

SUPPLEMENTARY MATERIAL

Characterization of Protocatechuate 4,5-Dioxygenase from *Pseudarthrobacter phenanthrenivorans* Sphe3 and *in situ* reaction monitoring in the NMR tube.

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[#]Equal contribution

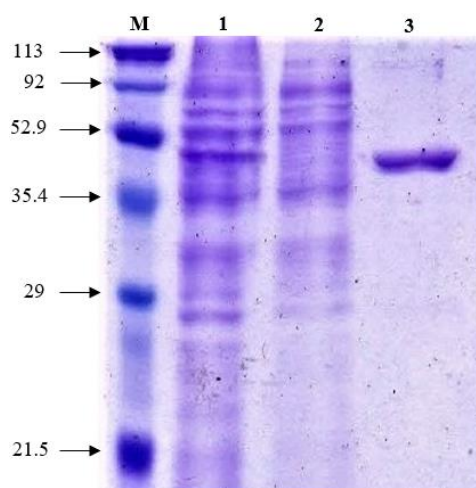
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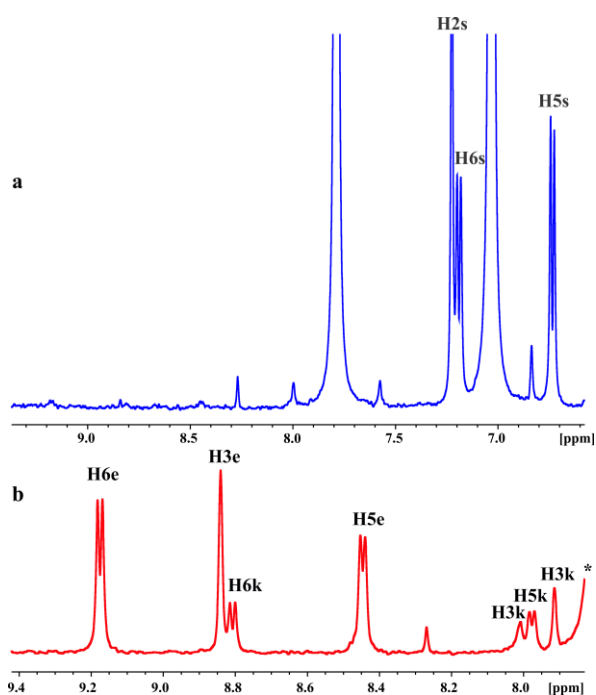
| | | |
|---------|---|-----|
| LigA | MTEKKERIDVHAYLAEFDDIPGTRVTTAQRARKGYNLNQFAMSLMKAENRRERFKADESAY | 60 |
| PcaA | -----MTLDKPYNDVPGTTIIFDADQARKGYNLNQFCMSLMKPKENRRERYLADRGAY | 50 |
| PcmA | -----MTLDKPYKDVPGTTIIFDADQARKGYNLNQFCMSLMKPKENRRERYLADRGAY | 50 |
| PmdA-T2 | -----MALEKPYLVDVPGTTIIFDAEQSRKGYNLNQFCMSLMKAENRRERFRADERAY | 50 |
| ProOa | -----MALEKPYLVDVPGTTIIFDAEQSRRRGYNLNQFCMSLMKAENRRDRFRANERAY | 50 |
| LigA-Rp | -----MPLDKPYKDVPGTTIIFDADMSRQGYHLNQFCMSLMKAENRRARFKADERAY | 50 |
| | : *:*:* : * : : : *:* ***** *:* : * : * | |
| | # # | |
| LigA | LDEWNLTPAAKAAVLARDYDINAMIDEGGNVYFLSKLFTSDGKSFQFAAGSMTGMTQEYYAQ | 120 |
| PcaA | LDEWPLNPVQQRQAVLDDIDLNTCIAEGGNIYFLAKIGATHGLSFQQMAGSMTGMSEAAAYRD | 110 |
| PcmA | LDEWPLNPVQQRQAVLDDIDLNTCIAEGGNIYFLAKIGATHGLSFQQMAGSMTGMSEAAAYRD | 110 |
| PmdA-T2 | LDEWAMTEEQQQAVLARDLNNCMRTGGNIYFLAKIGATDGKSFQQMAGSMTGMTTEEYRA | 110 |
| ProOa | LDEWAMTEEQQQAVLARDLNNCMRTGGNIYFLAKIGATDGKSFQQMAGSMTGMTTEEYRA | 110 |
| LigA-Rp | LDEWPMTEEQQQAVLDRDLNRCIALGGNIYFLAKIGATDGKSFQQMAGSMTGMTTEEYRN | 110 |
| | ***** : : *:* : * : : *:*:*:* : : *:* ***** : : * | |
| | A | |
| LigA | MMIDGGRSPAGVRSIKGGY----- | 139 |
| PcaA | MMIGGGRRPEGNNR----- | 123 |
| PcmA | MMIGGGRRPEGNNR----- | 123 |
| PmdA-T2 | MMMGGGRSAEGNRYVGEDGDAQAHHPQGSAGNQNKEGN | 149 |
| ProOa | MMMGGGRSAEGNRYVGEDGDAQAHHPQGSAGNQNKEGN | 149 |
| LigA-Rp | MMVGGGRPIEGNRYVGEKK----- | 129 |
| | *** :.*** ** | |

