Supporting Information

Discovery and heterologous expression of unspecific Peroxygenases

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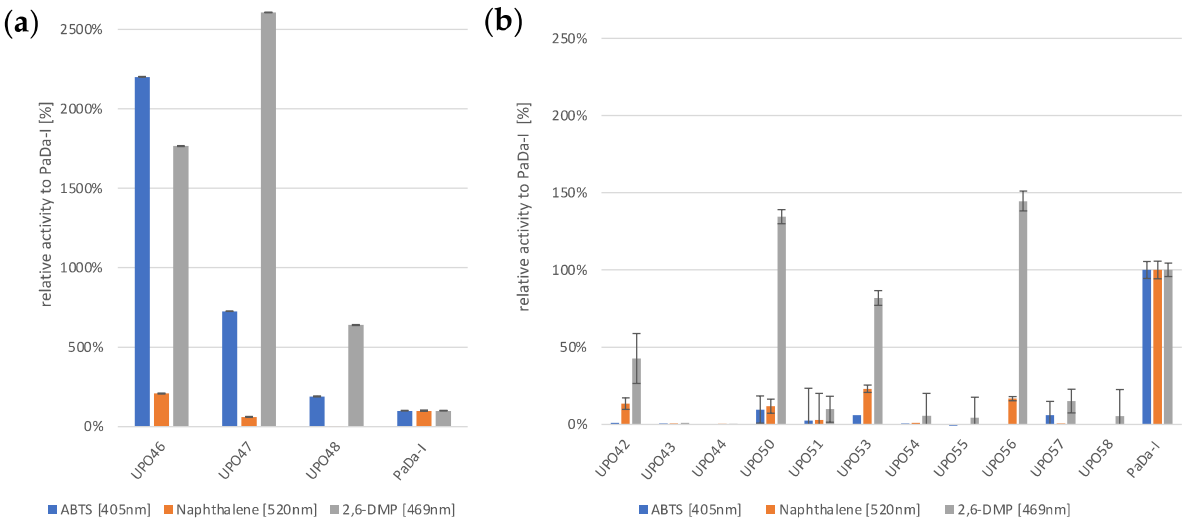

Figure S1. Initial screening of the supernatant of 12 pooled transformants for class II UPOs **(a)** UPO46, UPO47 and UPO48 **(b)** UPO42, UPO43, UPO44 and UPO50-UPO58. For each UPO target twelve individual clones were cultivated and enzymatic activities of the pooled supernatants were determined for substrates ABTS, 2,6-DMP and NBD at 405 nm, 469 nm and 425 nm, respectively. Values are depicted as change of absorbance per time [mAU/min] normalized against values of the PaDa-I, a mutant of the prototype enzyme *Aae*UPO (100%). Error bars represent variance of technical triplicate measurements. All values are corrected by the measurements for the control strain not expressing any target protein. Error bars over 50% omitted for clarity.

