

Supplementary Information

Structure- and computational-aided engineering of an oxidase to produce isoeugenol from a lignin-derived compound

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Supplementary Methods

Preparative scale conversion of 4-*n*-propylguaiacol

Preparative conversions were performed using both the purified PROGO as well as whole cells. A reaction volume of 60 mL containing 50 mM KPi pH 7.5, 18.5 mg (0.30 μ mol) purified PROGO, 10% (v/v) DMSO, and 0.5 gram (3.0 mmol) 4-*n*-propylguaiacol was added to a 500 mL flask. A 125 mL reaction volume containing 50 mM KPi pH 7.5, PROGO-expressing *E. coli* cells from a 150 mL culture (final OD₆₀₀ = 29), 10% (v/v) DMSO, and 1.27 gram (7.6 mmol) 4-*n*-propylguaiacol was added to another 500 mL flask. Both flasks were placed at 25 °C while shaking (6 x g). Samples were analyzed by HPLC as mentioned before to monitor conversion. After 48 h, both reaction mixtures were extracted with ethyl acetate. The organic layers were washed with water, brine and dried over anhydrous MgSO₄. After removal of the solvent under reduced pressure, the residues were purified by chromatography on a silica gel column with hexane-EtOAc (95/5) to afford isoeugenol as a mixture of *E* and *Z* isomers. The PROGO-catalyzed reaction yielded 328 mg (2.0 mmol, 66%, *E/Z* = 75/25), while the whole-cells catalyzed reaction afforded 524 mg (3.2 mmol, 42%, *E/Z* = 75/25) of isoeugenol as a yellow oil. NMR spectra are shown in Supplementary Figure 3.

¹H NMR (400 MHz, CDCl₃) δ (ppm): ***E*-isomer**: 6.93 – 6.81 (m, 3H, ArH), 6.33 (m, 1H, (C)CH), 6.09 (dq, *J* = 15.6, 6.6 Hz, 1H, CHCHCH₃), 5.61 (s, 1H, OH), 3.90 (s, 1H, OCH₃), 1.87 (dd, *J* = 6.6, 1.3 Hz, 3H, CH₃). ***Z*-isomer**: 6.93 – 6.81 (m, 3H, ArH), 6.33 (m, 1H, (C)CH), 5.71 (dq, *J* = 11.7, 7.2 Hz, 1H, CHCHCH₃), 5.65 (s, 1H, OH), 3.89 (s, 1H, OCH₃), 1.91 (dd, *J* = 7.2, 1.6 Hz, 3H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ (ppm): ***E*-isomer**: 146.52, 144.72, 130.70, 130.63, 123.41, 119.28, 114.32, 107.84, 55.81, 18.32. ***Z*-isomer**: 146.09, 144.24, 130.05, 129.60, 125.13, 122.06, 114.05, 111.38, 55.85, 14.62.

Supplementary List of sequence identifiers

List of sequence identifiers (Uniprot and Uniparc) of the sequences used for the multiple sequence alignment.

query: PDB 5FXD; Q0SBK1 (<https://rest.uniprot.org/uniprotkb/Q0SBK1>); UPI00020EEC90 (<https://rest.uniprot.org/uniparc/UP100020EEC90>); B8M1T5 (<https://rest.uniprot.org/uniprotkb/B8M1T5>); A4XDV9 (<https://rest.uniprot.org/uniprotkb/A4XDV9>); Q140Q7 (<https://rest.uniprot.org/uniprotkb/Q140Q7>); P56216 (<https://rest.uniprot.org/uniprotkb/P56216>); A7F673 (<https://rest.uniprot.org/uniprotkb/A7F673>); C7ZCV5 (<https://rest.uniprot.org/uniprotkb/C7ZCV5>); C7ZAL4 (<https://rest.uniprot.org/uniprotkb/C7ZAL4>); Q2G889 (<https://rest.uniprot.org/uniprotkb/Q2G889>); E1TAI0 (<https://rest.uniprot.org/uniprotkb/E1TAI0>); D5W7A0 (<https://rest.uniprot.org/uniprotkb/D5W7A0>); B6HN16 (<https://rest.uniprot.org/uniprotkb/B6HN16>); C7Z853 (<https://rest.uniprot.org/uniprotkb/C7Z853>); B2JKR1 (<https://rest.uniprot.org/uniprotkb/B2JKR1>); B6QEH9 (<https://rest.uniprot.org/uniprotkb/B6QEH9>); B6QKQ3 (<https://rest.uniprot.org/uniprotkb/B6QKQ3>); E3QPN3 (<https://rest.uniprot.org/uniprotkb/E3QPN3>); 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Supplementary Table 1. Distribution of mutants selected for thermostability according to their final score after visual inspection.

