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Supporting Information

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Self–sufficient Baeyer-Villiger Monooxygenases – Effective coenzyme regeneration for Biooxygenations by fusion engineering**

Supporting Information

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General Methods

Unless otherwise noted, all chemicals and enzymes were obtained from ACROS Organics, Jülich Fine Chemicals, Riedel-de Haën Fine Chemicals, Roche Applied Sciences, New England Biolabs and Finnzymes and used without further purification. Oligonucleotide primers were obtained from Sigma Genosys. DNA sequencing was done at GATC (Konstanz, Germany).
Harvesting of cells was realized by centrifugation using a Eppendorf 5804R centrifuge, Beckmann J2-21M/E centrifuge (JA-10 rotor) or Sigma 6K15 centifuge (rotor 372/C). Centrifugations for removal of cell debris were realized on a Beckmann J2-21M/E centrifuge (JA-17 rotor) or a Sigma 3K30 (rotor 19777).
Bacterial cultures were grown in incubators (Thermoshake, Gerhardt). Conversions containing cell-free extracts were shaken in an incubator (Heidolph Titramax 1000).
Enantiomeric excess was determined via GC using a BGB 175 column (30mx0.25mm ID, 0.25µm film) on a ThermoQuest Trace GC 2000 with FID detection (240°C), or using a BGB 173 column (30mx0.25mm ID, 0.25µm film) on a Thermo Finnigan Focus GC with FID detection (240°C). General conversion control and examination of products were performed with a standard capillary column DB5 (30m x 0.32mm ID, 1.0 µm film) on a GC-MS Voyager 8000 Top.
Ultrasonication was realized using a Bandelin Sonoplus HD 3200 (KE 76 probe).
Ketones for screening experiment were obtained from a substrate library of VUT and their synthesis was described previously elsewhere.

Cloning Strategy

Cloning vectors pCRE-X and pX-CRE were created by digestion of the pBADNK vector [1] with *Nde*I/*Xho*I, respectively *Pvu*II/*Hind*III and ligation of the double mutated *ptdh* gene containing complementary DNA overhangs. Subsequently, pPAMO-CRE was created by digestion of the pX-CRE vector with *Nde*I/*Xho*I and ligation of the amplified *pamo* gene with complementary DNA overhangs. In a similar way, expression vectors pCRE-PAMO, pCRE-CHMO and pCRE-CPMO were constructed by digestion of the pCRE-X vector with *Pvu*II/*Hind*III and ligation of the amplified *pamo*, *chmo* and *cpmo* genes, respectively. The *Hind*III restriction sites within the *chmo* and *cpmo* genes were removed prior to cloning by silently mutating one nucleotide.

Overexpression and Fusion Protein Purification

The bifunctional PAMO-CRE and CRE-PAMO enzymes were overexpressed in *E. coli* TOP10 at 30 °C in TB_{amp} medium supplemented with 0.2 % L-arabinose. CRE-CHMO and CRE-CPMO were overexpressed in *E. coli* TOP10 at 24 °C, respectively 17 °C in the same broth. Purification of the fusion enzymes was performed using a Q-sepharose anion exchange column and a Superdex 200 size-exclusion column. PAMO and the PTDH mutant E175A/A176R were purified as described previously.[2]

Determination of Kinetic Parameters and Conversion using Isolated Enzymes

Activities of the purified enzymes were determined spectrophotometrically by monitoring the increase or decrease of NADPH in time at 340 nm. The reaction mixture (1 mL) typically contained 50 mM Tris-HCl, pH 7.5, 100 µM coenzyme, 2 mM ketone or 5 mM Na_2HPO_3, 1 % (v/v) DMSO (not for CRE-CHMO) and 0.05-1 µM enzyme at 25 °C. Kinetic parameters were obtained by fitting the obtained data as described previously.[3] Conversions of phenylacetone by either PAMO-CRE or PAMO in the presence of PTDH were carried out in a 100 mL Erlenmeyer flask at 30 °C/200 rpm containing 25 mL 50 mM Tris-HCl, pH 7.5, 2.5 mM phenylacetone, 1 % (v/v) DMSO, 20 mM Na_2HPO_3, 0.13-0.6 µM enzyme and 0.2-100 µM $NADP^+$. During 3 hours, samples of 1 mL were taken, extracted and analyzed by GC.[2b]

