Electronic Supplementary Information - ESI

Chemo-enzymatic enantioselective Baeyer-Villiger oxidations: an opportunity offered by the oxygenase part of a two-component Baeyer-Villiger monooxygenase

R. Röllig [a,b], C. E. Paul [c], M. Claeys-Bruno [d], K. Duquesne [a], S. Kara [b] and V. Alphand [a,*]

Table of contents

Electronic Supplementary Information A – Material and methods

All chemicals were utilized as supplied without further purifications.

Cloning, expression, and purification of the synthetic 2,5-diketocamphane-monooxygenase

Design of the synthetic 2,5-diketocamphane-monooxygenase I (*Pseudomonas putida* ATCC 17453)

The gene of the 2,5-diketocamphane 1,2-monooxygenase I (2,5-DKCMO I) from *Pseudomonas putida* (*P. putida*) ATCC 17453 (NCIMB 10007) (*camE*$_{25\text{-}1}$, [*orf*4] on the CAM plasmid, *camP*, UniProtKB - Q6STM1 (25DK1_PSEPU)) was codon-optimized for *Escherichia coli* (*E. coli*) using the online tool GeneArt Strings DNA Fragments (Thermo Fisher Scientific) considering in the position 4-9 a *Nde*I [CATATG] and 1099-1104 a *Bam*HI [GGATCC] protecting side. *Xho*I [CTCGAG] restriction side motif was avoided in the construct. The synthetic gene had a size of 1107 bp (native *P. putida* 2,5-DKCMO: 1092 bp) and was dissolved in water at a concentration of 50 ng μL^{-1} and stored at -80 °C.

Cloning and transformation of the pET15b-*camE*$_{25\text{-}1}$ and the pET15b-*fre* construct

Ligation of the synthetic *camE*$_{25\text{-}1}$ gene into the multiple cloning site of the pET15b plasmid generating the pET15b-*camE*$_{25\text{-}1}$ construct. 300 ng of the synthetic gene was digested with *Nde*I for 1.5 hours at 37 °C and with *Bam*HI for two more hours at the same temperature. The same restriction protocol was applied for pET15b. The digested fragments were purified using commercial purification kits (Wizard SV Gel and PCR Cleanup) and ligated in 3:1, 10:1, and 20:1 ratios using T4 ligase for 14 hours at 16 °C and one hour at 37 °C. All ligations were kept at 65 °C for 15 minutes to inactivate the ligase. *Xho*I was added for two hours at 37 °C to digest self-ligated pET15b plasmids. The pET15b-*camE*$_{25\text{-}1}$ construct is shown in Fig. S2 (ESI B – Results).

Fre reductase from *E. coli* was selected[1] as the reductase counterpart of the 2,5-DKCMO. Whole DNA was isolated from *E. coli* BL21(DE3) by heating the cells for 60 minutes at 96 °C after cultivating the strain under standard conditions. The *fre* reductase gene was directly amplified from the genomic DNA *via* standard Phusion-PCR (1% v/v DMSO) applying the following primer with restriction sites for *Nde*I and *Bam*HI, respectively: AAACATATGACAACCTTAAGCTGTAAAG / AAAGGATCCTCAGATAAATGCAAACGC. The following PCR protocol was applied: 95 °C – 5 minutes / 30 cycles: 95 °C – 60 seconds, 51 °C – 30 seconds, 72 °C – 60 seconds / 72 °C – 5 minutes. The PCR product was purified using commercially available kits. After amplification and purification of the PCR product, the same digestion/ligation procedure as described for pET15b-*camE*$_{25\text{-}1}$ was applied for pET15b-*fre*.

pET15b-*camE*$_{25\text{-}1}$ and the pET15b-*fre* constructs were transformed independently into *E. coli* DH5α. Positive clones were selected from LB-Agar plates through pET15b mediated ampicillin resistance. For pET15b-*camE*$_{25\text{-}1}$ eight colonies, for pET15b-*fre* six colonies were chosen randomly for colony PCR using commercial PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Science) and T7 primers (TAATACGACTCACTATAGGG / pET-PR, TAGTTATTGCTCAGCGGTGG). The following PCR protocol was applied: 95 °C – 5 minutes / 30 cycles: 95 °C – 40 seconds, 55 °C – 30 seconds, 72 °C – 60 seconds / 72 °C – 5 minutes. The amplified colony PCR products were verified on a 1% agarose gel (100 V, 40 min), The results are shown in Fig. S3 (ESI B – Results). For pET15b-*camE*$_{25\text{-}1}$ three positive clones, for pET15b-*fre* two clones were chosen for plasmid isolation and sequencing (GENEWIZ). For the sequencing T7 forward and reverse primers were applied.

Expression, cell disruption, and purification

The correct construct was transformed into *E. coli* BL21(DE3) for heterologous expression. The corresponding protein (encoded by *camE*$_{25\text{-}1}$) was designed to have the amino acid sequence of the 2,5-DKCMO with an *N*-terminal poly-histidine-tag (6x His) attached by a ten amino acid linker. The expression was induced *via* 0.5 mM IPTG at an OD_{600} of 0.5 – 0.8 in liquid LB with the corresponding antibiotics for selection. After induction, the culture was cultivated at 25 °C for 14 hours and 180 rpm. In all cultivation steps, an ampicillin concentration of 100 mg L^{-1} was applied.