Final score	0	1	2	3	4	n/a
Number of mutants	72	185	185	45	4	5

As described in the experimental procedures, the mutants were assessed based on the physicochemical interactions between the new residues and their environment and the structural variability. A score of 1 was assigned to every detrimental effect observed per mutation. 72 mutations scored 0 and were selected for further analysis. In the last column (n/a), the number of mutants for which MD failed is listed.

Supplementary Table 2. Kinetic and thermostability properties of EUGO variants.

Variants	T_m (°C)	ΔT_m (°C)	K_M (μM) ^a	k_{cat} (s ⁻¹) ^a
Wild-type EUGO	66.5 ± 0.0	-	43.0 ± 2.2	6.8 ± 0.2
H434Y-EUGO	73.0 ± 0.4	+6.5	142.9 ± 12.6	2.7 ± 0.1
H434W-EUGO	70.0 ± 0.0	+3.5	124.5 ± 9.1	1.9 ± 0.1
S81H-EUGO	71.2 ± 0.3	+4.7	44.8 ± 3.4	4.8 ± 0.1
A423M-EUGO	69.2 ± 0.3	+2.7	75.4 ± 3.6	5.3 ± 0.1
S518P-EUGO	69.0 ± 0.0	+2.5	37.6 ± 2.5	4.6 ± 0.1
D202N-EUGO	69.0 ± 0.7	+2.5	7.4 ± 0.3	1.3 ± 0.0
I445D-EUGO	68.5 ± 0.0	+2.0	65.0 ± 6.0	5.7 ± 0.1
EUGO2X (S81H+A423M)	72.0 ± 0.0	+5.5	118.2 ± 10.9	6.8 ± 0.2
EUGO3X (S81H+A423M+ S518P)	73.5 ± 0.0	+7.0	73.1 ± 5.0	5.8 ± 0.2
EUGO4X (S81H+A423M+S518P+I445D)	74.0 ± 0.0	+7.5	92 ± 10.8	6.5 ± 0.3
EUGO5X (S81H+A423M+S518P+I445D+H434Y)	80.0 ± 0.0	+13.5	177.6 ± 15.8	4.8 ± 0.2

^a K_M and k_{cat} values are based on rates determined at 7 different substrate concentrations (each in duplicate); for each average, the error was smaller than 5%. Vanillyl alcohol was used as a substrate.

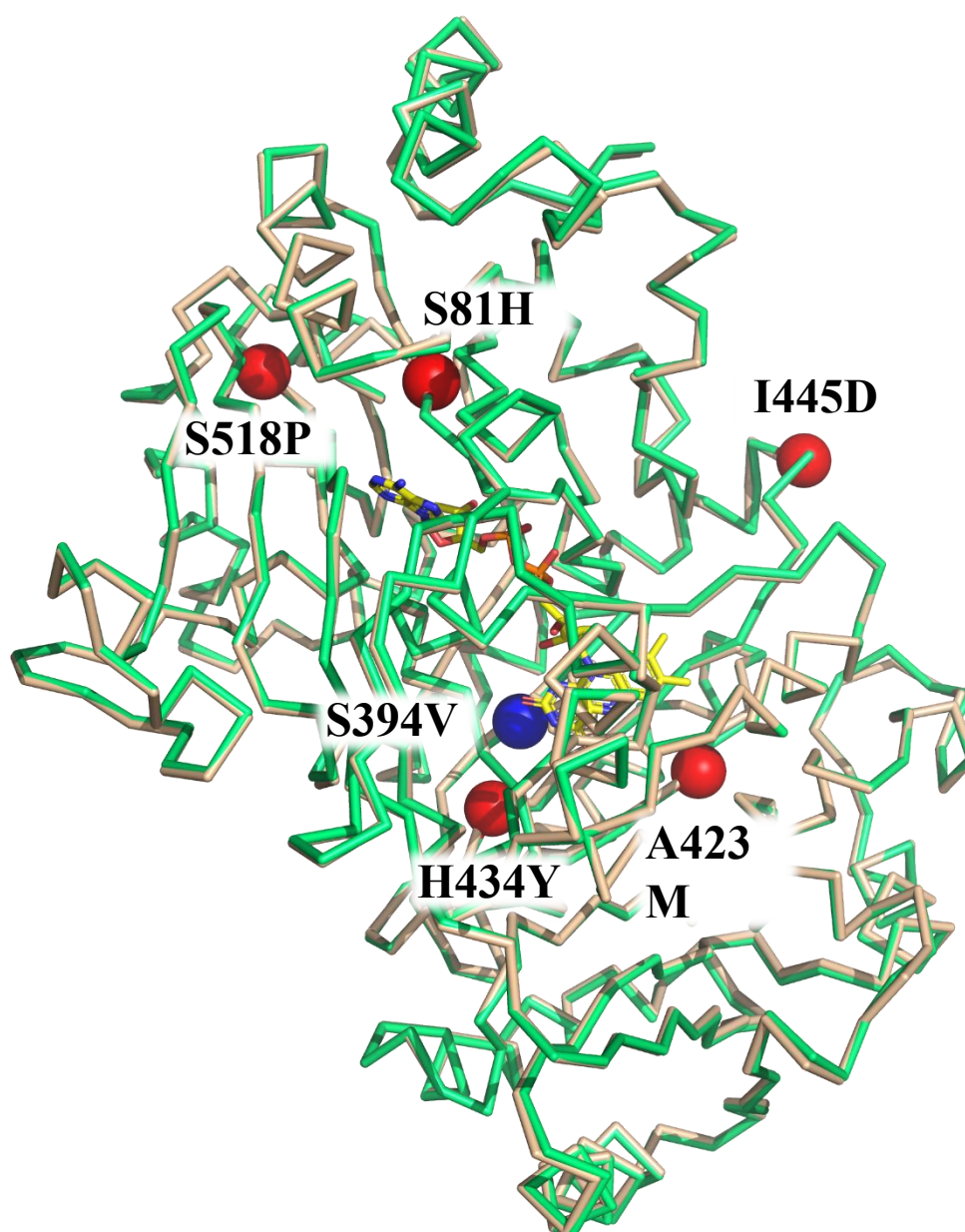
Supplementary Table 3. Data collection and refinement statistics for S394V-EUGO5X and PROGO.

