGCGGGGCGGGCTGGTGCAAAACCTCTATTCTTTTGCCAAAGCCGATGCCGCGCC
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AACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGC
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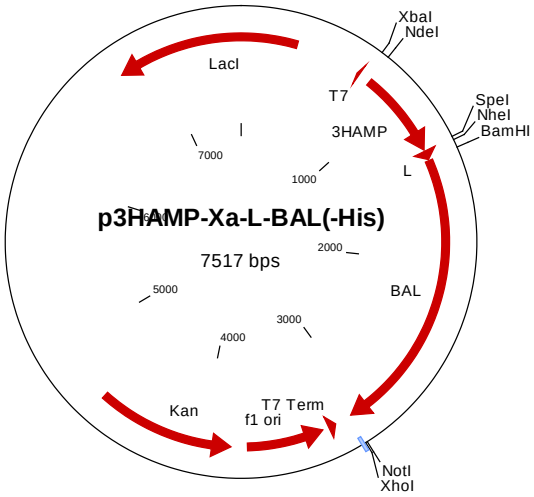
GGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAG
ATATTTATGCCAGC

2.4 Amino acid sequence of TDoT-*Pf*BAL

***Pf*BAL gen (black), linker (green), TDoT (orange), restrictions sites (blue)**

M I I N E T A D D I V Y R L T V I I D D R Y E S L K N L I T L R A D R L E M I I N D N V S T I L A S I T S I E G R A S G G G S
G G G S G G G S G S M A M I T G G E L V V R T L I K A G V E H L F G L H G A H I D T I F Q A C L D H D V P I I D T R H
E A A A G H A A E G Y A R A G A K L G V A L V T A G G G F T N A V T P I A N A W L D R T P V L F L T G S G A L R D D
E T N T L Q A G I D Q V A M A A P I T K W A H R V M A T E H I P R L V M Q A I R A A L S A P R G P V L L D L P W D I
L M N Q I D E D S V I I P D L V L S A H G A R P D P A D L D Q A L A L L R K A E R P V I V L G S E A S R T A R K T A L S
A F V A A T G V P V F A D Y E G L S M L S G L P D A M R G G L V Q N L Y S F A K A D A A P D L V L M L G A R F G L N
T G H G S G Q L I P H S A Q V I Q V D P D A C E L G R L Q G I A L G I V A D V G G T I E A L A Q A T A Q D A A W P D R
G D W C A K V T D L A Q E R Y A S I A A K S S S E H A L H P F H A S Q V I A K H V D A G V T V V A D G A L T Y L W L S
E V M S R V K P G G F L C H G Y L G S M G V G F G T A L G A Q V A D L E A G R R T I L V T G D G S V G Y S I G E F D T
L V R K Q L P L I V I I M N N Q S W G A T L H F Q Q L A V G P N R V T G T R L E N G S Y H G V A A A F G A D G Y H V
D S V E S F S A A L A Q A L A H N R P A C I N V A V A L D P I P P E E L I L I G M D P F A

2.5 Expression vector of 3HAMP-*Pf*BAL



2.6 DNA-sequence of the pET28a vector containing the gene fusion encoding for 3HAMP-*Pf*BAL

vector DNA (grey), start and stop codon of the *3hamp-pfbal*-ORF (red), *Pf*BAL gen (black), linker (green), 3HAMP (orange), restrictions sites (blue)

GCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGG

CACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGG
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GTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGC
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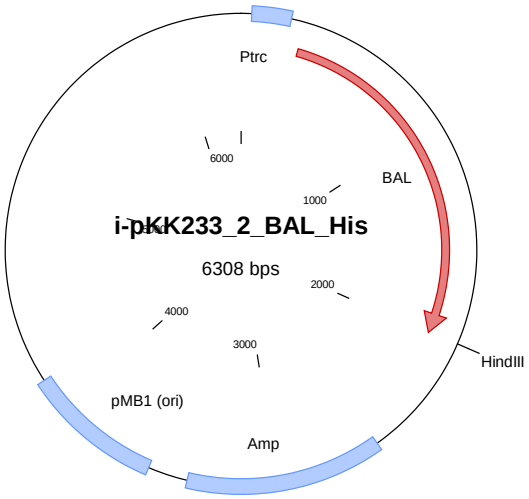
TTGCGCGAGAAGATTGT

2.7 Amino acid sequence of 3HAMP-*Pf*BAL

***Pf*BAL gen (black), linker (green), 3HAMP (orange), restrictions sites (blue)**

M G L F N A H A V A Q Q R A D R I A T L L Q S F A D G Q L D T A V G E A P A P G Y E R L Y D S L R A L Q R Q L R E Q
R A E L Q Q V E S L E A G L A E M S R Q H E A G W I D Q T I P A E R L E G R A A R I A K G V N E L V A A H I A V K M K
V V S V V T A Y G Q Q G N F E P L M D R L P G K K A Q I T E A I D G V R E R L R G A A E A T S A Q L A T A A Y N T S I E
G R A S G G G S G G G S G G G S G G G S G S M A M I T G G E L V V R T L I K A G V E H L F G L H G A H I D T I F Q A C L D H
D V P I I D T R H E A A A G H A A E G Y A R A G A K L G V A L V T A G G G F T N A V T P I A N A W L D R T P V L F L T
G S G A L R D D E T N T L Q A G I D Q V A M A A P I T K W A H R V M A T E H I P R L V M Q A I R A A L S A P R G P V
L L D L P W D I L M N Q I D E D S V I I P D L V L S A H G A R P D P A D L D Q A L A L L R K A E R P V I V L G S E A S R
T A R K T A L S A F V A A T G V P V F A D Y E G L S M L S G L P D A M R G G L V Q N L Y S F A K A D A A P D L V L M
L G A R F G L N T G H G S G Q L I P H S A Q V I Q V D P D A C E L G R L Q G I A L G I V A D V G G T I E A L A Q A T A
Q D A A W P D R G D W C A K V T D L A Q E R Y A S I A A K S S S E H A L H P F H A S Q V I A K H V D A G V T V V A D
G A L T Y L W L S E V M S R V K P G G F L C H G Y L G S M G V G F G T A L G A Q V A D L E A G R R T I L V T G D G S
V G Y S I G E F D T L V R K Q L P L I V I I M N N Q S W G A T L H F Q Q L A V G P N R V T G T R L E N G S Y H G V A A
A F G A D G Y H V D S V E S F S A A L A Q A L A H N R P A C I N V A V A L D P I P P E E L I L I G M D P F A

2.8 Expression vector of *Pf*BAL



2.9 DNA-sequence of the pKK233_2 vector containing the gene encoding for *Pf*BAL

vector DNA (grey), start and stop codon of the *pfbal*-ORF (red), *Pf*BAL gen (black), His-Tag (brown), restrictions sites (blue)

AATTCTCATGTTTGACAGCTTATCATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAG
GCAGCCATCGGAAGCTGTGGATTGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTC
GCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATATAAACGGTTCTGG
CAAATATTCTGAAATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATT
GTGAGCGGATAACAATTTCACACAGGAAACAGACCATGGCGATGATTACAGGCGGCGAAC
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CGTTGAGCGCGCCACGCGGGCCGGTGTTGCTGGATCTGCCGTGGGATATTCTGATGAACC
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CCGACCCTGCCGATCTGGATCAGGCTCTCGCGCTTTTGCGCAAGGCGGAGCGGCCGGTCA
TCGTGCTCGGCTCAGAAGCCTCGCGGACAGCGCGCAAGACGGCGCTTAGCGCCTTCGTGG
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CGCCAGATCTCGTGCTGATGCTGGGGGCGCGCTTTGGCCTTAACACCGGGCATGGATCTG
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TGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTG
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TCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAG
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AGGCGCCAGCAACGGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGA
GGATCCGG

2.10 Amino acid sequence of soluble *Pf*BAL

***Pf*BAL** gen (black), His-Tag (brown), restrictions sites (blue)

M A M I T G G E L V V R T L I K A G V E H L F G L H G A H I D T I F Q A C L D H D V P I I D T R H E A A A G H A A E G
Y A R A G A K L G V A L V T A G G G F T N A V T P I A N A W L D R T P V L F L T G S G A L R D D E T N T L Q A G I D Q
V A M A A P I T K W A H R V M A T E H I P R L V M Q A I R A A L S A P R G P V L L D L P W D I L M N Q I D E D S V I I
P D L V L S A H G A R P D P A D L D Q A L A L L R K A E R P V I V L G S E A S R T A R K T A L S A F V A A T G V P V F A
D Y E G L S M L S G L P D A M R G G L V Q N L Y S F A K A D A A P D L V L M L G A R F G L N T G H G S G Q L I P H S
A Q V I Q V D P D A C E L G R L Q G I A L G I V A D V G G T I E A L A Q A T A Q D A A W P D R G D W C A K V T D L A
Q E R Y A S I A A K S S S E H A L H P F H A S Q V I A K H V D A G V T V V A D G A L T Y L W L S E V M S R V K P G G F
L C H G Y L G S M G V G F G T A L G A Q V A D L E A G R R T I L V T G D G S V G Y S I G E F D T L V R K Q L P L I V I I
M N N Q S W G A T L H F Q Q L A V G P N R V T G T R L E N G S Y H G V A A A F G A D G Y H V D S V E S F S A A L A
Q A L A H N R P A C I N V A V A L D P I P P E E L I L I G M D P F G S H H H H H H

2.11 Long-term stability measurement in buffer

Stability of the soluble *Pf*BAL and the CatIBs were analyzed after incubation in TEA-buffer (50 mM, pH 7.5, 2.5 mM $MgSO_4$, 0.5 mM ThDP) at 30°C and 1000 rpm. Therefore, 0.6 mg ml^{-1} protein (calculated based on the protein content) of each enzyme (weight: 14.47 mg *Pf*BAL, 9.92 mg TDoT-*Pf*BAL, 41.76 mg 3HAMP-*Pf*BAL) was incubated in 600 µl volume in polypropylene reaction tubes (1.5 ml safe-lock tube, Eppendorf, Germany) and sampled at different points in time (0 h, 4 h, 24 h, 48 h, 72 h) to determine the initial rate activity (see sect. 4.7 in the main paper). Therefore the enzyme solutions were respectively diluted. This stability assay was performed as single measurement.

2.12 Solvent selection for the micro-aqueous reaction system

To select an optimal organic solvent, the conversion of the carboligation reaction of 100 mM 3,5-dimethoxybenzaldehyde (DMBA) by 0.6 U ml^{-1} TDoT-*Pf*BAL was measured (2.89 mg ml^{-1} protein concentration). Therefor micro-aqueous systems were prepared by adding 5 vol% TEA-buffer (1 M, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP) to 5 different organic solvents (cyclopentyl methyl ether (CPME), methyl *tert*-butyl ether (MTBE), cyclohexanone, dimethyl carbonate, 2-methyltetrahydro-furan). First the CatIBs were suspended in buffer and then the organic solvent containing the substrate was added. The reaction was performed in a volume of 1 ml in 2 ml glass reaction tubes (G1 clear, CS-Chromatographie Service GmbH, Germany) at 30 °C und 1400 rpm in a thermomixer (Thermomixer comfort, Eppendorf, Germany). 20 µl samples were taken from the organic phase after 1 h and 17.5 h, and were diluted 1:10 in 180 µl 2-methyltetrahydrofuran, thoroughly mixed and centrifuged at 15800 x *g* for 1 min (Centrifuge 5424, Eppendorf, Germany). Subsequently, 20 µl sample from the supernatant was diluted 1:10 in 180 µl n-heptane (incl. 4.3 mM acetophenone as internal standard), which was analyzed by HPLC (see sect. 4.13 in the main paper). To analyze evaporation effects, control samples were prepared in the same manner without CatIBs. Each solvent was tested once.

2.13 Optimization of the buffer content in the biphasic reaction system

The buffer content was optimized between 1 vol% - 20 vol% TEA-buffer (1 M, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP) added to either MTBE or CPME. The carboligation reaction of 50 mM DMBA was measured catalyzed by 0.6 U ml^{-1} TDoT-*Pf*BAL (2.9 mg ml^{-1} protein concentration). The reaction was performed as described in sect. 2.12. 20 µl samples taken at different points in time, were prepared as described in sect. 2.12, and analyzed by HPLC (see sect. 4.13 in the main paper). These reactions were performed in duplicate.

2.14 Determination of the reaction equilibrium in the biphasic reaction system

The reaction equilibrium was measured by the cleavage of 32 mM (*R*)-3,3´,5,5´-tetramethoxy benzoin (TMBZ) catalyzed by 6 U ml^{-1} 3HAMP-*Pf*BAL (1.1 mg ml^{-1} protein concentration) in 30 vol% TEA-buffer (50 mM, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP) in CPME. The reaction was performed as described in sect. 2.12. 20 µl samples taken after 1 h and, were prepared as described in sect. 2.12, and analyzed by HPLC (see sect. 4.13 in the main paper). These reactions were performed in as single measurement.