[illegible]

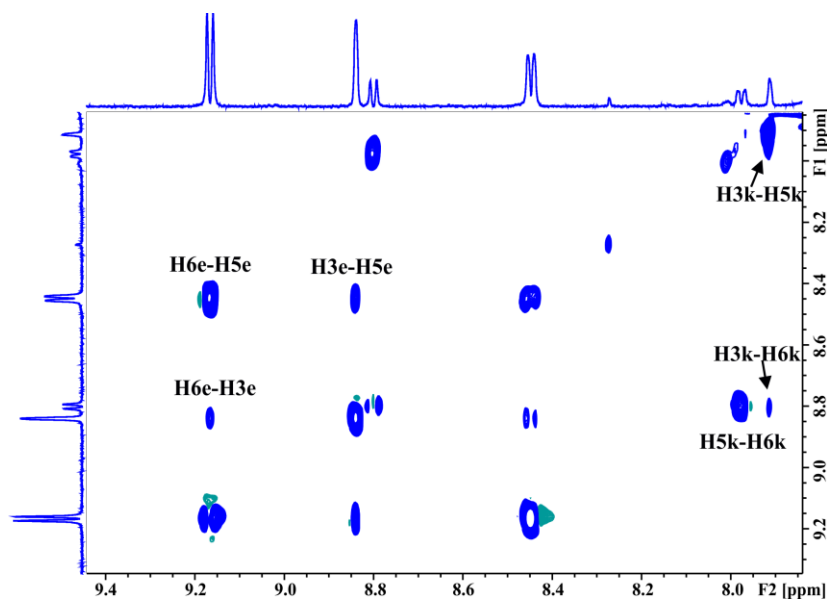
Supplementary Fig. S1: Multiple sequence alignment. Alignment (Clustal W) of the α (A) and β (B) subunits of PCA 4,5-dioxygenase (PcaA) from *P. phenanthrenivorans* Sphe3 with other PCA 4,5-dioxygenases of >60% identity. Although PcaA is a monomer, herein was considered as two subunits in order to facilitate the sequence comparisons. The linker sequence is underlined at the N terminus of the β subunit. Preserved histidine residues in all class II dioxygenases are also depicted in boldface. *Grey highlighting* indicates signature sequences for iron ligands observed in crystals of LigAB [39]. *Letters under the sequence* indicate residues as follows: A, nonspecific hydrophobic interaction with PCA; P, specific interaction with hydroxyl or carboxyl moieties of PCA; F, iron-atom coordination; *asterisks* conserved residues; *colons* conservative replacements. *Crosshatches* in A represent amino acids involved in the catalysis while in B they represent iron ligands identified for LigB [39]. The *black diamond* in B represents an absolutely conserved aspartate which provides a backbone contact crucial for delimiting the one side of the active site [14]. Sequences aligned: LigAB, from *S. paucimobilis* SYK-6 (M34835 [20]), PcaA (ADX75303.1, this study), PcmA from *A. keyseri* (AAK16524.1[30]), PmdAB-T2 from *C. testosteroni* (AF459635 [15]), ProOab from *P. straminea* (AB127969[17] and LigAB-Rp from *R. palustris* [WP_011160233.1, WP_011160234.1].



