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BLENDING BAEYER-VILLIGER MONOOXYGENASES: USING A ROBUST BVMO AS A SCAFFOLD FOR THE CREATION OF CHIMERIC ENZYMES WITH NOVEL CATALYTIC PROPERTIES

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Supporting Information

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1. General

Enzymes were obtained from New England Biolabs and Clontech. Oligonucleotides were synthesized by Sigma-Aldrich and sequencing of the created constructs was done at GATC (Konstanz, DE). pCRE2-PAMO was mutated into pCRE2-PAMO-RsrII using site-directed mutagenesis, introducing a silent mutation in the codon for G436. The resulting plasmid was cut with PvuII/RsrII or RsrII/HindIII to create the backbone for the pCRE-xxx-PAMO and pCRE-PAMO-xxx chimeras respectively. Inserts were made using PCR, with 15bp overlaps with the backbone to facilitate In-Fusion (Clontech, Mountain View, CA USA) cloning with the backbones, according to the manufacturer's instructions. The resulting constructs were transformed to $CaCl_2$ competent *E.coli* Top10 cells. For the chimeras based on metagenomic sequences, three pieces were synthesized by GeneArt (Germany), cut with RsrII/HindIII and ligated into the backbone using Quickligase (Roche, Basel, CH). Overexpression and purification of the enzymes was done as previously described.[1]

Substrates **1**-**2**, (±)-**3**-**4** and **5**, as well as all other reagents were purchased from Sigma-Aldrich, Acros or Alfa Aesar and were used as provided unless otherwise stated. Mass spectra were recorded on an AEI-MS-902 mass spectrometer. NMR spectra were obtained on Varian AMX400 spectrometer (^{1}H: 400 MHz; ^{13}C 100.59 MHz). Chemical shifts (δ) are given in parts per million (ppm) relative to the residual solvent peak, while the coupling constants (*J*) are given in Hz. Gas chromatography was carried out on a Hewlett-Packard 6890 Series using a flame ionization detector. For all the analyses, the injector temperature was 225 ºC and the FID temperature was 250 ºC. HPLC was performed on a Shimadzu LC-10ADVP equipped with a Shimadzu SPD-M10AVP diode array detector. UV-Vis spectra were obtained using a Hewlet-Packard HP 8543 FT spectrophotometer in a 1.0 cm quartz cuvette.

Racemic methyl phenyl sulfoxide and benzyl phenyl sulfoxide were prepared by treatment of the starting sulfides with H_2O_2 in methanol at room temperature (yields higher than 80%). Lactones were synthesized from the starting ketones by treatment with *m*CPBA in CH_2Cl_2 at 0ºC (yields range: 56-81%). All other reagents and solvents were of the highest quality grade available and were obtained from Sigma-Aldrich-Fluka and Acros Organics. The absolute configurations of the final sulfoxides and lactones is based on literature data.[2]

2. Experimental procedures

2.1. General procedure for the enzymatic oxidation of substrates 1-2, (±)-3-4 and 5 employing the native and chimeric BVMOs.

Unless otherwise state, substrates (5-10 mM) were dissolved in 50 mM Tris/HCl pH 7.5, containing 5% DMSO. Subsequently, 100 µM NADPH, 4 µM phosphite dehydrogenase, 10 mM sodium phosphite and 4 to 10 µM BVMO was added. The mixture was shaken on at 250 rpm at room temperature. Reactions were stopped by extraction with ethyl acetate (2 × 0.5 mL) with 0.1% mesitylene as internal standard, and the organic layer was dried over $MgSO_4$. Conversions and enantiomeric excesses of the oxidized products were determined by GC and HPLC.

3. GC and HPLC analyses.

The following columns were used for the determination of conversions and enantiomeric excesses of the sulfoxides by GC: A: GT-A (Alltech, 30 m x 0.25 mm x 0.25 µm), B: Hewlett Packard HP-1 (Agilent, 30m x 0.32 mm x 0.25µm) or C: Chiralsil Dex CB (Varian, 25 m × 0.32 mm × 0.25 µm).

Table S1. Determination of conversion and enantiomeric excess values by GC.