3 Results

3.1 Live cell images

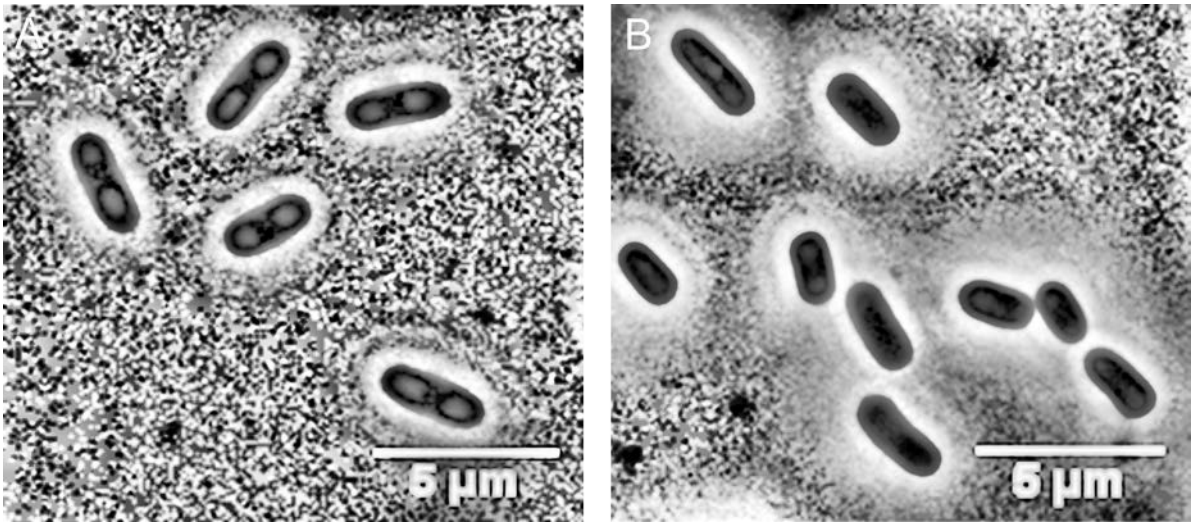


Figure S1: Live cell images of *E. coli* BL21(DE3) cells containing *Pf*BAL-CatIBs. A: TDoT-*Pf*BAL, B: 3HAMP-*Pf*BAL. Images were recorded using an inverted epifluorescence microscope in phase-contrast (see sect. 4.5 in the main paper) For better visualization the pictures were modified by image equalization with CorelDraw X6, version 16.0.0.707. 3HAMP-*Pf*BAL yielded rather diffuse particles at the cell poles, which are less clearly visible in unmodified phase-contrast images (main paper, Figure 1B). The corresponding particles can however be clearly detected after local image equalization (B), which involves increasing the contrast by resetting the darkest and lightest points and then evenly distributing the values across those two points.

3.2 Stability in buffer

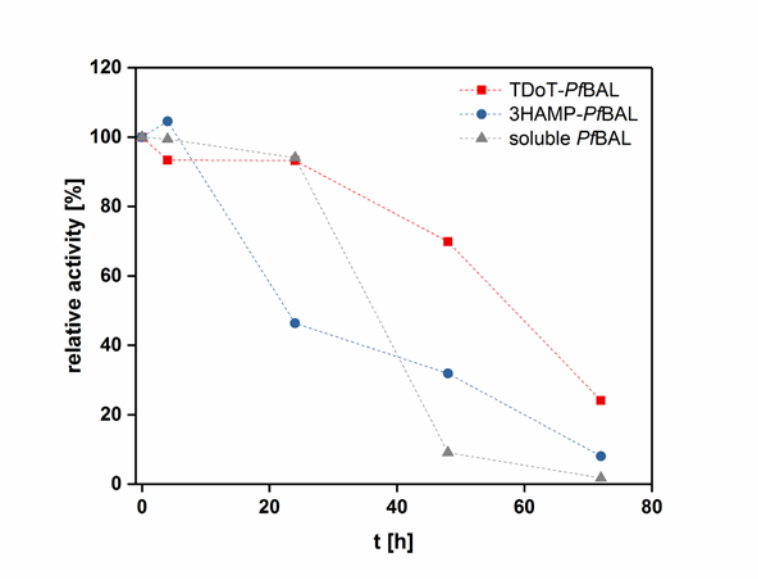


Figure S2: Stability of soluble *Pf*BAL, TDot-*Pf*BAL and 3HAMP-*Pf*BAL incubated in 50 mM TEA-buffer (pH 7.5). Incubation conditions: 50 mM TEA-buffer (pH 7.5, 2.5 mM $MgSO_4$, 0.5 mM ThDP), protein concentration for all enzyme variants: 0.6 mg ml^{-1}, 1000 rpm, T = 30 °C , V = 1 ml; n = 1. After distinct points in time, the initial rate activity was measured. For experimental details see ESI sect. 2.11. The half-lives decreased in the order TDoT-*Pf*BAL (57 h) > soluble *Pf*BAL (36 h) > 3HAMP-*Pf*BAL (23 h) and were estimated based on the deactivation curve.

3.3 EMR experiments

3.3.1 Overview of results obtained in EMR experiments

Table S4: Half-life and residual (*R*)-benzoin in the reaction chamber of *Pf*BAL, TDoT-*Pf*BAL, and 3HAMP-*Pf*BAL-CatIBs determined based on the experiments shown in Figure S5, Figure 3 in the main paper.
Half-lives were taken from the curve at 50% conversion from benzaldehyde to (*R*)-HPP. For experimental details see sect. 4.8 in the main paper. The residual (*R*)-benzoin was determined by transferring the whole suspension from the reaction chamber into a glass vessel, where methyltetrahydrofuran (m-THF) was added to the water-phase. The water-phase was extracted by m-THF. The reaction chamber was washed with m-THF and pooled with the m-THF phase. Finally the (*R*)-benzoin concentration in the m-THF phase was measured by HPLC and the amount of (*R*)-benzoin was calculated based on the measured concentration and the respective m-THF volume.

pH	reaction system	enzyme	half-life [h]	residual (*R*)-benzoin in the reaction chamber [mg]	(*R*)-benzoin conversion [%] in the reaction chamber calculated based on the total benzaldehyde amount (n/n)
7.5	buffer	TDoT-*Pf*BAL	48	n.d.	n.d.
		3HAMP-*Pf*BAL	10	n.d.	n.d.
		*Pf*BAL	7	7.2	2.5
7.5	30 vol% DMSO in buffer	TDoT-*Pf*BAL	131	n.d.	n.d.
		3HAMP-*Pf*BAL	59	0.036	0.0054
		*Pf*BAL	92	114.3	7.2
9.0	30 vol% DMSO in buffer	TDoT-*Pf*BAL	3	157.3	57.1
		3HAMP-*Pf*BAL	13	n.d.	n.d.
		*Pf*BAL	16	148.0	16.8

3.3.2 Absorption of benzaldehyde and (*R*)-HPP during the EMR experiments

Due to gaps in the mass balance and a maximum conversion of 80% (compare sect. 2.2 in the main paper), the absorption of benzaldehyde and (*R*)-HPP by the PEEK-(polyether ether ketone)-reactor material was tested in the EMR under reaction conditions (30 °C and 300 rpm, 10 kDa membrane) in TEA-buffer or the buffer-DMSO system with 30 vol% DMSO. Therefore, either a benzaldehyde (30 mM) or (*R*)-HPP (30 mM) solution was pumped through the reactor under conditions given in sect. 4.8 in the main paper in the absence of enzyme. After pumping overnight, samples of the efflux (direct at the output, without storage) and the reaction chamber were taken (Figure S3) and analyzed by HPLC (see sect. 4.13 in the main paper). Furthermore, samples were collected in open glass test tubes by a fraction collector, which were stored for the given period under the hood for evaporation analysis (Figure S4). In Figure S3 the relative difference between the initial concentration in the substrate reservoir and in the efflux or reaction chamber are shown in a box plot to visualize the significance of the differences compared to 0 (no difference). The whiskers indicate the minimum and maximum of the data set and the upper and lower quartiles 25% and 75%. Furthermore, outliers, the median and mean are given, whereas the median refers to the middle value of the data set and the mean is calculated by summarizing all values divided by the number of values. If the box (upper and lower quartiles) is on the doted zero line, the difference can be considered as not significant, so that there is no significant absorption of benzaldehyde or (*R*)-HPP by the reactor material, as can be seen in all cases for the buffer-DMSO system. However, in the buffer system without DMSO, benzaldehyde and (*R*)-HPP were absorbed by the reactor material. The open glass test tubes containing benzaldehyde or (*R*)-HPP solution were stored over different periods in time under the hood, which results in a more distinct evaporation of benzaldehyde in the buffer system without DMSO (A) than with 30 vol% DMSO (B) (Figure S4). The (*R*)-HPP concentration increased over a longer storage period due to evaporation of the solvent, which is more distinct for the buffer system without DMSO. (*R*)-HPP does not evaporate in both reaction systems. Table S5 shows the absorption of benzaldehyde by polypropylene (PP) reaction tubes over the time.