Supplementary Fig. S2: SDS-PAGE protein analysis. Purification of overexpressed PcaA from *P. phenanthrenivorans* Sphe3. Lane M, protein molecular mass marker in kDa; lane 1, cell-free crude extract from *E. coli* BL21/ pET29c::pcaA; lane 2, Ni⁺-NTA column chromatography flow-through; lane 3, purified PcaA protein (48 kDa).



Supplementary Fig. S3. *In situ* monitoring of the biotransformation of PCA by PcaA, inside the NMR tube ¹H NMR spectra (a) 0 hours and (b) 24 hours after the addition of substrate.



Supplementary Fig. S4. 2D TOCSY NMR spectrum of keto-enol forms of 4-carboxy-2-hydroxymuconate-6-semialdehyde reaction product (number of scans = 64, mixing time = 0.06s, total experimental time = 4h 40min).

The keto/enol equilibrium of the CHMS product was described by the equilibrium constant K_{eq}

$$K_{eq} = [\text{enol}]/[\text{keto}] \quad (1)$$

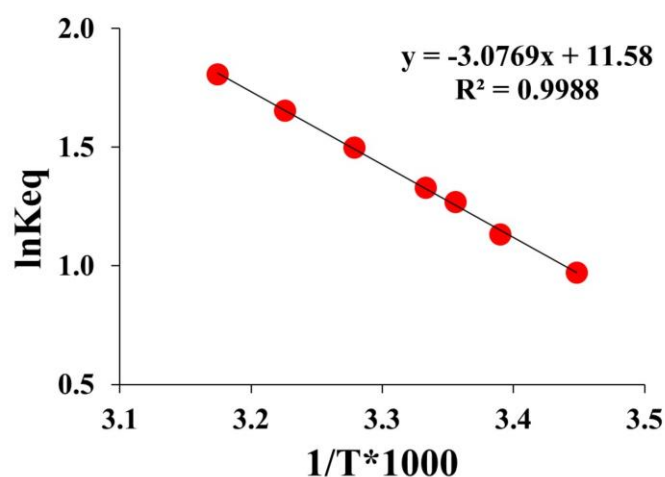
which is directly related to the Gibbs energy ΔG°