The cells were harvested by centrifugation at 4 °C for 45 minutes at 5000 rpm. The pellet was washed twice with 20 mL potassium phosphate buffer (20 mM, pH 7.5) and stored at -20 °C. For cell disruption, cells were

resuspended in 10 mL NPI-10 buffer (50 mM potassium phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 8.0) and sonicated on ice pulse-wise for 7 minutes (30% amplitude, 4 seconds sonication, 6 seconds break). No lysozyme was added. The disrupted cell fragments were removed from the soluble protein fraction by centrifugation for 15 minutes at 4 °C and 15000 rpm.

For purification from the soluble fraction, a Protino® Ni-NTA agarose bead column was equilibrated with NPI-10 buffer (ten column volumes). The supernatant remained on the column for 1.5 hours at 7 °C to bind the poly-histidine-tagged 2,5-DKCMO or Fre, respectively, to the beads. Washing of the unbound components and the elution of the protein of interest was executed at 7 °C according to the manufacture's protocol (polyhistidine-tagged proteins under native conditions). Dialysis with a membrane cut-off of 10-12 kDa was performed in 10 L Tris-HCl buffer (20 mM, pH 7.5) for 14 hours at 7 °C. The procedure was repeated once. The enzyme solution was aliquoted and used for bioconversions or frozen in liquid nitrogen and stored at -80 °C, or lyophilized, respectively.

All cultures and purifications were examined in terms of qualitative and semi-quantitative protein expression using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% separation gel, 100 V, 80 minutes). Typically, 8 µL of soluble protein (containing sample and loading dye) and 4 µL Euromedex protein ladder 10-250 kDa were loaded into the gel and separated at 100 V and for 80 minutes. Additional quantitative protein analysis was done at 280 nm using a Thermo Scientific Nanodrop ™ 2000.

Bioconversion

Bioconversions were performed in closed 2 mL glass vials with a total volume of 1 mL, or 0.5 mL respectively. Alice Guarneri (Wageningen University, The Netherlands) kindly provided the nicotinamide coenzyme biomimetics (NCBs) after the synthesis as described in literature[2,3] adapted from Mauzerall and Westheimer.[4] All substrates were supplied from stock solutions in ethanol. The reactions took place in an Eppendorf® New Brunswick™ Innova® 42 Incubator Shaker under controlled conditions at 160 rpm and various temperatures.

Samples were taken from the reaction medium and directly extracted in ethyl acetate as described in the manuscript. All experiments were performed in independent duplicates, if not stated differently.

Gas chromatography

Gas chromatography (GC) analyses were carried out on a Shimadzu GC-14A with hydrogen as the gas phase in 50/50 split mode and a flame ionization detector (FID). Separation was achieved on a Cyclosil-B column (Agilent J&W GC Columns). Separation of bicyclo[3.2.0]hept-2-en-6-one and its corresponding lactones was performed at 125 °C with the following retention times: (-)-(1*S*,5*R*)-bicyclo[3.2.0]hept-2-en-6-one: 2.3 minutes / (+)-(1*R*,5*S*)-bicyclo[3.2.0]hept-2-en-6-one: 2.5 minutes / tridecane: 5.3 minutes / (-)-(1*R*,5*S*)-3-oxa-bicyclo[3.3.0]oct-6-en-2-one: 10.2 minutes / (+)-(1*R*,5*S*)-2-oxabicyclo[3.3.0]oct-6-en-3-one: 11.2 minutes / (-)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one: 12.1 minutes / (+)-(1*S*,5*R*)-3-oxa-bicyclo[3.3.0]oct-6-en-2-one: 12.5 minutes. Separation of the *rac*-camphor (1,7,7-trimethyl-bicyclo[2.2.1]heptan-2-on) was performed on the same Cyclosil-B column at 90 °C obtaining the following retention times: (1*S*,4*S*)-(-)-camphor: 16.9 minutes / (1*R*,4*R*)-(+)-camphor: 17.4 minutes / tridecane: 23.8 minutes. The corresponding lactone(s) were separated at 160 °C with the following retention times: *rac*-camphor: 2.8 minutes / tridecane: 3.3 minutes / corresponding lactone(s) from (1*R*,4*R*)-(+)-camphor Baeyer-Villiger (BV) oxidation: 11.4 minutes. *rac*-norcamphor (*rac*-bicyclo[2.2.1]heptan-2-one) separation was carried out at 100 °C under the same conditions. The retention times of the two ketones were 7.0 minutes, and 7.4 minutes respectively and for tridecane 14.8 minutes.

Design of Experiments

Design of Experiments (DoE) was executed using AZURAD software (www.azurad.fr) applying a polynomial model with linear effects for the DoE on the screening of the effect of various parameter or linear, quadratic, and interaction effects for the second DoE on the optimization. Coefficient's estimations were calculated by multilinear regression. Table S1 shows the matrix of the DoE to screen the effect of various parameters, as results are displayed in Fig. 4 in the manuscript.

Table S1 DoE matrix of the screening of the effect of various parameters.[a]

experiment	X1	X2	X3	X4	X5	X6	X7
1	0	0	0	0	0	0	0
2	1	1	0	1	1	0	1
3	2	2	0	2	0	1	1
4	3	3	0	0	1	1	0
5	0	1	1	2	1	1	0
6	1	0	1	0	0	1	1
7	2	3	1	0	1	0	1
8	3	2	1	1	0	0	0
9	0	2	2	0	1	0	1
10	1	3	2	2	0	0	0
11	2	0	2	1	1	1	0
12	3	1	2	0	0	1	1
13	0	3	0	1	0	1	1
14	1	2	0	0	1	1	0
15	2	1	0	0	0	0	0
16	3	0	0	2	1	0	1

[a] The tested factors (X1 – X7) were investigated for two, three, or four levels (0 to 3). Herein, the factors and corresponding level (listed in increasing numbers) are the following: X1… FMN concentration / 25, 50, 100, 150 µM, X2… 2,5-DKCMO concentration / 50, 100, 150, 200 µg mL^{-1}, X3… temperature / 15, 20, 30 °C, X4… NCB concentration / 10, 25, 50 mM, X5… NCB type / AmNAH, BNAH, X6… pH / 7.5, 8.5, X7… buffer / Tris-HCl, Tricine. Experiment 7 was performed in triplicates to validate the results within the variation range of the factors.