General Protocol for Whole-Cell Screening Experiments

Plates with either 12 or 24 wells were utilized. Each well was charged with LB_{amp} medium (5.0 g peptone, 2.5 g yeast extract, 5.0 g sodium chloride, 500 mL deion. water, 2 mL of ampicilline stock solution – 50 mg/mL) (2 mL or 1 mL resp.) and inoculated with 1% of an overnight preculture of recombinant *E. coli* overexpressing BVMOs. A plate was incubated at 120 rpm at 37°C on an orbital shaker for 2 hours. L-arabinose was added (final concentration of 0.02%) together with substrate (final concentration of 3-6 mM). The plate was shaken at RT. After 24 hours samples for GC measurement were taken (700 µL of culture were centrifuged and supernatant was extracted with 700 µL of EtOAc supplemented with methylester of benzoic acid as internal standard).

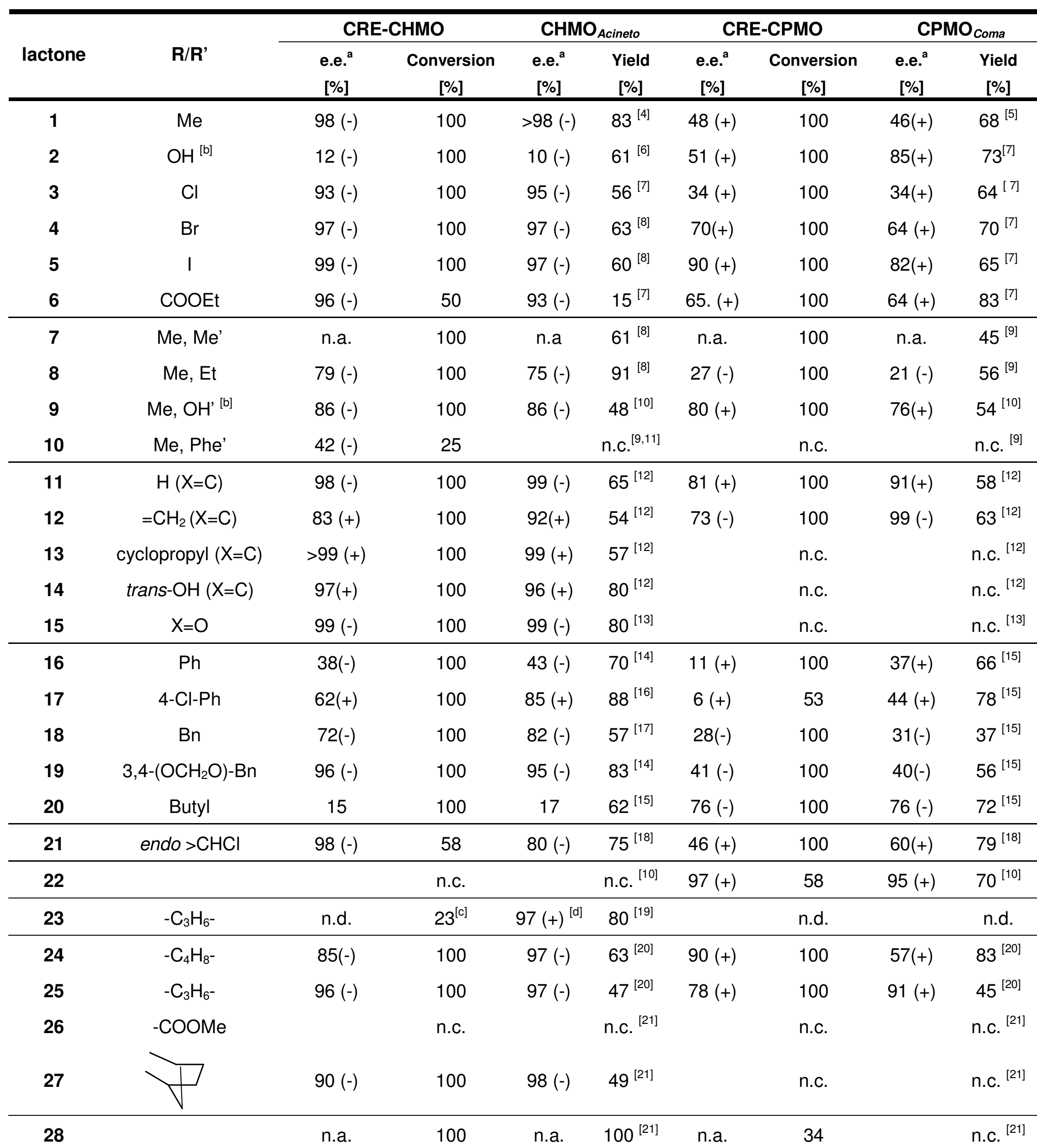
Fusion Protein Crude Cell Extract (CE)