$$\Delta G^\circ = -RT \ln K_{eq} \quad (2)$$

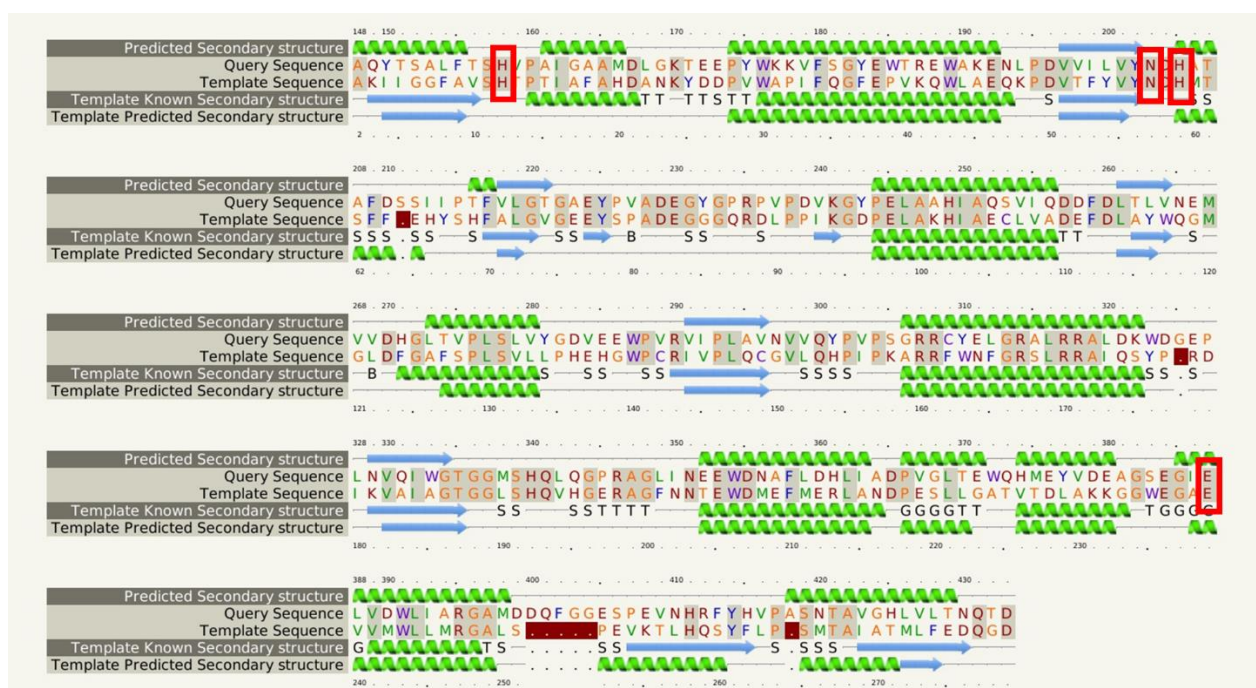
Once the equilibrium constant can be determined at several temperatures, then, according to the Van't Hoff equation

$$\ln K_{eq} = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (3)$$

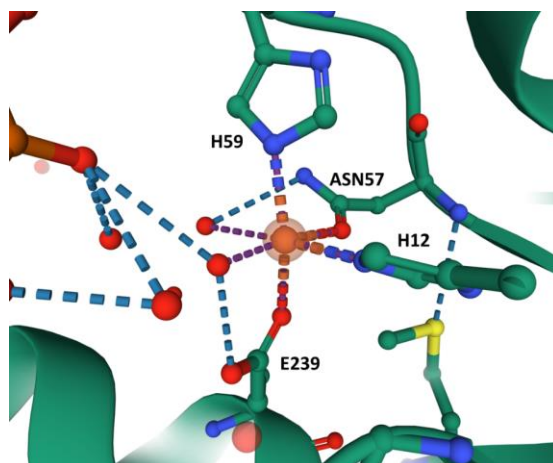
the ΔH° and ΔS° terms can be obtained by the plot of $\ln K_{eq}$ versus $1/T$. The line represents the best fit to eqs (2) and (3) with a correlation coefficient of 0.9988.



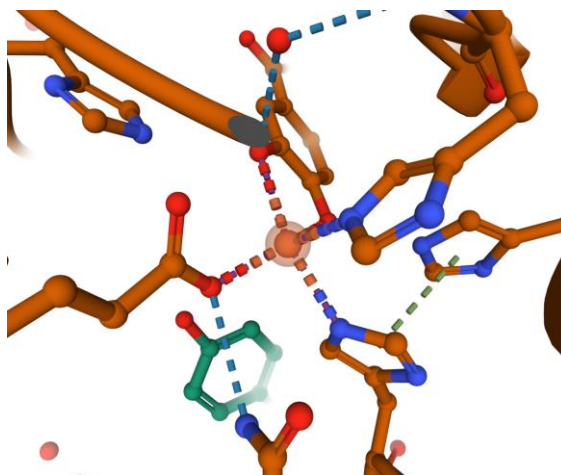
Supplementary Fig. S5. Representative Van't Hoff plot based on the enol/keto equilibrium NMR integration data of H3 protons in NMR buffer 90% H_2O (50mM Tris-HCl, 50mM NaCl, 0.25mM DTT, pH=8) with 10% $DMSO-d_6$. The solid line represents the best fit to eq (3).