Table S5: Absorption of benzaldehyde by polypropylene (PP) reaction tubes (1.5 ml safe-lock tube, Eppendorf, Germany) after different incubation times. Benzaldehyde (33.5 mM) was dissolved in TEA-buffer (50 mM, pH 7.5, 2.5 mM $MgSO_4$, 0.5 mM ThDP) in a glass flask and 500 µl were transferred into a PP reaction tube and incubated with closed lid at room temperature without shaking. Subsequently, the concentration of benzaldehyde in the PP tube was determined by HPLC (sect. 4.13 in the main paper) after 2, 5 and 10 min. Errors correspond to the standard deviation of the mean obtained from technical triplicate.

time (min)	benzaldehyde (mM)	standard deviation (%)
0	33.5	
2	31.0	7.4
5	30.9	7.8
10	30.9	7.8

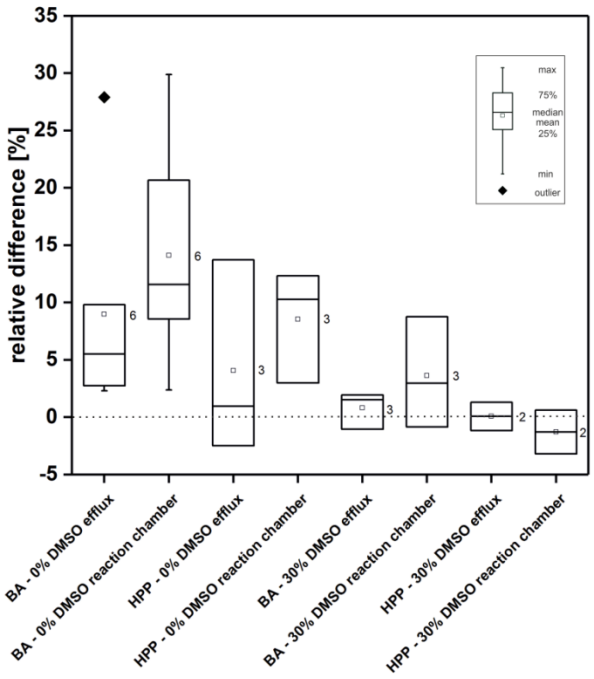
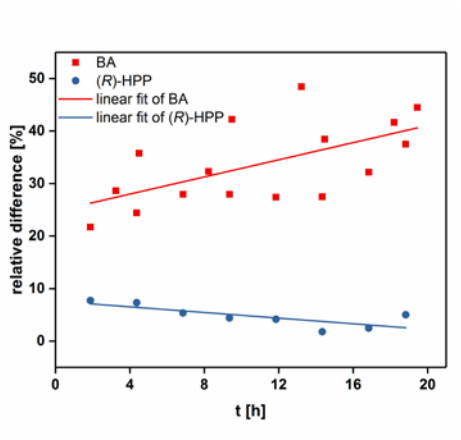


Figure S3: Absorption of benzaldehyde (BA) and (*R*)-2-hydroxy-1-phenylpropanone (HPP) by the PEEK (polyether ether ketone) material of the enzyme membrane reactor. Conditions: 30 mM benzaldehyde or 30 mM (*R*)-HPP in TEA-buffer (50 mM, pH 7.5, 2.5 mM $MgSO_4$, 0.5 mM ThDP), in the presence and absence of 30 vol% DMSO, 300 rpm, T = 30 °C , $V_{reactor}$ = 3 ml, residence time: 30 min, flow: 0.1 ml min^{-1}, membrane: regenerated cellulose (YM10 Milipore, 10 kDa cut-off). The number of repetitions is given next to the box. For experimental details see sect. 4.8 in the main paper.

A



B

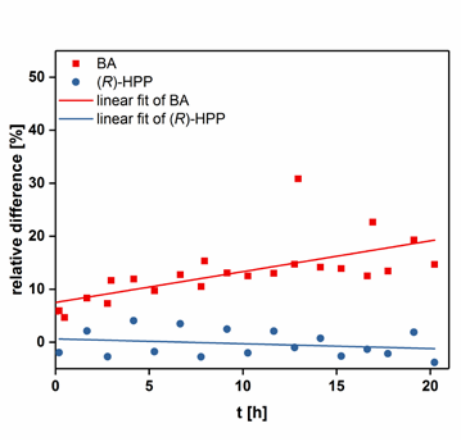


Figure S4: Evaporation of benzaldehyde (BA) and (*R*)-2-hydroxy-1-phenylpropanone (HPP) dissolved in TEA-buffer (A) without DMSO and (B) with 30 vol% DMSO after being pumped through the enzyme membrane reactor and stored in open glass test tubes over a time period. Conditions: 30 mM benzaldehyde or 30 mM HPP in TEA-buffer (50 mM, pH 7.5, 2.5 mM $MgSO_4$, 0.5 mM ThDP), in the presence and absence of 30 vol% DMSO, 300 rpm, T = 30°C , $V_{reactor}$ = 3 ml, residence time: 30 min, flow: 0.1 ml min^{-1}, PEEK (polyether ether ketone) - enzyme membrane reactor (EMR) with regenerated cellulose membrane (YM10 Milipore, 10 kDa cut-off); n = 1-3. For experimental details see sect. 4.8 in the main paper.