The experimental matrix of the DoE for FMN concentration and temperature optimization during the enzymatic BV oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one, *rac*-camphor, and *rac*-norcamphor by 2,5-DKCMO is shown in Table S2. The results are displayed in the manuscript in Fig. 5.

Table S2 DoE matrix of the optimization of FMN concentration and temperature for the BV oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one, *rac*-camphor, and *rac*-norcamphor by 2,5-DKCMO.[a]

experiment	temperature [°C]	FMN [µM]	substrate [5 mM]
1	12	10	S2
2	12	10	S3
3	30	10	S1
4	30	10	S2
5	12	25	S1
6	12	25	S3
7	30	25	S3
8	12	25	S2
9	12	17.5	S2
10	30	17.5	S2
11	21	10	S2
12	30	10	S3
13	30	25	S1
14	21	25	S2
15	30	25	S2
16	21	17.5	S1
17	12	10	S1
18	21	17.5	S3
19	17	16	S1
20	25	16	S2
21	21	21	S3

[a] The substrates were applied in a concentration of 5 mM as shown in the table coded by S1 for *rac*-bicyclo[3.2.0]hept-2-en-6-one, S2 for *rac*-camphor, and S3 for *rac*-norcamphor. Experiment 16 was performed in triplicates. Validation of the results was executed by experiments 19, 20, 21.

Electronic Supplementary Information B – Results

Cloning and expression of the synthetic *camE$_{25\text{-}1}$* gene, as well as translation and purification of the synthetic 2,5-DKCMO I

The successful restriction and ligation of the *E. coli*-harmonized *camE$_{25\text{-}1}$* with pET15b created the corresponding pET15b-*camE$_{25\text{-}1}$* construct and is shown in Fig. S1.

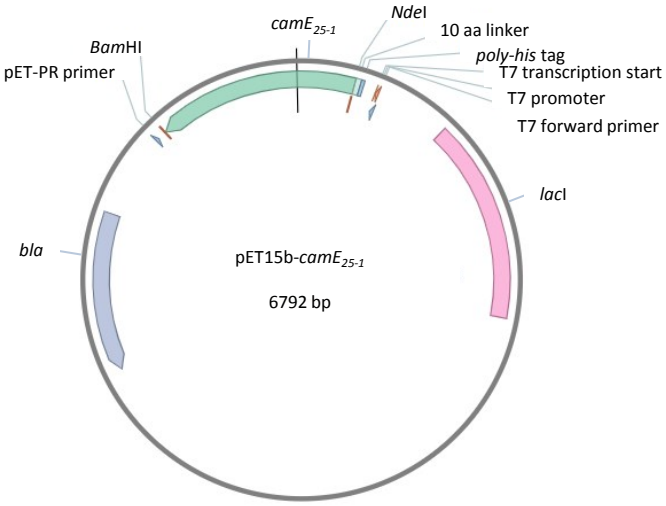


Fig. S1 Plasmid map of the *pET15b-camE$_{25\text{-}1}$* construct. The nucleotide sequence of the synthetic *pET15b*-camE$_{25\text{-}1}$ was integrated into the pET15b vector. The corresponding protein (2,5-DKCMO) has a *N*-terminal poly-histidine tag spaced by a 10 amino acid linker (10 aa linker). The location of the pET-PR primer and T7 forward primer to sequence the construct after transformation into *E. coli* DH5α and the restriction sides of *Bam*HI and *Nde*I are labelled on the plasmid map. *bla* encodes for the ampicillin resistance. *lac*I is essential for the IPTG induction of the transcription of *camE$_{25\text{-}1}$*.

The transformation of pET15b-*camE$_{25\text{-}1}$*, or pET15b-*fre* in *E. coli* DH5α and the subsequent selection on LB-agar plates for plasmid-born ampicillin resistance resulted in a substantial number of colonies. Eight, or six colonies, respectively, were taken randomly for colony PCRs. The PCR products were loaded on a 1% agarose gel and are shown in Fig. S2.