Fresh LB_{amp} medium (200 mL) was inoculated with 1% of an overnight preculture of recombinant *E. coli* TOP10 overexpressing pCRE-CHMO or pCRE-CPMO in a baffled 1 L Erlenmeyer flask. The L-arabinose was added to a final concentration 0.02% and culture was incubated at 120 rpm at 24 °C on an orbital shaker for 24 hours. Cells were harvested by centrifugation (6000 x g, 10 min, 4 °C, Sigma centrifuge). The cell pellets were resuspended in 10 mM PBS buffer (pH=7.4) (double volume of cells) and disintegrated by ultrasonication using a Bandelin Sonoplus HD 3200 (KE76 probe, 50% amplitude, 5sec on and 30s off, 12 cycles, 4°C). Cell debris was removed by centrifugation (15000 x g, 20 min, 4 °C, Sigma). The cell-free extract was divided into 0.5 mL aliquots and stored at -20 °C until needed.
Protein concentration of crude extract was estimated by Bradford method using Protein Assay (Bio-Rad). Bovine serum albumin was used as standard for the calibration curve.

General Protocol for CE Biotransformations

Biotransformations using crude extract were realized in regular 96-well plates. Each well was charged with 100 µL of CE with a total protein concentration of approx. 20 mg/mL. To determine optimal concentration of sodium phosphite, Na_2HPO_3 was added in different concentrations in the range between 0-1000 mM. Substrate was added in the range between 5-50 mM for substrate inhibition studies. The mixture was incubated at 700 rpm at 24 °C in a multi-well plate incubator (Heidolph Titramax 1000). In time samples were taken, extracted by EtOAc supplemented with an internal standard (1 mM of methyl ester of benzoic acid), and analyzed by GC.

Table 1. Biooxidation of functionalized prochiral ketones by whole-cells of *E. coli* expressing CRE-CHMO or CRE-CPMO.