3.3.3 EMR experiment in the buffer-DMSO system at pH 9

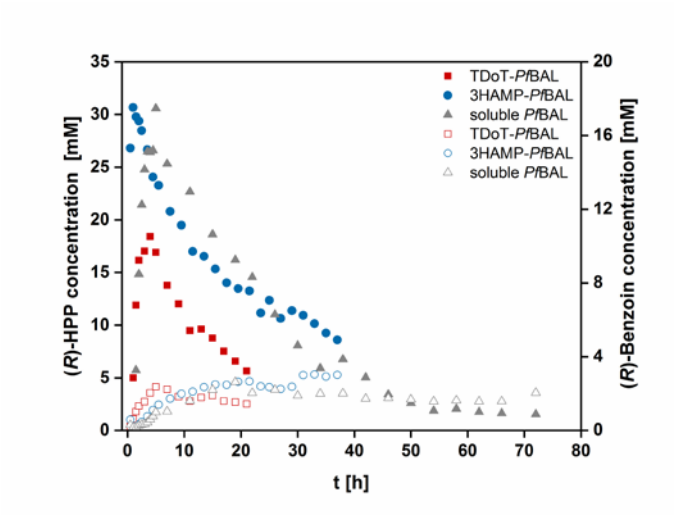


Figure S5: Carboligation of benzaldehyde and acetaldehyde to (*R*)-2-hydroxy-1-phenylpropanone (HPP) in an EMR catalyzed by *Pf*BAL, TDoT-*Pf*BAL, and 3HAMP-*Pf*BAL-CatIBs, respectively. Filled symbols refer to (*R*)-HPP and empty symbols to (*R*)-benzoin concentration. Half-life was deduced from the point in time where 50 % conversion to (*R*)-HPP (approx. 15 mM) was reached. Reaction conditions: 30 mM benzaldehyde, 90 mM acetaldehyde, TEA-buffer (50 mM, pH 9, 2.5 mM $MgSO_4$, 0.5 mM ThDP), 30 vol% DMSO, 28 U ml^{-1} protein concentrations of the enzymes: TDoT-*Pf*BAL (56.8 mg ml^{-1}), 3HAMP-*Pf*BAL (5.9 mg ml^{-1}), *Pf*BAL (0.94 mg ml^{-1}), 300 rpm, T = 30 °C , $V_{reactor}$ = 3 ml, residence time: 30 min, flow: 0.1 ml min^{-1}, PEEK (polyether ether ketone) - enzyme membrane reactor (EMR) with regenerated cellulose membrane (YM10 Milipore, 10 kDa cut-off); n = 1. For experimental details see sect. 4.8 in the main paper. The halve-lives decreased in the order *Pf*BAL (16 h) >3HAMP-*Pf*BAL (13 h) > TDoT-*Pf*BAL (3 h) and were taken from at 50% conversion from benzaldehyde to (*R*)-HPP.

3.4 Results in the biphasic reaction system

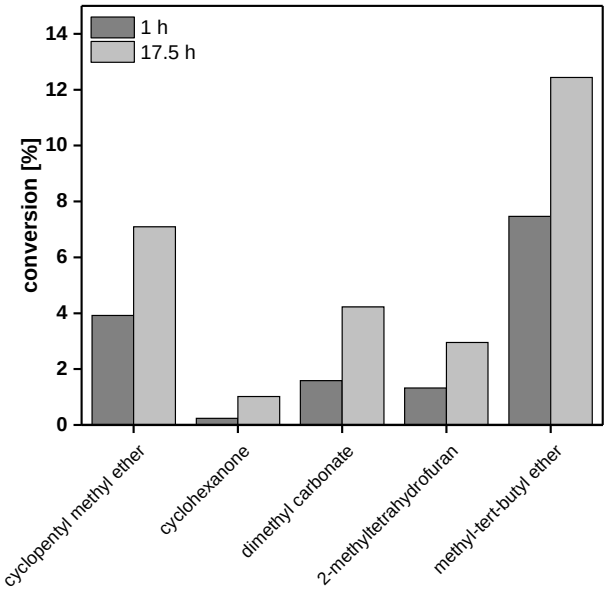


Figure S6: Solvent screening in the micro-aqueous reaction system for the carboligation of DMBA to TMBZ using TDot-*Pf*BAL-CatIBs. Reactions of DMBA to TMBZ were performed in the respective organic solvents (cyclopentyl methyl ether (CPME), cyclohexanone, dimethyl carbonate, 2-methyltetrahydrofuran, methyl *tert*-butyl ether (MTBE)) with 5 vol% TEA-buffer (1 M, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP), 100 mM DMBA, 0.6 U ml^{-1} TDoT-*Pf*BAL (2.9 mg ml^{-1} protein concentration) in 2 ml glass vials at T = 30°C, 1400 rpm, V = 1 ml, in a thermomixer, n = 1. For experimental details see sect. 2.12, n = 1.

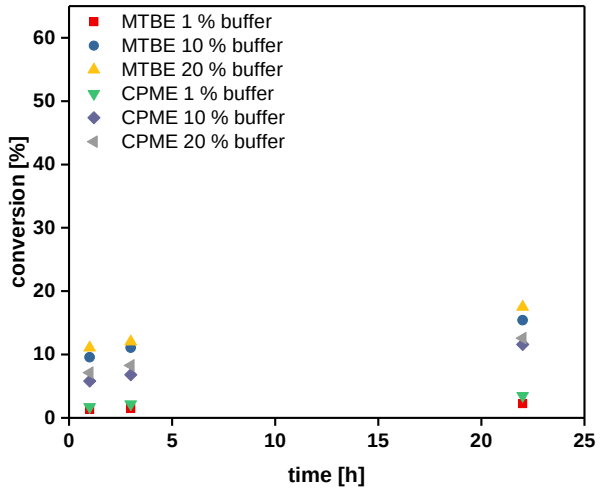


Figure S7: Optimization of the buffer content in MTBE/CPME in the aqueous-organic two-phase system for the carboligation of DMBA to TMBZ using TDoT-*Pf*BAL-CatIBs. Reactions were performed in CPME or MTBE with 1-20 vol% TEA-buffer (1 M, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP), 100 mM DMBA, 0.6 U ml^{-1} TDoT-*Pf*BAL (2.9 mg ml^{-1} protein concentration) in 2 ml glass vials at T = 30°C, 1400 rpm, V = 1 ml, in a thermomixer, n = 1. For experimental details see sect. 2.13. A higher buffer content of 20 vol% is necessary to obtain a higher conversion. Reaction in MTBE with 20 vol% buffer revealed the best results.

A B

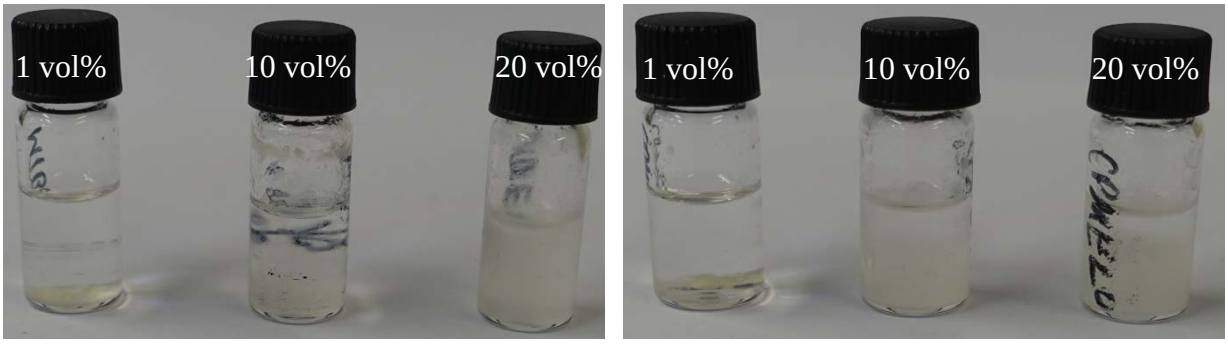


Figure S8: Emulsion formation of TDoT-*Pf*BAL-CatIBs in MTBE (A) or CPME (B) with 1 vol% 10 vol%, or 20 vol% buffer (from left to right) in the aqueous-organic two-phase system after carboligation of DMBA to TMBZ. Reactions were performed in CPME or MTBE with 1-20 vol% TEA-buffer (1 M, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP), 100 mM DMBA, 0.6 U ml^{-1} TDoT-*Pf*BAL (2.9 mg ml^{-1} protein concentration) in 2 ml glass vials at T = 30°C, 1400 rpm, V = 1 ml, in a thermomixer, n = 1. For experimental details see sect. 2.13.