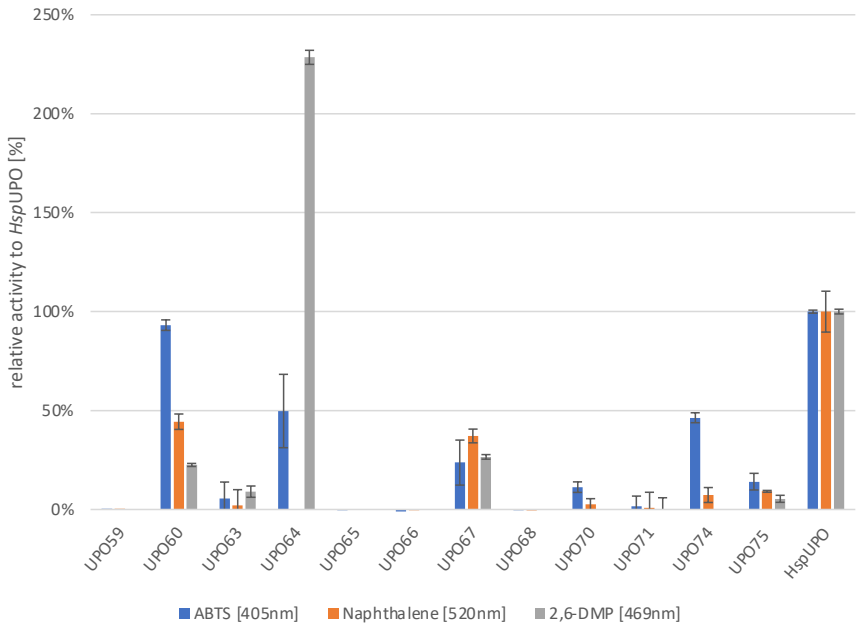

Figure S2. Initial screening of the supernatant of 12 pooled transformants for class I UPOs, UPO59-UPO75. For each UPO target twelve individual clones were cultivated and enzymatic activities of the pooled supernatants were determined for substrates ABTS, 2,6-DMP and NBD at 405 nm, 469 nm and 425 nm, respectively. Values are depicted as change of absorbance per time [mAU/min] normalized against values of the prototype enzyme *Hsp*UPO (100%). Error bars represent variance of technical triplicate measurements. All values are corrected by the measurements for the control strain not expressing any target protein. Error bars over 50% omitted for clarity.

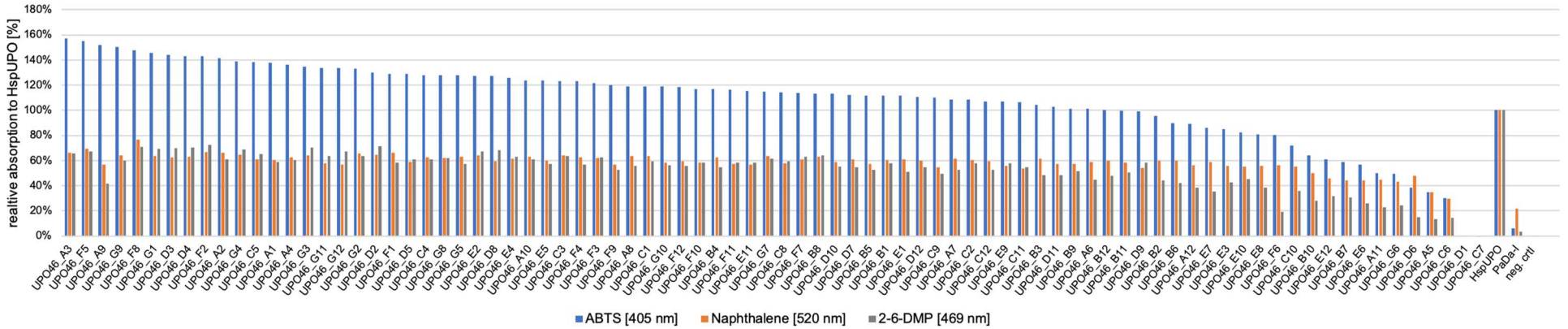

Figure S3. UPO46 landscape sorted according to enzymatic activity for oxidation of ABTS. Values were corrected by the corresponding optical cell density of the culture at 600 nm (OD_{600}) and are depicted as change of absorbance per time [mAU/min] normalised against values of the *Hsp*UPO (100%).