Supplementary Fig. S6: Sequence alignment of PcaA to the sequence of DesB. DesB is a type II extradiol dioxygenase catalyzing a ring opening reaction of gallate, derived from *Sphingobium* sp. SYK-6 [41]. Residues of DesB that coordinate to the non-heme Fe (II) are boxed (red). Relevant residues are conserved in PcaA and it is expected to share the same coordination geometry for Fe (II) (refer to Figure S7).



Supplementary Fig. S7: Coordination geometry of the non-heme Fe (II) in DesB (pdbid: 3WRB). H12, H59, N57 and E239 are conserved in PcaA to H35 β , N80 β , H82 β and E264 β , respectively.



Supplementary Fig. S8: Coordination geometry of the non-heme Fe (II) in LigAB in complex with PCA (pdbid: 1B4U). H12, H61, N59 and E242 are conserved in PCA to H35 β , H82 β , N80 β and E264 β , respectively.

The pairwise alignment of the PcaA sequence with the sequences of known structures used for the modelling can be found in the [embedded.pdf](#).

Supplementary Table S1: Oligonucleotides used in this study. Highlighted nucleotides

indicate the restriction sites for *Nde*I (5'-CA/TATG-3') and *Xho*I (5'-C/TCGAG-3') that facilitated pET29c cloning.

| Designation | Oligonucleotide sequence | Purpose | Size (bp) |
|-----------------------------------|------------------------------------|----------------|-----------|
| pca45diox _{for} | 5'-CCCGAAGGAGTATCATATGACACTCGAC-3' | pET29c cloning | 1302 |
| pca45diox _{rev} | 5'-ATGCGGTCACTCGAGCTGGTTC-3' | pET29c cloning | |
| pca45α _{for} | 5'-ACACCTCGGCACTATTACAC-3' | RT-qPCR | 162 |
| pca45α _{rev} | 5'-CGTTGTACACCAGGATGACG-3' | RT-qPCR | |
| pca _{reg} _{for} | 5'-ACGCCTTGGCACAAATAGAC-3' | RT-qPCR | 178 |
| pca _{reg} _{rev} | 5'-TCGAGCCGTTCTACCTGAAT-3' | RT-qPCR | |
| pcaRT1 _{for} | 5'-AAT ACG TCC GAT CCC ATG AC-3' | RT-PCR | 583 |
| pcaRT1 _{rev} | 5'-TCA TGA GCT TGA GCT GGT TG-3' | RT-PCR | |
| pcaRT2 _{for} | 5'-CATCGACCTTCTACCAAGG-3' | RT-PCR | 450 |
| pcaRT2 _{rev} | 5'-GTTGTCACCAGGCTGTAGCA-3' | RT-PCR | |
| pcaRT3 _{for} | 5'-GATAACGAGGAAGCCAAACG-3' | RT-PCR | 362 |
| pcaRT3 _{rev} | 5'-CGCCTGTACGATGACGTT C-3' | RT-PCR | |
| pcaRT4 _{for} | 5'-GTCGACTACATCCCGCAGAT-3' | RT-PCR | 438 |
| pcaRT4 _{rev} | 5'-CCGATCATCATGTCTCGGTA-3' | RT-PCR | |
| pcaRT5 _{for} | 5'-GAATGGCAGCACATGGAGTA-3' | RT-PCR | 515 |
| pcaRT5 _{rev} | 5'-ACGGAATCTCGACCTGCAC-3' | RT-PCR | |
| pcaRT6 _{for} | 5'-ACGGTATTGAGCTGCAGGAT-3' | RT-PCR | 358 |
| pcaRT6 _{rev} | 5'-CGTCCATCGATGACTTTCAC-3' | RT-PCR | |
| pcaRT7 _{for} | 5'-CACCTGCGTGAGAACATGG-3' | RT-PCR | 470 |
| pcaRT7 _{rev} | 5'-CTCGTCGGCATTCGTAAAG-3' | RT-PCR | |