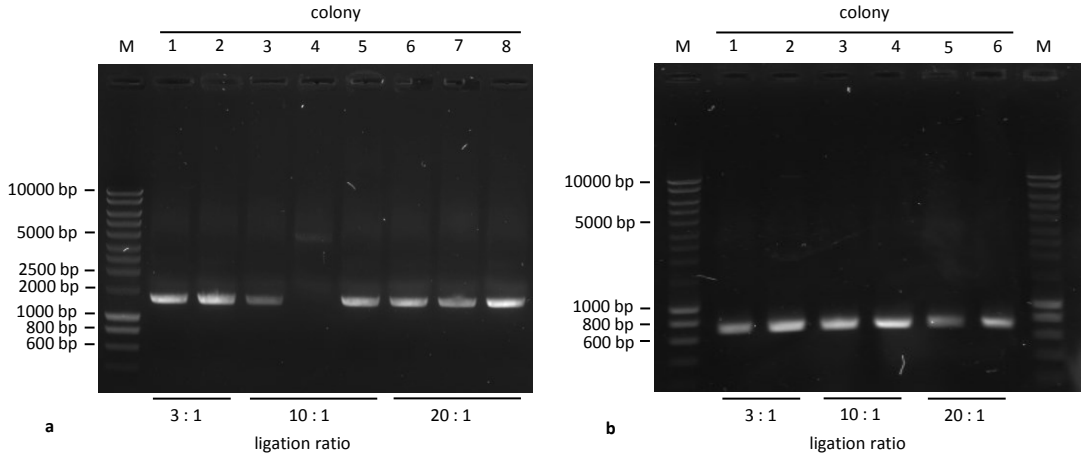


Fig. S2 Agarose gel after PCR from randomly picked *E. coli* DH5α colonies after the transformation of the pET15b-camE$_{25\text{-}1}$ in (a), and the pET15b-*fre* construct in (b). Commercial T7 forward and reverse (pET-PR) primers were applied for the amplification.

For pET15b-*camE$_{25\text{-}1}$* seven of the eight colonies tested showed the expected result after successful PCR. Fig. S2a shows the appearance of the DNA fragments at the expected size (approx. 1.3 kbp). The analogue results for the pET15b-*fre* construct are shown in Fig. 2b. Sequencing (genewiz) confirmed the correct nucleotide

sequence (data not shown). The transformation of the construct into *E. coli* BL21(DE3) gave excellent expression results. Both 2,5-DKCMO and Fre were part of the soluble protein fraction.

Fig. S3a shows the SDS-PAGE with cell-free extract of *E. coli* BL21(DE3) without the pET15b-*camE*$_{25\text{-}1}$ construct on the left (as a control) and *E. coli* BL21(DE3) with pET15b-*camE*$_{25\text{-}1}$ on the right. Both cultures were cultivated under the same conditions e.g. IPGT concentration as mentioned in section A. For the culture with the pET15b-*camE*$_{25\text{-}1}$ construct, we saw the intensity of the band at the expected 2,5-DCKMO size of approx. 41 kDa increasing with time, resulting in very high expression levels. A longer cultivation period than 22 hours did not result in higher protein expression. Therefore, overnight inductions (approx. 14 hours) was applied for further protein expressions. SDS-PAGE confirmed the successful purification of the oxygenase as shown in Fig. S3b.

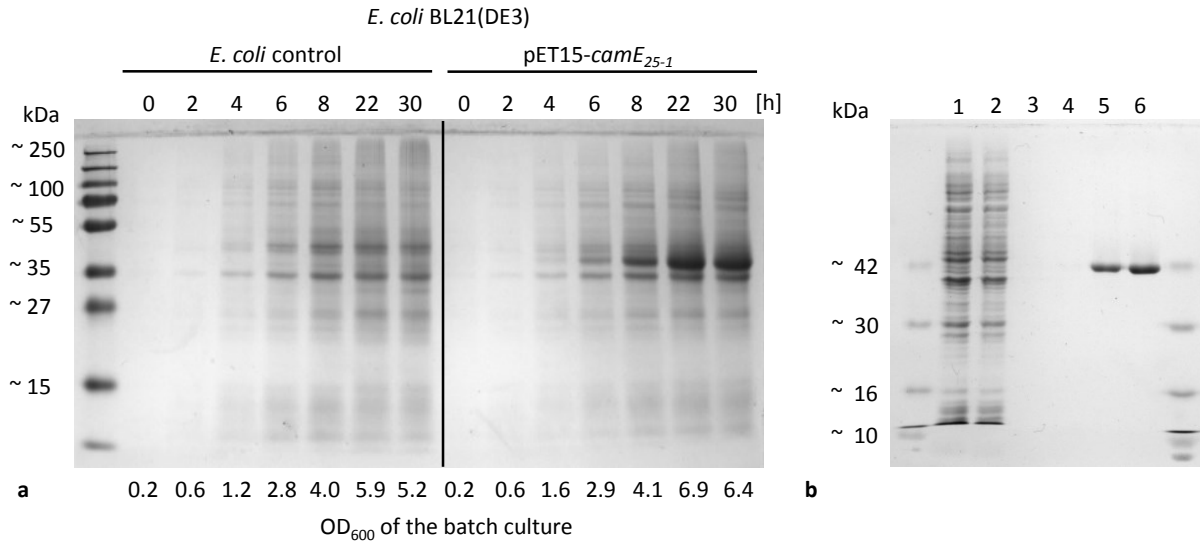
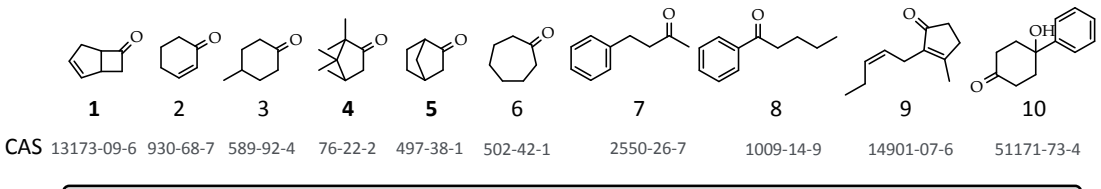


Fig. S3 SDS-PAGE of the IPTG induced expression of *camE*$_{25\text{-}1}$ in *E. coli* BL21(DE3) over 30 hours in (a). For the *pET15b*-camE$_{25\text{-}1}$ strain, this resulted in the translation of 2,5-DKCMO (right side). (b) shows the SDS-PAGE of the purification of 2,5-DKCMO *via* Ni-NTA agarose beads. (b) 1… cell-free extract of *E. coli* BL21(DE3) for 2,5-DKCMO translation 16 hours after induction (75 µg), 2… flow-through from the column (75 µg), 3… begin of the wash steps with NPI 20 (same volume as in 2)), 4… end of the wash steps with NPI 20 (same volume as in 2), 5… begin of the elution (discarded), 6… 2,5-DCKMO after purification and dialysis (0.75 µg).