	<i>S394V-EUGO5X</i>	<i>PROGO</i>
Space group	P2 ₁	I2
Unit cell axes (Å)	149.64, 79.43, 189.18	114.19, 142.40, 288.52
Unit cell angles (°)	90, 96.25, 90	90, 100.41, 90
Resolution (Å)	2.8	2.4
PDB code	7YWU	7YWV
R _{sym} ^{a,b}	0.166 (1.099)	0.129 (1.264)
CC _{1/2}	0.974 (0.468)	0.992 (0.466)
Completeness ^b (%)	97.8 (98.8)	99.3 (100.0)
Unique reflections	106726	175685
Redundancy	3.5 (3.7)	3.1 (3.2)
I/ σ ^b	6.6 (1.3)	7.8 (1.1)
N° of non-hydrogen atoms	32569/8x53	33568/8x53
protein/FAD	8x13	8x13
ligand	33	313
water		
Average B value (Å ²)	55.77	58.80
R _{crys} ^c (%)	22.9	22.23
R _{free} ^c (%)	27.4	26.16
Rms bond length (Å)	0.014	0.014
Rms bond angles (°)	1.81	1.77

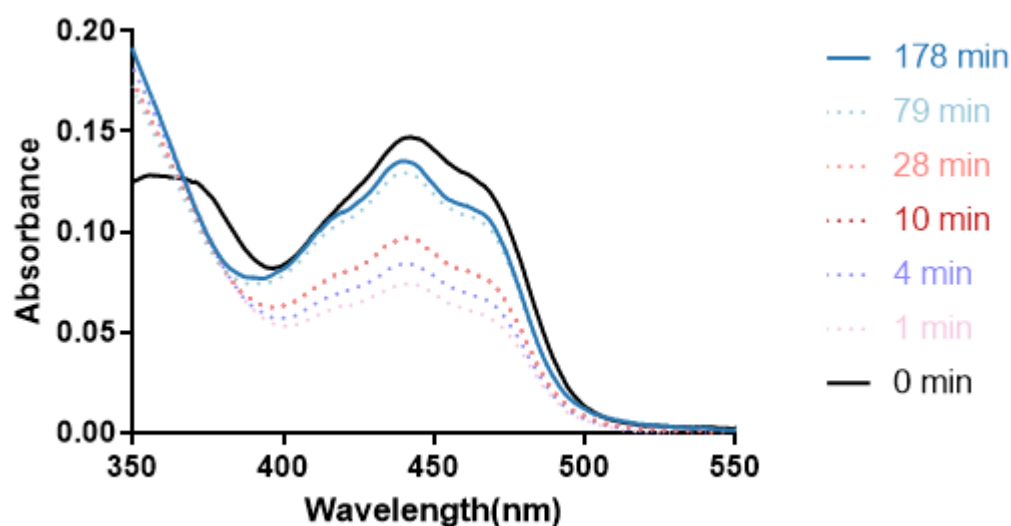
^a $R_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of i^{th} observation and $\langle I \rangle$ is the mean intensity of the reflection.

^b Values in parentheses are for reflections in the highest resolution shell.