A B

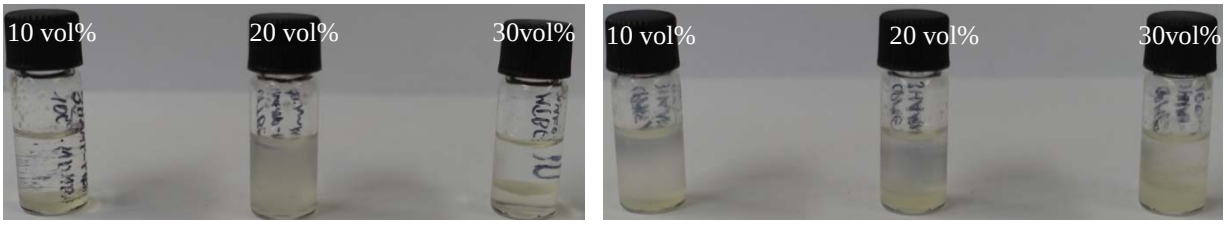


Figure S9: 3HAMP-*Pf*BAL-CatIBs show emulsion formation in (A) MTBE and (B) CPME with 10 vol% 20 vol%, or 30 vol% buffer (from left to right). Carboligation reactions of DMBA to TMBZ were performed in MTBE with 10-30 vol% TEA-buffer (1 M, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP), 50 mM DMBA, 6 U ml^{-1} 3HAMP-*Pf*BAL (1.1 mg ml^{-1} protein concentration) in 2 ml glass vials at T = 30°C, 1400 rpm, V = 1 ml, in a thermomixer. Image was recorded 24 h after the last measuring point. In between they were stored at room temperature without shaking. At 30 vol% buffer, an emulsion was formed after 0.5 hours of reaction. Since the photo was taken 24 hours later, the 3HAMP-*Pf*BAL accumulated at the interface of the phases. n=1. For experimental details see sect. 2.13.

Table S6: Equilibrium constant K_{eq} for the carboligation of DMBA to TMBZ by 3HAMP-*Pf*BAL-CatIBs in a biphasic system with different DMBA concentrations. K_{eq} was calculated based on data presented in the given figures.

Figure no.	DMBA		TMBZ		
	start concentration [mM]	end concentration [mM]	start concentration [mM]	end concentration at [mM]	K_{eq} [mM^{-1}]
4A, main paper	50	25.0	0	12.5	0.02
4B, main paper	70	34.2	0	17.9	0.02
4C, main paper	85	46.8	0	19.1	0.01
S10, ESI	0	26.0	32	19.0	0.02

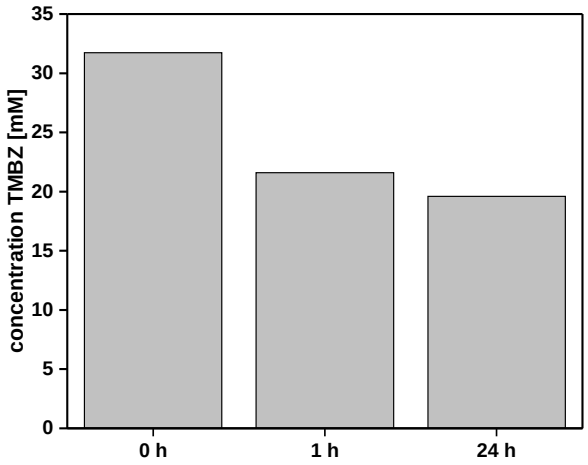


Figure S10: Analysis of the reaction equilibrium of TMBZ synthesis by 3HAMP-*Pf*BAL-CatIBs in the aqueous-organic two-phase system. The reaction equilibrium was determined by the conversion of 32 mM TMBZ to DMBA by 6 U ml^{-1} 3HAMP-*Pf*BAL (1.1 mg ml^{-1} protein concentration) in CPME with 30 vol% TEA-buffer (50 mM, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP), in 2 ml glass vial at T = 30°C, 1400 rpm, V = 1 ml, in a thermomixer, n = 1. For experimental details see sect. 2.14. It could be demonstrated that the reaction equilibrium is at about 50% conversion with a K_{eq} = 0.01 mM (compare Table S7).

Table S7: Initial rate actvitiy of the carboligation of benzaldehyde or DMBA to the respective benzoin catalyzed by 3HAMP-*Pf*BAL or soluble *Pf*BAL in biphasic reaction system. Conditions: 6 U ml^{-1} 3HAMP-*Pf*BAL (0.84 mg ml^{-1} protein concentration), 2.6 U ml^{-1} *Pf*BAL (0.34 mg ml^{-1} protein concentration), 70 mM benzaldehyde or 70 mM DMBA in 1 ml reaction volume composed of 30 vol% TEA-buffer (50 mM, 2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 7.5); 70 vol% CPME, 1400 rpm, 30 °C, n = 3. Calculations are based on Figure 5 in the main paper. SD: standard deviation.

	3HAMP-*Pf*BAL		soluble *Pf*BAL	
	DMBA	benzaldehyde	DMBA	benzaldehyde
k_{cat} [s^{-1}]	4.1	2.6	1.4	2.0
SD	0.1	0.2	0.3	0.3