Simultaneously, the pET15b-*camE*$_{25\text{-}1}$ co-transformation was performed applying pG-KJE8, pGro7, pKJE7, pG-Tf2, pTf16 plasmids (TaKaRa Chaperone Plasmid Set #3340), but none of the co-expressed systems could improve the translation of the protein of interest quantitatively (data not shown).

Chemo-enzymatic BV oxidations and gas chromatographic analysis

Purified 2,5-DCKMO was tested in a reductase-free reaction system with NADH as the hydride donor for the FMN reduction on 13 ketones for its ability to perform BV oxidation. The tested ketones are shown in Fig. S4. The typical reaction took place in 250 µL Tris-HCl buffer (50 mM, pH 7.5), 25 mM NADH, 20 µM FMN, and 5 mM substrate supplied from 100 mM stock in ethanol. Moreover, 2.5 mg mL^{-1} catalase and 0.78 mg mL^{-1} 2,5-DCKMO were applied. No reductase component was applied as the FMN reduction was carried out directly by NADH oxidation. Surprisingly the enzyme showed a very narrow substrate spectrum, as it only converts three of the tested 11 ketones. In this context, it is noteworthy to mention that, in the conditions we tested, purified 2,5-DKCMO was only able to convert (1*R*,4*R*)-(+)-camphor, but not the enantiomeric counterpart, which is consistent with previous studies.[1]



	1	2	3	4	5	6	7	8	9	10
CAS	13173-09-6	930-68-7	589-92-4	76-22-2	497-38-1	502-42-1	2550-26-7	1009-14-9	14901-07-6	51171-73-4
	+ + +	-	-	+ + *	+	-	-	-	-	-

1 **(±)-cis-bicyclo[3.2.0]hept-2-en-6-one**
2 cyclohex-2-en-1-one
3 4-methylcyclohexanone
4 ***rac*-camphor**
5 ***rac*-norcamphor**
6 cycloheptanone
7 4-phenyl-2-butanone
8 valerophenone
9 jasmone
10 4-hydroxy-4-phenylcyclohexan-1-one

-	no conversion
+	low conversion
++	moderate conversion
*	only (*1R,4R*)-camphor / (+)-camphor conversion
+++	high conversion

Fig. S4 Overview of the tested substrates used for 2,5-DKCMO catalyzed BV oxidation. The oxygenase was tested to catalyze the BV oxidation for 11 potential substrates, and for three an enzymatic ketone conversion was observed in different qualities (low, moderate, high conversion). The substrates in bold are further investigated in the study.

Based on these results *rac*-bicyclo[3.2.0]hept-2-en-6-one was selected as the model substrate. A typical gas chromatogram for the chemo-enzymatic enantioselective BV oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one by the 2,5-DKCMO is shown in Fig. S5.