lactone	R/R'	CRE-CHMO		$CHMO_{Acineto}$		CRE-CPMO		$CPMO_{Coma}$	
		e.e.[a] [%]	Conversion [%]	e.e.[a] [%]	Yield [%]	e.e.[a] [%]	Conversion [%]	e.e.[a] [%]	Yield [%]
1	Me	98 (-)	100	>98 (-)	83 [4]	48 (+)	100	46(+)	68 [5]
2	OH [b]	12 (-)	100	10 (-)	61 [6]	51 (+)	100	85(+)	73[7]
3	Cl	93 (-)	100	95 (-)	56 [7]	34 (+)	100	34(+)	64 [7]
4	Br	97 (-)	100	97 (-)	63 [8]	70(+)	100	64 (+)	70 [7]
5	I	99 (-)	100	97 (-)	60 [8]	90 (+)	100	82(+)	65 [7]
6	COOEt	96 (-)	50	93 (-)	15 [7]	65. (+)	100	64 (+)	83 [7]
7	Me, Me'	n.a.	100	n.a	61 [8]	n.a.	100	n.a.	45 [9]
8	Me, Et	79 (-)	100	75 (-)	91 [8]	27 (-)	100	21 (-)	56 [9]
9	Me, OH' [b]	86 (-)	100	86 (-)	48 [10]	80 (+)	100	76(+)	54 [10]
10	Me, Phe'	42 (-)	25		n.c.[9,11]		n.c.		n.c. [9]
11	H (X=C)	98 (-)	100	99 (-)	65 [12]	81 (+)	100	91(+)	58 [12]
12	$=CH_2$ (X=C)	83 (+)	100	92(+)	54 [12]	73 (-)	100	99 (-)	63 [12]
13	cyclopropyl (X=C)	>99 (+)	100	99 (+)	57 [12]		n.c.		n.c. [12]
14	*trans*-OH (X=C)	97(+)	100	96 (+)	80 [12]		n.c.		n.c. [12]
15	X=O	99 (-)	100	99 (-)	80 [13]		n.c.		n.c. [13]
16	Ph	38(-)	100	43 (-)	70 [14]	11 (+)	100	37(+)	66 [15]
17	4-Cl-Ph	62(+)	100	85 (+)	88 [16]	6 (+)	53	44 (+)	78 [15]
18	Bn	72(-)	100	82 (-)	57 [17]	28(-)	100	31(-)	37 [15]
19	3,4-(OCH_2O)-Bn	96 (-)	100	95 (-)	83 [14]	41 (-)	100	40(-)	56 [15]
20	Butyl	15	100	17	62 [15]	76 (-)	100	76 (-)	72 [15]
21	*endo* >CHCl	98 (-)	58	80 (-)	75 [18]	46 (+)	100	60(+)	79 [18]
22			n.c.		n.c. [10]	97 (+)	58	95 (+)	70 [10]
23	$-C_3H_6-$	n.d.	23[c]	97 (+) [d]	80 [19]		n.d.		n.d.
24	$-C_4H_8-$	85(-)	100	97 (-)	63 [20]	90 (+)	100	57(+)	83 [20]
25	$-C_3H_6-$	96 (-)	100	97 (-)	47 [20]	78 (+)	100	91 (+)	45 [20]
26	-COOMe		n.c.		n.c. [21]		n.c.		n.c. [21]
27		90 (-)	100	98 (-)	49 [21]		n.c.		n.c. [21]
28		n.a.	100	n.a.	100 [21]	n.a.	34		n.c. [21]

[a] sign of specific rotation in parentheses [b] isolated as rearranged product (see ref. [6]). [c] conversion based on GC/MS analysis [d] biooxidation utilizing isolated enzyme. n.a.: not applicable; n.c. no conversion; n.d. not determined.

Table 2. Regiodivergent biooxidation by whole-cells of *E. coli* expressing CRE-CHMO or CRE-CPMO.

ketone	CRE-CHMO		$CHMO_{Acineto}$		CRE-CPMO		$CPMO_{Coma}$	
	Conversion[%]/ ratio[a]	e.e. [b] [%]	Yield[%]/ ratio[a]	e.e.[b] [%]	Conversion[%]/ ratio[a]	e.e.[b] [%]	Yield [%]/ ratio[a]	e.e.[b] [%]
29	100	81	74 [22]	95	100	0	61 [10]	0
	55:45	*99*	51:49	*>99*	98:2	*99*	97:3	*>99*
30	100	77	78 [22]	>99	100	89	79[23]	94
	70:30	*>99*	65:35	*96*	54:46	*96*	51:49	*96*
31	100	73	85 [23]	60	100	15	80 [23]	0
	44:56	*>99*	45:55	*>99*	94:6	*>99*	96:4	*>99*
32	100	39	84 [23]	44	100	10	89 [23]	14
	70:30	*98*	70:30	*>99*	87:13	*97*	87:13	*>99*

[a] ratio between normal and abnormal lactone [b] value of abnormal lactone is given in italics.