Substrate	Program[a]	Column	t_R (min) substrates	t_R (min) products
1	40/5/10/200	A	7.3	13.6 (*R*); 15.6 (*S*)
2	100/5/5/200/5	B	23.1	28.9
(±)-**3**	35/0/5/130/0/15/200	A	9.7	16.9 (normal) 17.1 (abnormal)
(±)-**3**	130 isotherm	C	--	10.8 (1*R*,5*S*)-abn 11.3 (1*R*,5*S*)-norm 11.6 (1*S*,5*R*)-abn 11.9 (1*S*,5*R*)-norm
(±)-**4**	40/10/3/180/5	A	23.5 (*R*) 23.6 (*S*)	30.6 (*S*) 30.9 (*R*)
5	190/1/4/235/5/30/300/3	B	19.4	18.9

[a] Program: initial T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min).

For the determination of the enantiomeric excesses of the benzyl phenyl sulfoxide by HPLC, a Chiralcel OD (0.46 cm x 25 cm) column from Daicel was employed.

Table S2. Determination of enantiomeric excess values by HPLC.

Substrate	Flow rate (mL min^{-1})	T (ºC)	Eluent[a]	t_R (min)
2	0.5	25	*n*-hexane-IPA 9:1	19.2 (*R*); 22.4 (*S*)

[a] The experiments was performed with isocratic eluent.

4. Kinetics measurements

Oxidation rates for the three wild type enzymes as well as for the three chimeric enzymes were determined by observing the depletion of NADPH, monitoring its absorbance at 340 nm in a solution containing 0.05 to 0.5 µM BVMO, 100 µM NADPH and 0 to 1 mM phenylacetone (employed as model substrate) dissolved in 50 mM Tris-HCl pH 7.5. Observed rates were fitted to the Michaelis-Menten equation, using the SigmaPlot software package.

Table S3. Determination of the catalytic constants of the different BVMOs employing phenylacetone as substrate.

Enzyme	k_{cat} (s^{-1})	K_M (µM)
PAMO	2.3	80
STMO	0.6	106
CHMO	1.5	633
PASTMO	0.6	163
PACHMO	n/a[a]	n/a[a]
PAMEMO1	0.5	39

[a] Observed rate similar to uncoupling rate (0.1 s^{-1}).

5. ThermoFAD method[4]

Experiments were performed using a BioRad (Hercules, CA, USA) RT-PCR machine and BioRad RT-PCR plates. The excitation wavelength was set between 470 and 500nm and a SYBR-green filter (523–543 nm) was used for measuring fluorescence emission. Unfolding curves were measured between 20 °C and 95 °C (1 °C min^{-1}) with fluorescence intensity measured every 0.5 °C after a 10 s delay for temperature stabilization. The fluorescence intensity was plotted against the temperature to obtain a sigmoidal curve. The reported T_m values were determined as the maximum of the derivative of this sigmoidal curve. 20 µl solutions of 5 µM enzyme in 50 mM Tris/HCl pH 7.5 were used.

5. Protein sequences

All proteins were created as fusion enzymes, linked at the N-terminus to PTDH,[3] with a N-terminal poly-His tag.

>PAMO, *Thermobifida fusca*, YP_289549, GI:72161892
MAGQTTVDSRRQPPEEVDVLVVGAGFSGLYALYRLRELGRSVHVIETAGDVGGVWYWNRYPGARCDIESI
EYCYSFSEEVLQEWNWTERYASQPEILRYINFVADKFDLRSGITFHTTVTAAAFDEATNTWTVDTNHGDR
IRARYLIMASGQLSVPQLPNFPGLKDFAGNLYHTGNWPHEPVDFSGQRVGVIGTGSSGIQVSPQIAKQAA
ELFVFQRTPHFAVPARNAPLDPEFLADLKKRYAEFREESRNTPGGTHRYQGPKSALEVSDEELVETLERY
WQEGGPDILAAYRDILRDRDANERVAEFIRNKIRNTVRDPEVAERLVPKGYPFGTKRLILEIDYYEMFNR
DNVHLVDTLSAPIETITPRGVRTSEREYELDSLVLATGFDALTGALFKIDIRGVGNVALKEKWAAGPRTY
LGLSTAGFPNLFFIA**G**PGSPSALSNMLVSIEQHVEWVTDHIAYMFKNGLTRSEAVLEKEDEWVEHVNEIA
DETLYPMTASWYTGANVPGKPRVFMLYVGGFHRYRQICDEVAAKGYEGFVLT