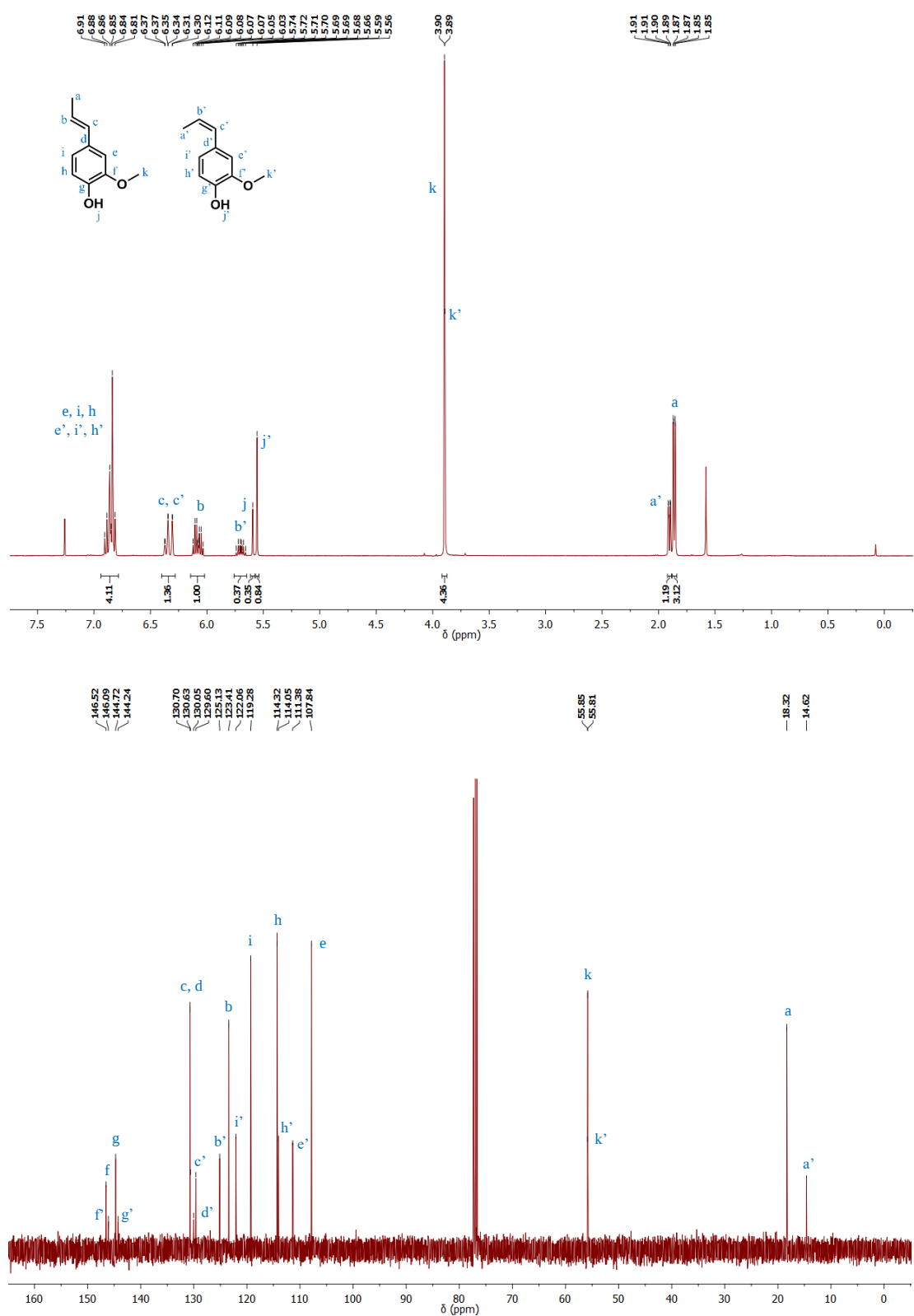
^c $R_{\text{cryst}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively. R_{cryst} and R_{free} were calculated using the working and test sets, respectively.



Supplementary Figure 1. Comparison between wild-type EUGO and S394V-EUGO5X. Superposition of the backbone of wild-type EUGO (wheat) and S394V-EUGO5X (green). The five stabilizing mutation sites are represented with red spheres. The chemoselectivity-affording S394V mutation is indicated as a blue sphere. The FAD is shown in yellow sticks.



Supplementary Figure 2. Time-course absorbance spectra of an incubation of 200 μM 4-n-propylguaiacol with 10 μM S394V-EUGO5X (50 mM KPi, pH 7.5, 25°C). Absorbance spectra were collected at indicated time intervals. The fully oxidized enzyme ($t = 0$) is also included (black line) by collecting the absorbance spectrum before addition of substrate. Upon adding the substrate, the absorbance of the flavin cofactor at around 450 nm is largely reduced, indicative for a partial reduced or modified flavin cofactor. At the end of the incubation, conversion of 4-n-propylguaiacol is complete and the oxidized flavin cofactor is restored.



Supplementary Figure 3. ¹H NMR (top) and ¹³C (bottom) spectra of E/Z isoeugenol isolated from whole-cells catalyzed reaction.