3.5 ^{1}H-NMR spectrum

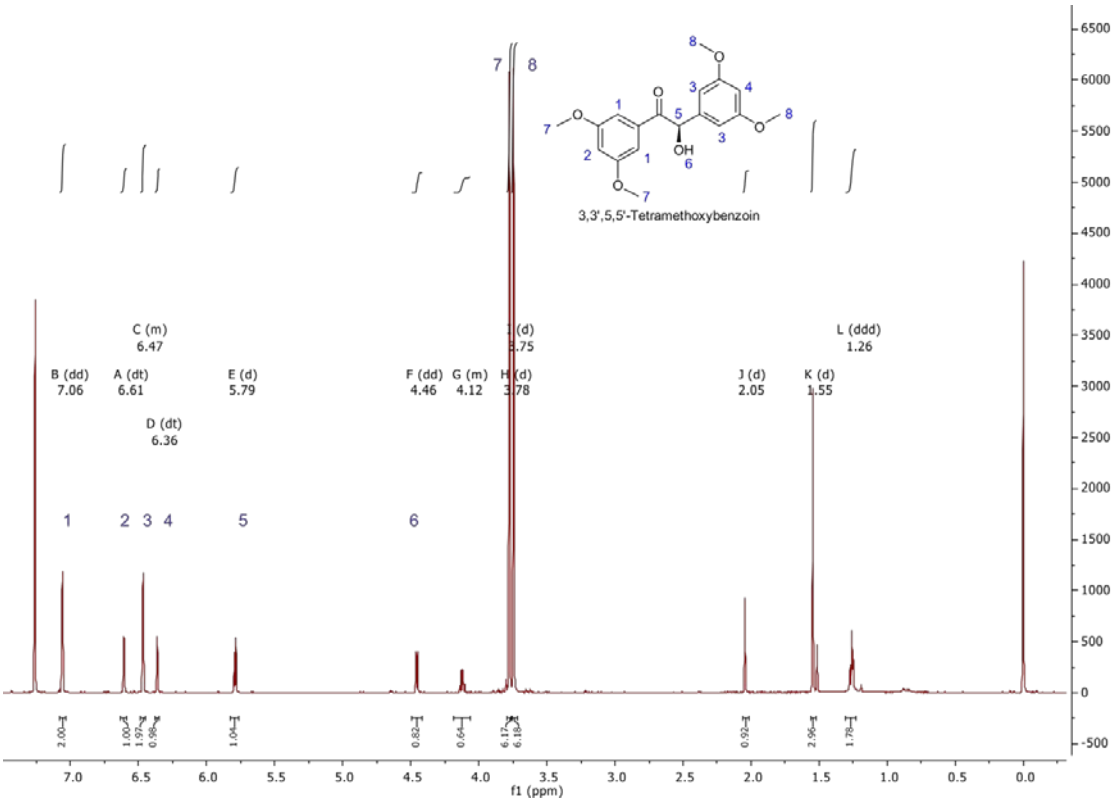


Figure S11: ^{1}H-NMR spectrum (600 MHz, $CDCl_3$) of (*R*)-3,3´,5,5´-tetramethoxy benzoin (TMBZ), which also includes 18.0-19.2 vol% ethyl acetate.

3.6 HPLC calibration curves and analysis

A **B**

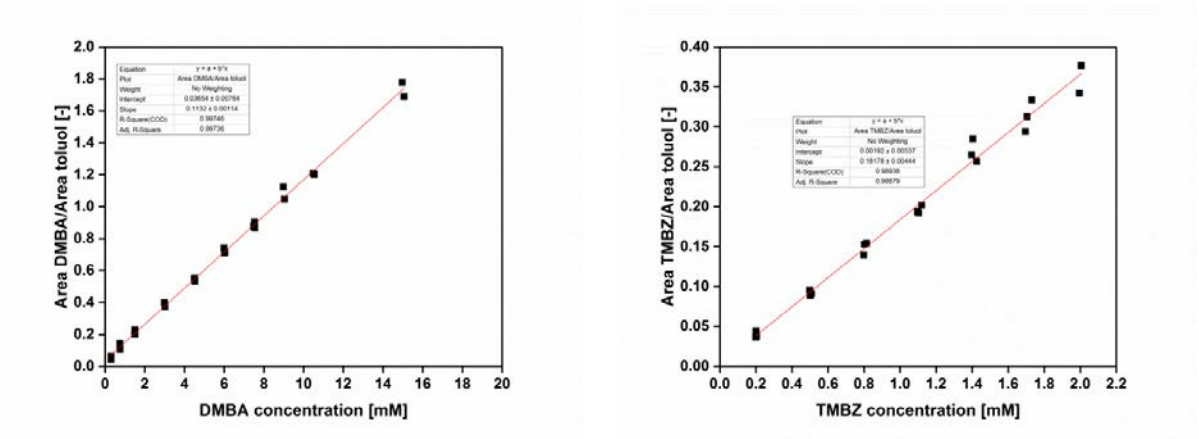


Figure S12: Calibration curve for (A) 3,5-dimethoxybenzaldehyde (DMBA) or (B) (*R*)-3,3‘,5,5‘-tetramethoxy benzoin (TMBZ) for initial rate determination. Conditions: TMBZ (4 mg) and DMBA (25 mg) were dissolved in 2 ml DMSO, respectively. Samples were diluted with TEA-buffer (50 mM, pH 7.5, 2.5 mM $MgSO_4$, 0.1 mM ThDP) and DMSO to a final percentage of 80 vol% TEA-buffer and 20 vol% DMSO. DMBA (25 mg) was dissolved in 1 ml DMSO and samples were diluted with TEA-buffer and DMSO to a final percentage of 80 vol% TEA-buffer and 20 vol% DMSO. The so prepared samples were diluted 1:10 with 180 µl methanol (incl. 4.7 mM toluene as internal standard) according to the protocol in sect. 4.7 in the main paper. HPLC analysis was performed with acetonitrile/water as the mobile phase (see sect. 4.13 in the main paper).