>CHMO, *Acinetobacter* sp. NCIMB 9871, BAA86293.1 GI:6277322
MSQKMDFDAIVIGGGFGGLYAVKKLRDELELKVQAFDKATDVAGTWYWNRYPGALSDTETHLYCYSWDKE
LLQSLEIKKKYVQGPDVRKYLQQVAEKHDLKKSYQFNTAVQSAHYNEADALWEVTTEYGDKYTARFLITA
LGLLSAPNLPNIKGINQFKGELHHTSRWPDDVSFEGKRVGVIGTGSTGVQVITAVAPLAKHLTVFQRSAQ
YSVPIGNDPLSEEDVKKIKDNYDKIWDGVWNSALAFGLNESTVPAMSVSAEERKAVFEKAWQTGGGFRFM
FETFGDIATNMEANIEAQNFIKGKIAEIVKDPAIAQKLMPQDLYAKRPLCDSGYYNTFNRDNVRLEDVKA
NPIVEITENGVKLENGDFVELDMLICATGFDAVDGNYVRMDIQGKNGLAMKDYWKEGPSSYMGVTVNNYP
NMFMVLGPNGPFTNLPPSIESQVEWISDTIQYTVENNVESIEATKEAEEQWTQTCANIAEMTLFPKAQSW
IFGANIPGKKNTVYFYLGGLKEYRSALANCKNHAYEGFDIQLQRSDIKQPANA

>STMO, *Rhodococcus rhodochrous* BAA24454.1 GI:2804298
MNGQHPRSVVTAPDATTGTTSYDVVVVGAGIAGLYAIHRFRSQGLTVRAFEAASGVGGVWYWNRYPGARC
DVESIDYSYSFSPELEQEWNWSEKYATQPEILAYLEHVADRFDLRRDIRFDTRVTSAVLDEEGLRWTVRT
DRGDEVSARFLVVAAGPLSNANTPAFDGLDRFTGDIVHTARWPHDGVDFTGKRVGVIGTGSSGIQSIPII
AEQAEQLFVFQRSANYSIPAGNVPLDDATRAEQKANYAERRRLSRESGGGSPHRPHPKSALEVSEEERRA
VYEERWKLGGVLFSKAFPDQLTDPAANDTARAFWEEKIRAVVDDPAVAELLTPKDHAIGAKRIVTDSGYY
ETYNRDNVELVDLRSTPIVGMDETGIVTTGAHYDLDMIVLATGFDAMTGSLDKLEIVGRGGRTLKETWAA
GPRTYLGLGIDGFPNFFNLTGPGSPSVLANMVLHSELHVDWVADAIAYLDARGAAGIEGTPEAVADWVEE
CRNRAEASLLNSANSWYLGANIPGRPRVFMPFLGGFGVYREIITEVAESGYKGFAILEG

>PASTMO chimera
MAGQTTVDSRRQPPEEVDVLVVGAGFSGLYALYRLRELGRSVHVIETAGDVGGVWYWNRYPGARCDIESI
EYCYSFSEEVLQEWNWTERYASQPEILRYINFVADKFDLRSGITFHTTVTAAAFDEATNTWTVDTNHGDR
IRARYLIMASGQLSVPQLPNFPGLKDFAGNLYHTGNWPHEPVDFSGQRVGVIGTGSSGIQVSPQIAKQAA
ELFVFQRTPHFAVPARNAPLDPEFLADLKKRYAEFREESRNTPGGTHRYQGPKSALEVSDEELVETLERY
WQEGGPDILAAYRDILRDRDANERVAEFIRNKIRNTVRDPEVAERLVPKGYPFGTKRLILEIDYYEMFNR
DNVHLVDTLSAPIETITPRGVRTSEREYELDSLVLATGFDALTGALFKIDIRGVGNVALKEKWAAGPRTY
LGLSTAGFPNLFFIAGPGSPSVLANMVLHSELHVDWVADAIAYLDARGAAGIEGTPEAVADWVEECRNRA
EASLLNSANSWYLGANIPGRPRVFMPFLGGFGVYREIITEVAESGYKGFAILEGKLGPEQKLISEEDLNS
AVDHHHHHH