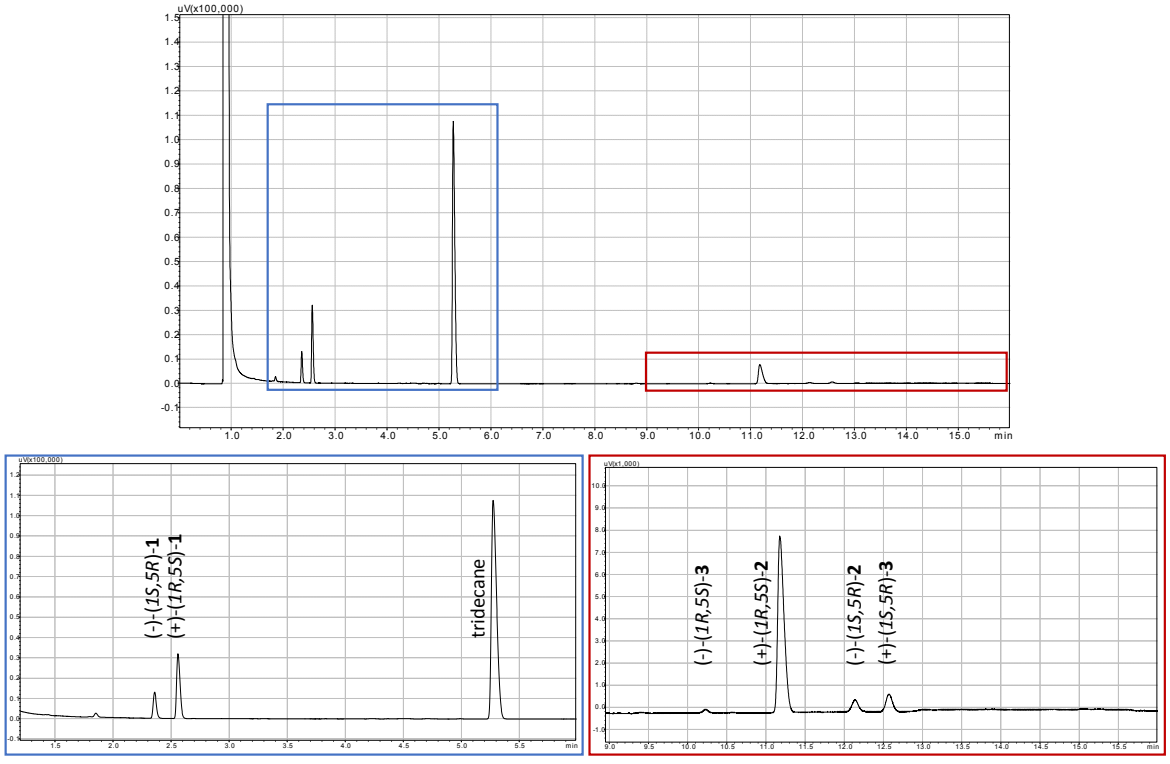


Fig. S5 Gas chromatogram of the chemo-enzymatic conversion of the model substrate *rac*-bicyclo[3.2.0]hept-2-en-6-one. 5 mM was converted by the 2,5-DCKMO and extraction from an aqueous phase using ethyl acetate and 0.5 g L^{-1} tridecane as an internal standard. Both substrates (*rac*-bicyclo[3.2.0]hept-2-en-6-one, *rac*-**1**), the standard, and their retention times at the GC are displayed in the blue box. In the red box the products (2-oxabicyclo[3.3.0]oct-6-en-3-one, **2**, and 3-oxa-bicyclo[3.3.0]oct-6-en-2-one, **3**) at their corresponding retention times are shown.

To facilitate the experimental procedure, the catalytic performance of the 2,5-DKCMO was characterized after storing the enzyme at –20 ° C for 21 days as well as after lyophilization and storage for the same period. Fig. S6 shows the conversion of 5 mM of the model substrate after 30 and 120 minutes, respectively. Herein, the enzyme remained its activity to a remarkable extent (approx. 80% of the lyophilized, and 70% of the frozen form, respectively, compared to the fresh enzyme after two hours), which justified its usage of the lyophilized form.

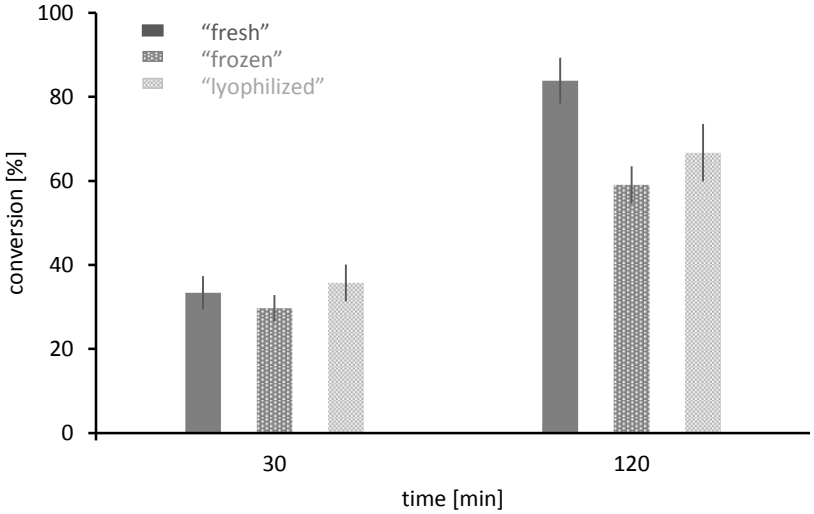


Fig. S6 Total conversion [%] from 3.2 mM *rac*-bicyclo[3.2.0]hept-2-en-6-one by the 2,5-DKCMO after purification or stored for 21 days in its frozen and lyophilized form. 0.39 mg mL^{-1} 2,5-DKCMO was applied in three formulations: fresh, frozen, and lyophilized in 50 mM Tris-HCl buffer, pH 7.5. FMN was added to a final concentration of 50 µM, likewise 25 mM AmNAH for hydride transfer to the flavin. 3.0 mg mL^{-1} catalase was added to the reactions to prevent hydrogen peroxide accumulation. The error bars indicate the standard deviation from triplicates after 30 and 120 minutes.

In the next set of experiments different FMN and NADH concentrations were tested for the BV oxidation of our model substrate. The results of the *rac*-bicyclo[3.2.0]hept-2-en-6-one BV oxidation are shown in Table S3 as total substrate conversion after one and seven hours.

Table S3 Total conversion of *rac*-bicyclo[3.2.0]hept-2-en-6-one by the 2,5-DKCMO with NADH as hydride donor in different FMN concentrations in a reductase-free reaction system.[a]

reaction condition		remaining ketones [%]	
FMN [µM]	**NADH [mM]**	**one hour**	**seven hours**
20	**2**	86	72
	5	76	48
	25	28 ± 1	1
100	**5**	60 ± 3	27 ± 2
	25	4 ± 1	4

[a] The reaction took place in a closed 2 mL glass vial in 0.5 mL of 50 mM Tris-HCl buffer pH 7.5, shaking at 160 rpm and 30 °C. 2,5-DKCMO was applied directly after dialysis (375 µg mL^{-1}) for the BV oxidation of 5 mM *rac*-bicyclo[3.2.0]hept-2-en-6-one. No catalase was utilized. Experiments have been done in duplicates.

In Table S4 the results of the screening of different FMN concentrations and hydride donors are shown. These data already indicate the importance to balance the FMN reduction/(re)oxidation for the system, and the suitability of the two tested NCBs, from which the AmNAH is the better hydride donor in our conditions as described in Fig. 3a in the manuscript.