Optimization of Operational Parameters for CE Biotransformations

Optimization of sodium phosphite concentration

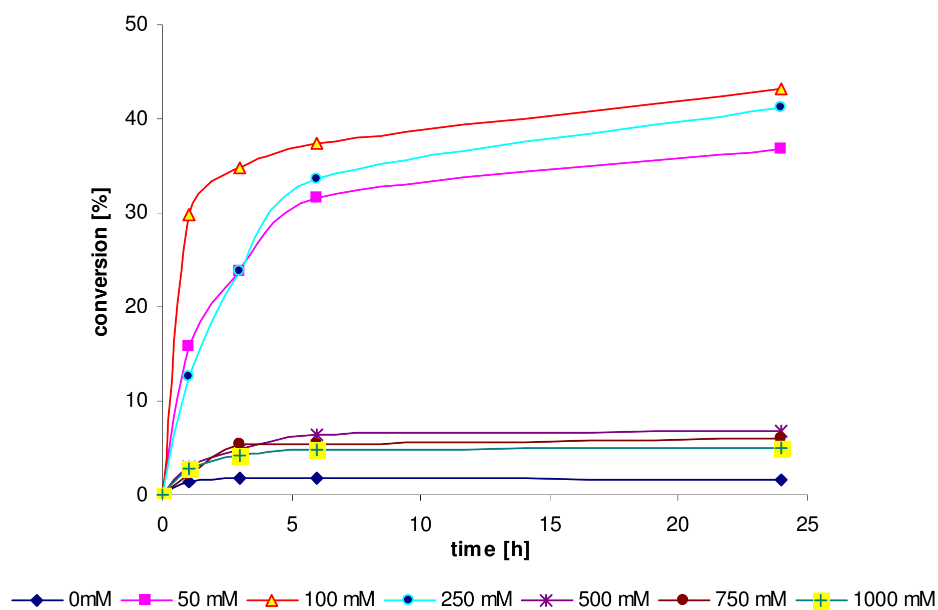


Figure 1: Conversion of 4-methyl cyclohexanone (10 mM) with CE of *E. coli* TOP10/pCRE-CHMO in the presence of varying concentrations sodium phosphite (0-1000 mM).

Substrate inhibition study

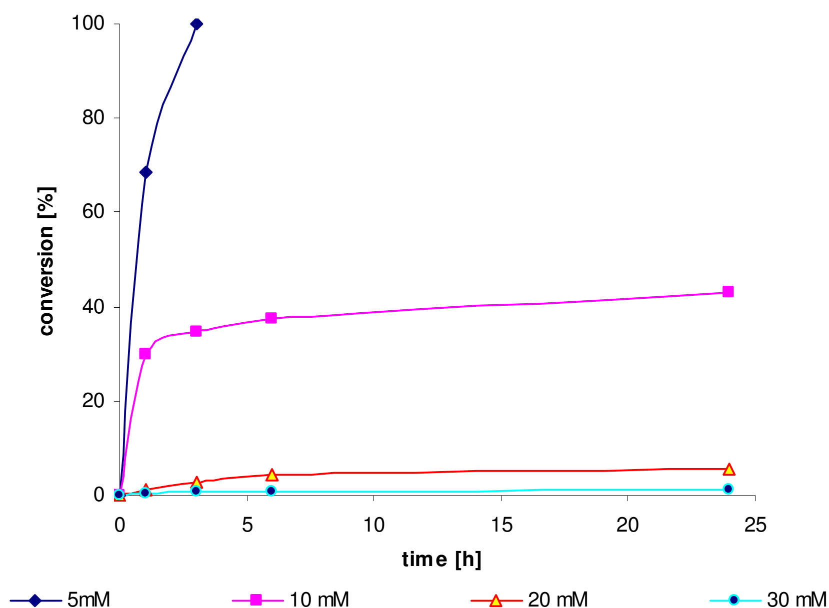


Figure 2: Conversion of 4-methyl cyclohexanone (5, 10, 20, 30 mM) with CE of *E. coli* TOP10/pCRE-CHMO in the presence of 100 mM sodium phosphite.

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