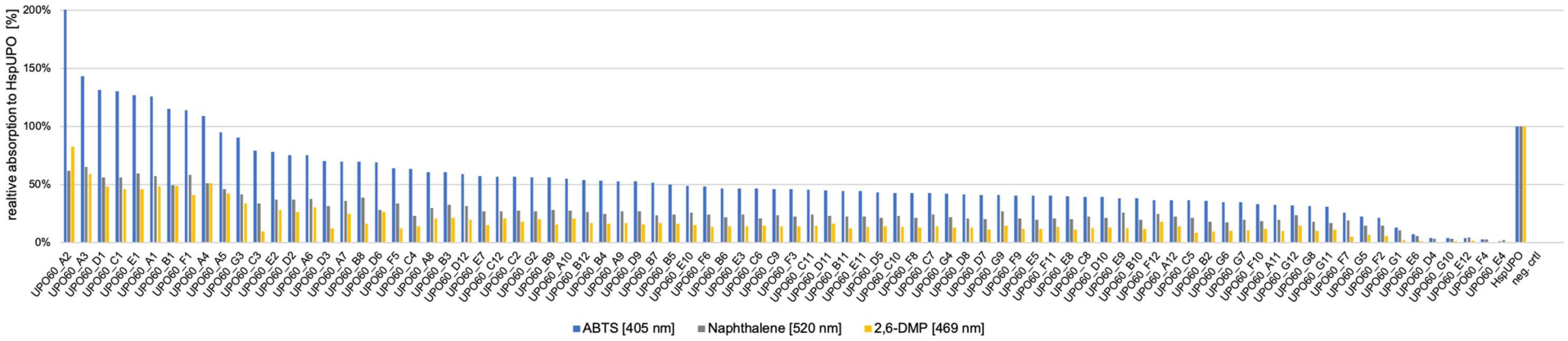

Figure S4. UPO60 landscape sorted according to enzymatic activity for oxidation of ABTS. Values were corrected by the corresponding optical cell density of the culture at 600 nm (OD_{600}) and are depicted as change of absorbance per time [mAU/min] normalised against values of the *Hsp*UPO (100%)