Table S4 Evaluation of different hydride donors for FMN reduction and further enzymatic BV oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one by the 2,5-DKCMO.[a]

		***ee* ketone**		**remaining (-)-ketone**	
FMN [µM]	**[hydride donor]**	**two hours**	**four hours**	**two hours**	**four hours**
1000	5 mM NADH	1.6	1.5	47.9	46.4
100	5 mM NADH	4.4	6.0	38.6	37.0
	2.5 mM NADH	2.6	3.2	41.1	37.8
10	5 mM NADH	1.4		43.1	
	5 mM BANH	16.8		29.2	
	5 mM AmNAH	10.7		33.6	

[a] Enantiomeric excess of the (+)-(1*R*,5*S*)-bicyclo[3.2.0]hept-2-en-6-one (*ee* ketone), as well as the conversion of the (-)-(1*S*,5*R*)-bicyclo[3.2.0]hept-2-en-6-one, are shown to evaluate the performance of the enzymatic BV oxidation of our model substrate. 5 mM *rac*-bicyclo[3.2.0]hept-2-en-6-one were utilized in a closed 2 mL glass vial in 1 mL of 50 mM Tris-HCl buffer pH 7.5, shaking at 160 rpm and 30 °C. 550 µg mL^{-1} lyophilized 2,5-DKCMO, as well as 6.5 U mL^{-1} catalase, was utilized. The remaining (-)-ketone [%] refers to the racemic ketone mix, therefore the remaining (-)-ketone is 50% when the reaction starts.

Control reaction without FMN resulted in a negligible amount of lactone formation. A slight excess of one lactone over the other indicates an enzymatic conversion of the precursor ketone. We address this observation to traces of FMN/$FMNH_2$, which remained in the enzyme after purification and are recycled in the reaction.

Design of Experiments – Experimental results, calculations and model

Screening

The DoE output for the screening of the effect of various parameters was calculated based on the i) enantiomeric excess of the (+)-ketone at three hours and ii) total conversion after 22 hours. The data (experimental results) implemented in the calculation are listed in Table S5.

Table S5 DoE input for the screening of the effect of various parameters on reductase-free 2,5-DKCMO-mediated enantioselective BV oxidations of *rac*-**1**.[a]

experiment	**i) *ee* ketone at three hours**	**ii) conversion at 22 hours**
1	6.9	68.2
2	2.1	75.5
3	12.2	52.3
4	1.8	59.8
5	1.6	79.9
6	10.8	71.9
7 (1-3)	2.5 / 2.3 / 2.4	82.3 / 82.1 / 87.8
8	9.1	71.0
9	10.6	75.1
10	26.9	31.0
11	0.8	85.2
12	6.2	100.0
13	39.1	36.1
14	2.5	57.1
15	3.4	62.9
16	1.5	37.8

[a] *ee* and conversion are shown in %. For further information see Table S1.

.

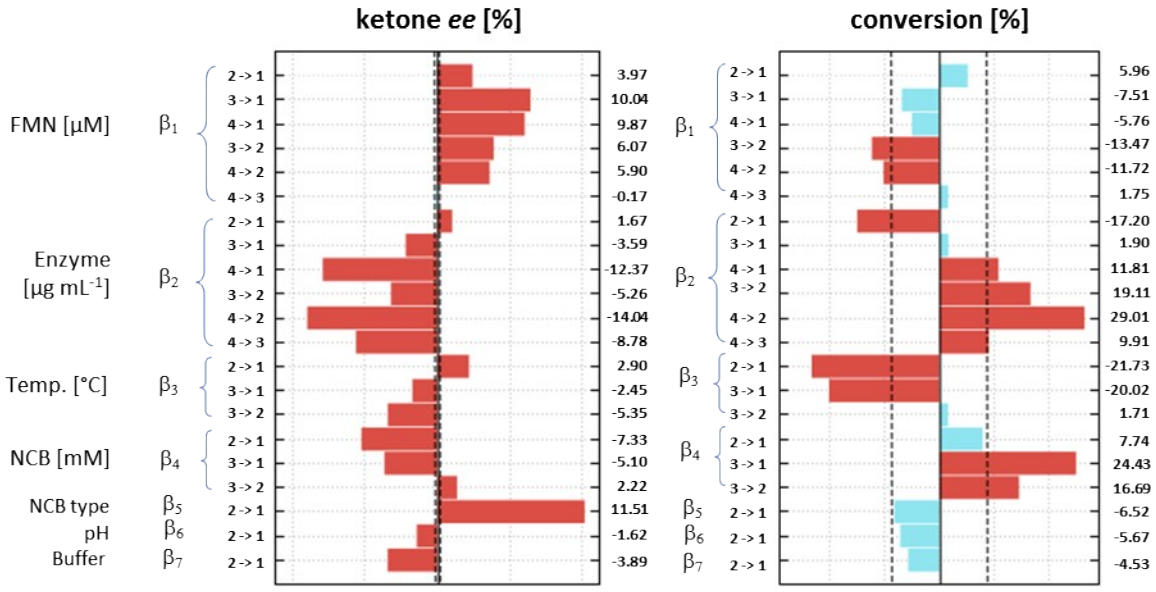


Fig. S7: Graph of effects. Vertical dashed lines represent the limits of significance. Significant values: red bars, non-significant values: blue bars.