>PACHMO chimera
MAGQTTVDSRRQPPEEVDVLVVGAGFSGLYALYRLRELGRSVHVIETAGDVGGVWYWNRYPGARCDIESI
EYCYSFSEEVLQEWNWTERYASQPEILRYINFVADKFDLRSGITFHTTVTAAAFDEATNTWTVDTNHGDR
IRARYLIMASGQLSVPQLPNFPGLKDFAGNLYHTGNWPHEPVDFSGQRVGVIGTGSSGIQVSPQIAKQAA
ELFVFQRTPHFAVPARNAPLDPEFLADLKKRYAEFREESRNTPGGTHRYQGPKSALEVSDEELVETLERY
WQEGGPDILAAYRDILRDRDANERVAEFIRNKIRNTVRDPEVAERLVPKGYPFGTKRLILEIDYYEMFNR
DNVHLVDTLSAPIETITPRGVRTSEREYELDSLVLATGFDALTGALFKIDIRGVGNVALKEKWAAGPRTY
LGLSTAGFPNLFFIAGPNGPFTNLPPSIESQVEWISDTIQYTVENNVESIEATKEAEEQWTQTCANIAEM
TLFPKAQSWIFGANIPGKKNTVYFYLGGLKEYRSALANCKNHAYEGFDIQLQRSDIKQPANA

>PAMEMO1 chimera – containing a part from ABEF01054497
MAGQTTVDSRRQPPEEVDVLVVGAGFSGLYALYRLRELGRSVHVIETAGDVGGVWYWNRYPGARCDIESI
EYCYSFSEEVLQEWNWTERYASQPEILRYINFVADKFDLRSGITFHTTVTAAAFDEATNTWTVDTNHGDR
IRARYLIMASGQLSVPQLPNFPGLKDFAGNLYHTGNWPHEPVDFSGQRVGVIGTGSSGIQVSPQIAKQAA

ELFVFQRTPHFAVPARNAPLDPEFLADLKKRYAEFREESRNTPGGTHRYQGPKSALEVSDEELVETLERY
WQEGGPDILAAYRDILRDRDANERVAEFIRNKIRNTVRDPEVAERLVPKGYPFGTKRLILEIDYYEMFNR
DNVHLVDTLSAPIETITPRGVRTSEREYELDSLVLATGFDALTGALFKIDIRGVGNVALKEKWAAGPRTY
LGLSTAGFPNLFFIAGPGSPSVLSNMPVSIEQHIDWIADLLQHMREHDIKSVEAEADAEKAWVVHVNEVA
EPTMFMQANSWYLGANIPGKPRVFMPYAGGVGTYRKKCNEVADNGYEGFILGAGRRGAETVKS

6. Supporting references

(1) M. W. Fraaije, J. Wu, D. P. H. M. Heuts, E. W. van Hellemond, J. H. Lutje Spelberg, D. B. Janssen, *Appl. Microbiol. Biotechnol*. 2005, **66**, 393.

(2) (a) H. Dudek, G. de Gonzalo, D. E. Torres Pazmiño, Piotr Stepniak, L. S. Wyrwicz, L. Rychlewski, M. W. Fraaije, *Appl. Environ. Microbiol.* 2011, **77**, 5730; (b) F. Zambianchi, P. Pasta, G. Ottolina, G. Carrea, S. Colonna, N. Gaggero, J. M. Ward, *Tetrahedron: Asymmetry* 2000, **11**, 3653; (c) V. Alphand, R. Furstoss, S. Pedragosa-Moreau, S. M.Roberts, A. J. Willetts, *J. Chem. Soc. Perkin Trans. 1* 1996, 1867.

(3) D. E. Torres Pazmiño, A. Riebel, J. de Lange, F. Rudroff, M. D. Mihovilovic, M. W. Fraaije, *ChemBioChem* 2009, **10**, 2595.

(4) F. Forneris, R. Orru, D. Bonivento, L. R. Chiarelli, A. Mattevi, *FEBS J.* 2009, **276**, 2833.