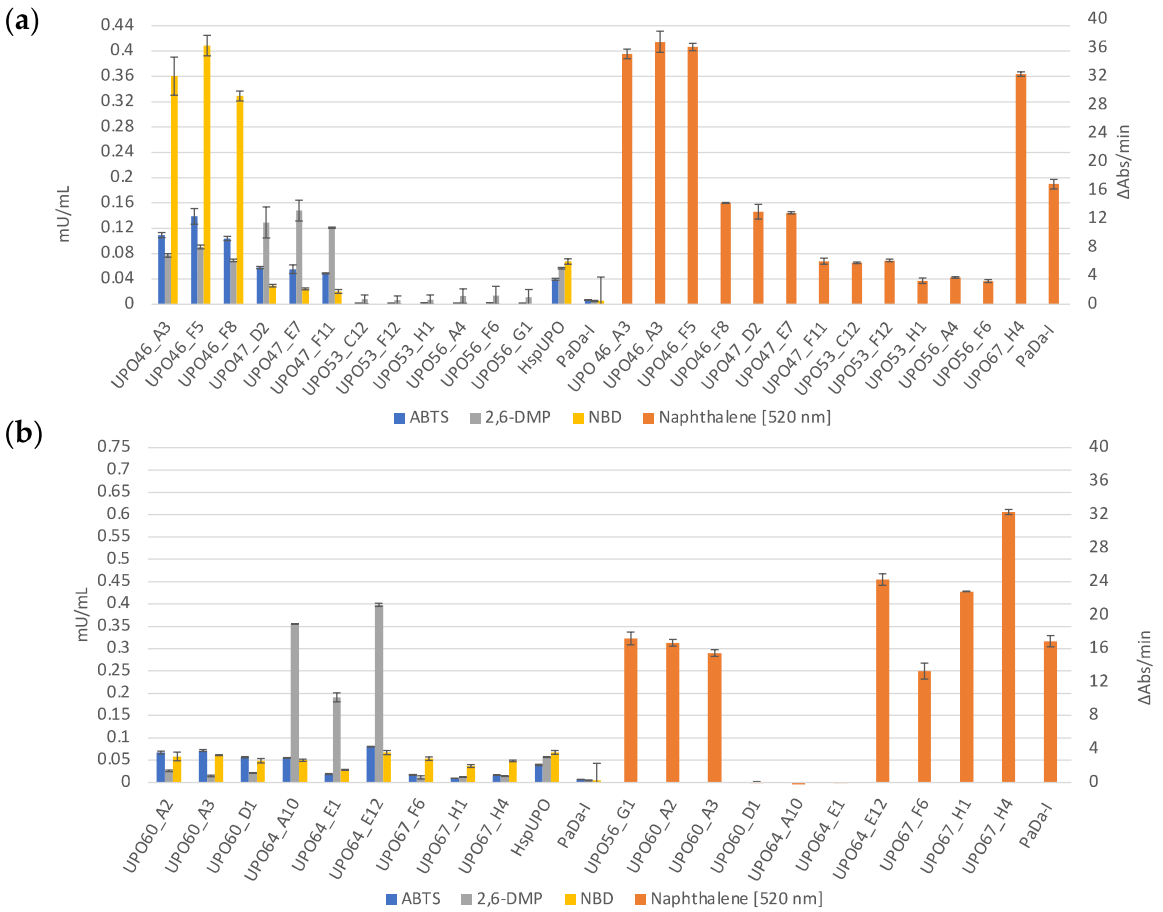

Figure S5. Microscale rescreening of *K. pfaffii* strains expressing selected novel (a) class II (long) and (b) class I (short) UPOs. For each UPO target three different strains were cultivated in biological triplicates and enzymatic activities of supernatants were determined for substrates ABTS, 2,6-DMP and NBD. For substrates ABTS, 2,6-DMP and NBD values are depicted as units volumetric activity in [mAU/mL] using the extinction coefficients of the reaction products, as described in literature (ABTS: ε(405) = 36,000 1/M*cm [33] 2,6-DMP: ε(469) = 27,500 1/M*cm [34], NBD: ε(425) = 9,700 1/M*cm [32]). For conversion of naphthalene, enzymatic activities are gives as change in absorbance at 520 nm over time [ΔAbs/min]. Error bars represent standard deviation of technical triplicate measurements. All values are corrected by the measurements for the control strain not expressing any target protein.

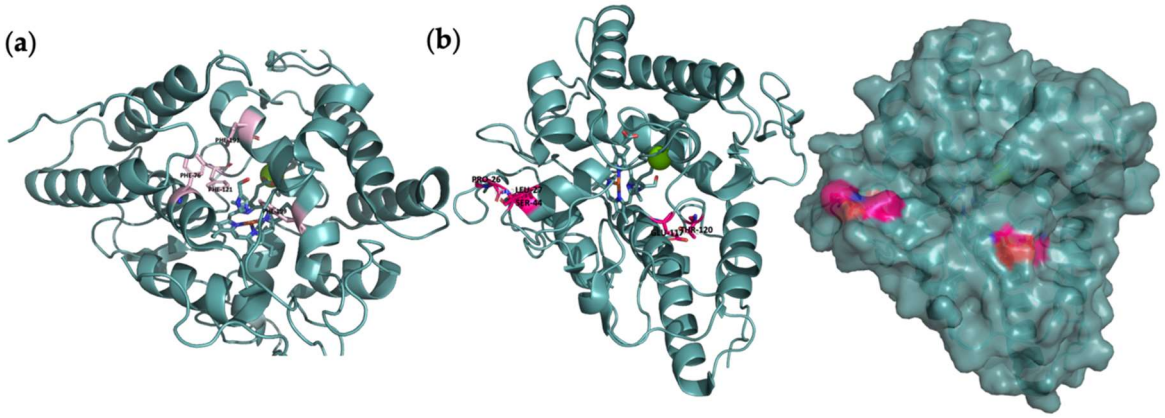

Figure S6. Crystal structure of *Aae*UPO (PDB: 2YORA) as published by Piontek *et al*. [18] with motifs selected for the discovery of novel family II UPOs highlighted. **(a)** M4F motif: F76, F121, F191 and F199 in *Aae*UPO PDB numbering corresponding to 3DM numbering F73, F118, F188 and F196. All residues are on the inside of the protein. **(b)** Positions of the PLSTV motif in the *Aae*UPO; 3DM positions 22, 23, 40, 114 and 117 corresponding to *Aae*UPO PDB numbering P26, L27, S44, E117 and T120. All residues are on the surface of the protein.