In screening study, the effects of factors are supposed to be additive. This hypothesis implies that all the interaction effects were negligible. In our case, mixed-level factors were studied and the reduced reference state model used for 2 factors (parameters) with 4 levels (X_1, X_2), 2 factors with 3 levels (X_3, X_4) and 3 factors with 2 levels (X_5, X_6, X_7) is expressed as following:

$$\eta=\beta_0+\beta_{1A}X_{1A}+\beta_{1B}X_{1B}+\beta_{1C}X_{1C}+\beta_{2A}X_{2A}+\beta_{2B}X_{2B}+\beta_{2C}X_{2C}+\beta_{3A}X_{3A}+\beta_{3B}X_{3B}+\beta_{4A}X_{4A}+\beta_{4B}X_{4B}+\beta_{5A}X_{5A}+\beta_{6A}X_{6A}+\beta_{7A}X_{7A}$$

with $X_{ij}=0$ in absence of variables, $X_{ij}=1$ if the level j of the variable i is present.

- β_{1A}, β_{1B} and β_{1C} (and β_{2A}, β_{2B} and β_{2C}) represent the variation of the response when the factor changes from the highest level taken as reference (here level 3) to level 0 (A), 1 (B) and 2 (C) respectively.
- β_{3A} and β_{3B} (and β_{4A} and β_{4B}) represent the variation of the response when the factor changes from the highest level taken as reference (here level 2) to level 0 (A) and 1 (B) respectively.
- Coefficients β_{5A}, β_{6A} and β_{7A} represent the variation of the response when the factor changes from level 1 (reference) to level 0 (A).

The estimations of the model coefficients using multilinear regression have been calculated for *ee* ketone at three hours and conversion at 22 hours, with as parameters: X1 (FMN concentration), X2 (2,5-DKCMO concentration), X3 (temperature), X4 (NCB concentration), X5 (NCB type), X6 (pH) , X7 (buffer).

Ee ketone = $15{,}0 + 9.87\,X_{1A} + 5.90X_{1B} - 0.16X_{1C} - 12.37X_{2A} - 14.04X_{2B} - 8.78X_{2C} - 2.45X_{3A} - 5.35X_{3B} - 5.10X_{4A} + 2.22X_{4B} + 11.51X_{5A} - 1.61X_{6A} - 3.89X_{7A}$

Conversion = $59.45 - 5.76X_{1A} - 11.72X_{1B} + 1.75X_{1C} + 11.81X_{2A} + 29.01X_{2B} + 9.91X_{2C} - 20.02X_{3A} + 1.71X_{3B} + 24.43X_{4A} + 16.69X_{4B} - 6.52X_{5A} - 5.67X_{6A} - 4.52X_{7A}$

As example, with regard to ee output:

- If all factors are taken at their reference level ($X_{ij}=0$), then ee = β_0 = 15%, the value corresponding to the black bars
- If FMN concentration is changed from 150 mM (reference level) to 25 mM, ee will increase of 9.87 % (=β_{1A})
- If FMN concentration is changed from 150 mM (reference level) to 100 mM, ee will decrease of 0.16 % (=β_{1C})

Optimization

Table S6 shows the experimental results implemented in the calculation of the DoE for the optimization of FMN concentration and temperature for the BV oxidation by the 2,5-DKCMO of the three substrates as mentioned in the manuscript.

Table S6 DoE input for the optimization of FMN concentration and temperature for the BV oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one, *rac*-camphor, and *rac*-norcamphor by 2,5-DKCMO.

experiment	conversion at two hours	*ee* ketone at two hours
1	22.8	26.2
2	2.2	1.7
3	65.7	80.1
4	32.5	27.6
5	54.4	87.0
6	0	1.4
7	13.3	2.1
8	10.5	10.1
9	19.6	26.8
10	31.2	22.1
11	35.6	38.4
12	14.8	4.7
13	65.4	82.6
14	16.3	11.4
15	27.3	10.3
16	62.8	87.2
17	85.6	100.0
18	66.7	97.1
19	56.8	91.5
20	11.7	2.4
21	93.0	74.6

[a] *ee* and conversion are shown in %. For further information see Table S2.

The complete model describing the results (conversion and ee ketone) was a multiplicative particular model for quantitative variables (X_1=Temperature and X_2= FMN Concentration) and qualitative variable (X_3=Substrat with 3 levels) in order to take into account the interactions.

Conversion = $14.96 + 6.88X_1 - 2.18X_2 - 4.13X^2_1 - 6.36X^2_2 + 1.08X_1X_2 + 59.31X_{3A} + 17.89X_{3B} - 4.19X_{3A}X_1 + 0.48X_{3A}X_2 - 0.25X_{3B}X_1 - 4.10X_{3B}X_2$

Ee ketone = $5.54 + 0.94X_1 - 1.02X_2 - 2.96X^2_1 - 1.55X^2_2 + 0.19X_1X_2 + 84.17X_{3A} + 19.46X_{3B} - 3.18X_{3A}X_1 + 1.28X_{3A}X_2 - 1.45X_{3B}X_1 - 9.03X_{3B}X_2$

References

1 M. Kadow, K. Balke, A. Willetts, U. T. Bornscheuer and J. - E. Bäckvall, *Appl. Microbiol. Biotechnol.,* 2014, **98**, 3975–3986.

2 C. E. Paul, S. Gargiulo, D. J. Opperman, I. Lavandera, V. Gotor - Fernández, V. Gotor, A. Taglieber, I. W. C. E. Arends and F. Hollmann, *Org. Lett.*, 2013, **15**, 180–183.

3 A. Guarneri, A. H. Westphal, J. Leertouwer, J. Lunsonga, M. C. R. Franssen, D. J. Opperman, F. Hollmann, W. J. H. Berkel and C. E. Paul, *ChemCatChem*, 2020, **12**, 1368–1375.

4 D. Mauzerall and F. H. Westheimer, *J. Am. Chem. Soc.*, 1955, **77**, 2261–2264.