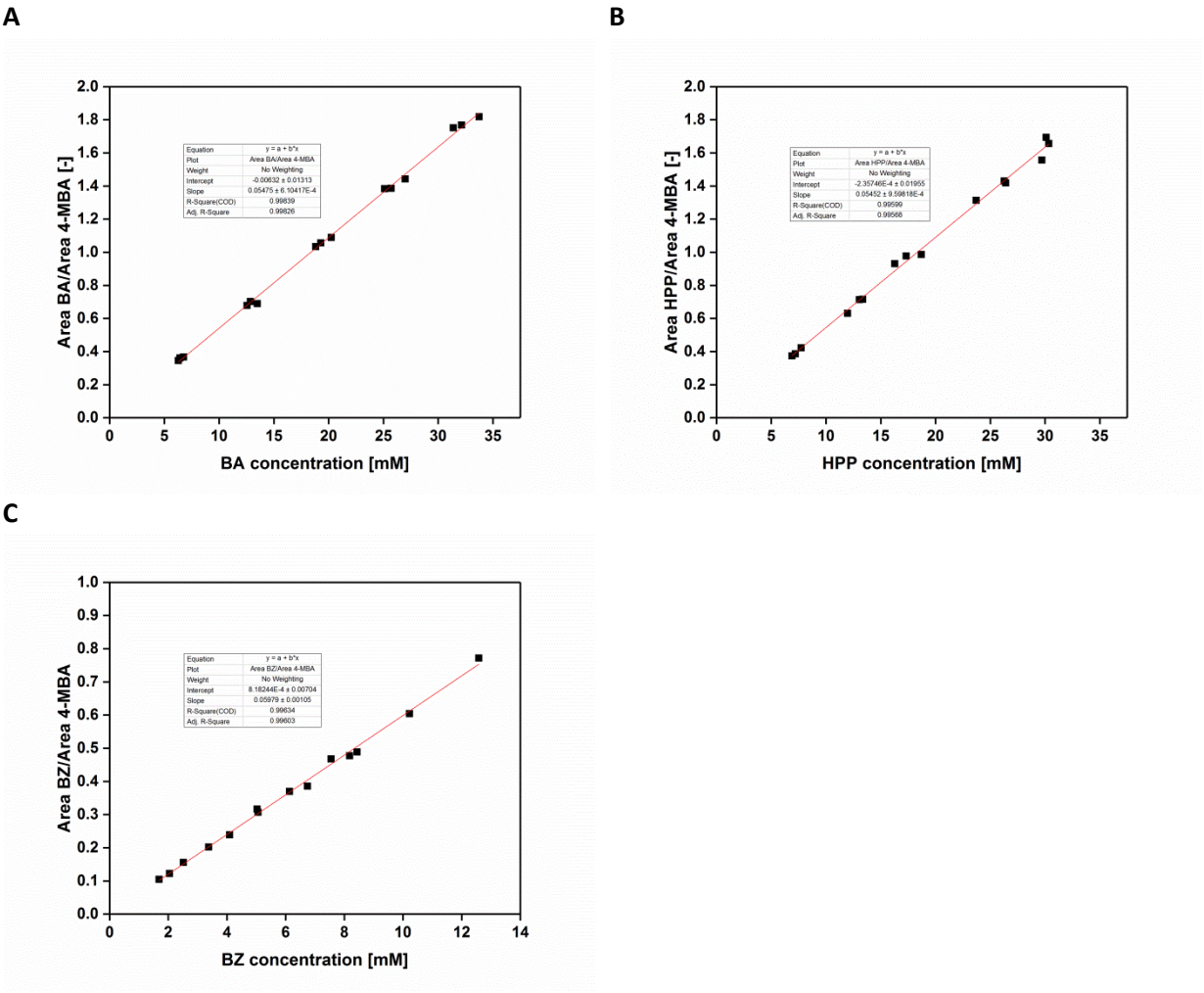


Figure S13: Calibration curve for (A) benzaldehyde (BA), (B) (*R*)-2-hydroxy-1-phenylpropanone (HPP), and (C) (*R*)-benzoin (BZ) to determine conversion in the EMR. Conditions: BA (17-18 mg) was dissolved in 5 ml TEA-buffer (50 mM, pH 7.5, 2.5 mM $MgSO_4$, 0.1 mM ThDP) and diluted with TEA-buffer by a factor of 1,25, 1.66, 2.5 and 5. HPP (2-9 mg) and BZ (0.7-5.3 mg) were dissolved in 2 ml TEA-buffer, respectively. All samples were diluted 1:20 according to the protocol in sect. 4.8 in the main paper. HPLC analysis was performed with acetonitrile/water as the mobile phase (see sect. 4.13 in the main paper).

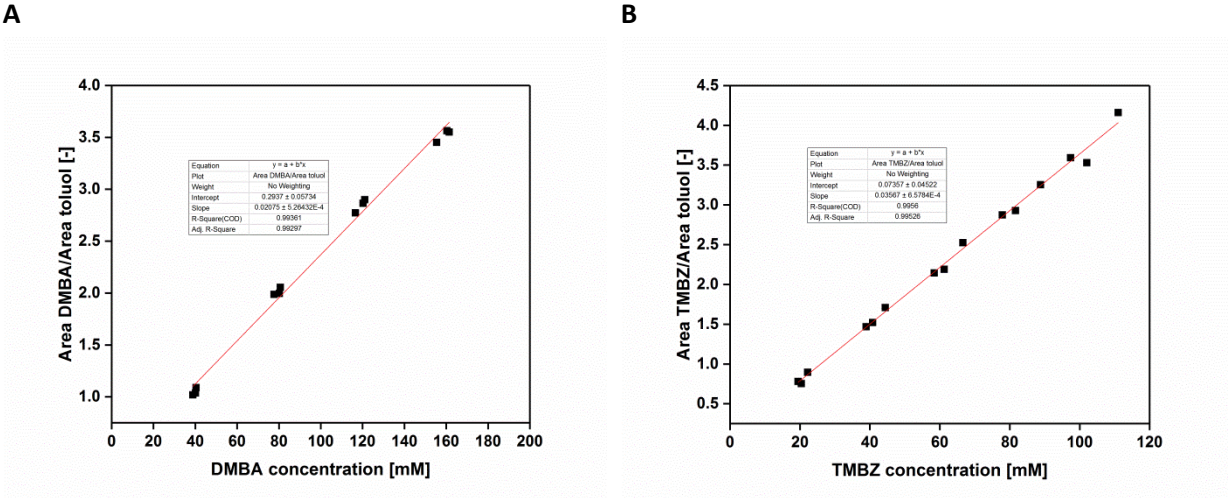


Figure S14: Calibration curve for (A) 3,5-dimethoxybenzaldehyde (DMBA) or B) (*R*)-(3,3‘,5,5‘)-tetramethoxy benzoin (TMBZ) to determine conversion in the biphasic batch system. Conditions: DMBA (33 mg) and TMBZ (38-44 mg) were dissolved in 1 ml 2-methyltetrahydrofuran and diluted with 2-methyltetrahydrofuran by the

factor of 1,25, 1.66, 2.5 and 5, respectively. All samples were diluted 1:10 in 2-methyltetrahydrofuran, and subsequently 1:10 diluted in 180 µl n-heptane (incl. 4.3 mM acetophenone as internal standard) according to the protocol in sect. 4.9 in the main paper., HPLC analysis was performed with n-heptane/isopropanol as the mobile phase (see sect. 4.13 in the main paper).

Table S8: HPLC conditions to determine substrate and (side-)product concentrations: 3,5-dimethoxy-benzaldehyde (DMBA), (*R*)-(3,3‘,5,5‘)-tetramethoxy benzoin (TMBZ), (*R*)-2-hydroxy-1-phenylpropanone (HPP), benzaldehyde (BA) and (*R*)-benzoin (BZ) and the initial standards: toluene, 4-methoxybenzaldehyde (4-MBA), acetophenone. dd: double desalted; ACN: acetonitrile. For the gradient applied in the continuous reaction experiment see Table S10. For HPLC analysis see sect. 4.13 in the main paper)

		initial rate activity determination	**continuous reaction in an EMR**	**reaction in biphasic system**
mobile phase	A	50 vol% (dd) H_2O	gradient of (dd) H_2O	70 vol% *n*-heptane
	B	50 vol% ACN	gradient of ACN	30 vol% 2-propanol
	flow (ml min^{-1})	1.0	0.9	1.5
retention times (min)	DMBA (215 nm)	7.6		4.2
	TMBZ (215 nm)	9.4		11.8
	HPP (245 nm)		7.9	
	BA (245 nm)		9.4	
	BZ (245 nm)		13.8	
	toluene (215 nm)	6.9		
	4-MBA (270 nm)	6.1	11.9	
	acetophenone (215 nm)			3.5

Table S9: Gradient of the mobile phase for HPLC determination and separation of substrates/products during a continuous reaction in an EMR. A is double desalted (dd) H_2O and B acetonitrile (ACN). For HPLC analysis, see sect. 4.13 in the main paper).

time (min)	**A (%)**	**B (%)**
0-7	65	35
7-8	65-40	35-60
8-14	40	60
14-15	40-65	60-35
15-20	65	35

4 References

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