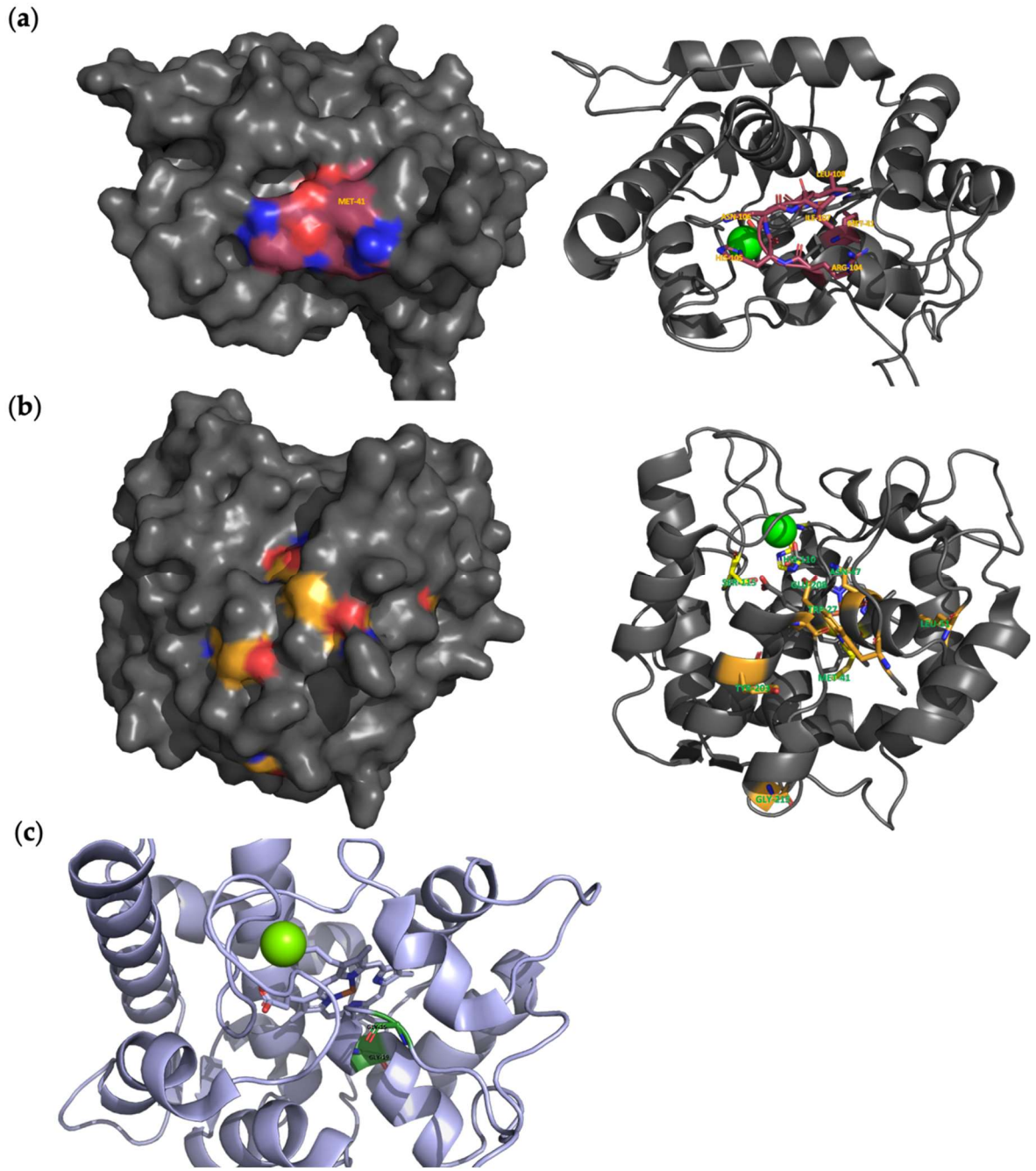

Figure S7. Crystal structure of **(a, b)** *Hsp*UPO (PDB: 7O2G) and **(c)** *Mro*UPO (PDB: 5FUK) as resolved by Rotilio *et al*. [12] and Piontek *et al*. (unpublished), respectively, with motifs selected for the discovery of novel family I UPOs highlighted. **(a)** M34, R114, H115, N116, I117, L118 motif (3DM numbering) shown as M41, R104, H105, N106, I107, L108 in *Hsp*UPO PDB numbering. All residues but M41 are on the surface of the protein. **(b)** WMNLHSYEG motif: in *Hsp*UPO PDB numbering W27, M41, N47, L51, H110, S115, Y203, E208 and G215 corresponding to 3DM numbering W20, M34, N40, L44, H120, S125, Y218 and E223. All residues but M41, H110 and S125 are on the surface of the protein. **(C)** G30, G34 motif: in *Mro*UPO PDB numbering G15 and G19 corresponding to 3DM numbering G30 and G34. All residues are on the inside of the protein.

References

12. Rotilio, L.; Swoboda, A.; Ebner, K.; Rinnofner, C.; Glieder, A.; Kroutil, W.; Mattevi, A. Structural and Biochemical Studies Enlighten the Unspecific Peroxygenase from Hypoxylon Sp. EC38 as an Efficient Oxidative Biocatalyst. *ACS Catal.* **2021**, *11*, 11511–11525. https://doi.org/10.1021/acscatal.1c03065.
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