Supporting Information

Systematic Evolution of Decoy Molecules for the Highly Efficient Hydroxylation of Benzene and Small Alkanes Catalyzed by Wild-Type Cytochrome P450BM3

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Materials and methods

All chemical reagents were purchased from commercial sources (e.g. Wako, TCI, Merck and Aldrich) and used without further purification unless otherwise specified. Following Fmoc-amino acids were used for the preparation of 400 Z-dipeptide library: Fmoc-Gly, Fmoc-Ala, Fmoc-Val, Fmoc-Leu, Fmoc-Ile, Fmoc-Pro, Fmoc-Phe, Fmoc-Tyr(*t*Bu), Fmoc-Trp(Boc), Fmoc-Ser(*t*Bu), Fmoc-Thr(*t*Bu), Fmoc-Asn(Trt), Fmoc-Gln(Trt), Fmoc-Lys(Boc), Fmoc-Arg(Pbf), Fmoc-His(Trt), Fmoc-Asp(O*t*Bu), Fmoc-Glu(O*t*Bu), Fmoc-Cys(Trt), Fmoc-Met. $^{12}C_2H_6$ gas (99.99%) were purchased from Taiyo Nippon Sanso Corp. $^{13}C_2H_6$ gas (99 atom%) was purchased from ICON Isotopes.

Measurement

Ultraviolet-visible spectra were recorded on a Shimadzu UV-2600 spectrophotometer and a Shimadzu UV-2450 spectrophotometer. High performance liquid chromatography (HPLC) analyses were performed using an COSMOSIL $5C_{18}$-MS-II column (4.6 mm × 250 mm; nacalai tesque, Inc., Kyoto, Japan) installed on a Shimadzu SCL-10Avp system controller equipped with a Shimadzu LC-10AD pump systems, an SPD-10AVVP UV/Vis Detector, an SIL-20A autosampler, a Shimadzu CTO-10Avp column oven and a Shimadzu DGU-$20A_3$ degasser. Gas Chromatography (GC)-MS analyses were performed with GC-MS-QP2010 SE equipped with a Rtx-1 column (Restek corporation, 60.0 m × 0.32 mm). ^{1}H NMR spectra were measured on a JNM-A400 spectrometer (JEOL). ^{1}H NMR chemical shifts were reported versus tetramethylsilane (TMS) and referenced to residual solvent peaks (DMSO-d_6: 2.50 ppm at 25 °C). ESI-TOF-MS spectra were measured by micrOTOF II (BRUKER ANALYTIC). Microplate was handled by TECAN® Infinite M200 PRO Multimode Microplate Reader (Tecan Ltd.).

Expression and purification of P450BM3

Expression and purification of cytochrome P450BM3 was performed according to previously described methods[1–4]. The details of P450BM3 purification are described below. The purity of protein was checked by SDS-PAGE and the enzyme concentration was determined by pyridine hemochromagen assay.

Escherichia coli cells expressing P450BM3 were suspended in 20 mM Tris-HCl (pH 7.4) and disrupted using an ultrasonicator at 4 °C. After removing cell debris by centrifugation, the supernatant was applied to a CELLUFINE A*500 anion-exchange column (JNC). Weakly bound impurities were removed with 20 mM Tris-HCl containing 50 mM KCl (pH 7.4) and tightly bound proteins including P450BM3 were eluted in 250 mM KCl (pH 7.4) and fractions containing P450BM3 were pooled and desalted by spin centrifugation-dialysis using an Amicon® Ultra Centrifuge Filter Ultracel® (Millipore,Co.) with a MWCO of 30 kDa, followed by further purification using a DEAE 650S anion-exchange column (TOSOH). P450BM3 was eluted in Tris-HCl buffer over a KCl concentration gradient ranging from 0 to 120 mM. Eluted fractions were pooled and concentrated before applying to a Sephacryl S-300 gel-filtration column (GE Healthcare), equilibrated with 20 mM Tris buffer and 100 mM KCl (pH 7.4 and the P450BM3 fraction was collected).

General procedure for the preparation of *N*-substituted-Xaa-Yaa molecules (3CPPA-Pip-Phe as an example)

N-substituted-Xaa-Yaa molecules were synthesized by general solid-phase peptide synthesis method.[5] Details are as following:

Coupling of Yaa to resin

Cl-Trt(2-Cl) resin (50 mg, 1.60 mmol g^{-1}) was dispersed in dichloromethane and left to swell for at least one hour in a reaction vessel. Solvent was removed from the resin and Fmoc-L-Phenylalanine (Fmoc-Phe, 46.5 mg, 0.12 mmol, for Yaa), *N*,*N*-diisopropylethyl amine (DIPEA, 41.8 μL, 0.24 mmol), and dichloromethane (2 mL) were added to resin and the reaction vessel was shaken for 60 min. After the reaction, solvent was removed and the resin was washed thrice with dichloromethane. Methanol/dichloromethane (1/1, v/v) (3 mL) was added to the resin and the reaction mixture was shaken for 40 min. Solvent was removed and the resin was washed with dichloromethane and *N*,*N*-dimetylformamide (DMF) five times each.

Fmoc deprotection

Piperidine/DMF (1/4, v/v) (2 mL) was added to the resin. After 20 min shaking, solution was removed and resin was washed with DMF 10 times.

Coupling of Xaa or *N*-substituent

Fmoc-Pipecolic acid (Fmoc-Pip, 84.3 mg, 0.24 mmol, for Xaa) or 3-cyclopentylpropionic acid (34.1 mg, 0.24 mmol, for *N*-substituent), HOBt•H_2O (54.1 mg, 0.40 mmol), *N*,*N*'-diisopropylcarbodiimide (DIC, 62.6 μL, 0.40 mmol), and DMF (3 mL) were added to the resin and stirred for 90 min. The Kaiser or chloranil test was performed to confirm completion of coupling. When coupling was not completed, double or further couplings were performed until coupling was completed.

Cleavage of *N*-substituted-Xaa-Yaa from the resin

Cleavage was carried out in 4 mL of 20 % AcOH in dichloromethane for at least 2 hours. The resin was separated and washed thrice with dichloromethane (6 mL) and then thrice with MeOH (6 mL). Filtrates were combined and evaporated. When the amino acid residues are protected by any protecting group, following deprotection procedure was performed. Obtained products were used for the screening without further purifications. The compounds which were used for estimation of turnover frequency (TOF), total turnover number and coupling efficiency were characterized by ^{1}H NMR and ESI-MS.

Deprotection of protecting group on *N*-substituted-Xaa-Yaa which contains sidechain-protected Xaa or Yaa.

Cleaved peptides were treated with deprotecting solution (95 % trifluoroacetic acid (TFA), 2.5 % triisopropylsilane, 2.5 % water, 3 mL) for 3 hours. Solution was evaporated and obtained products were used for screening without further purifications.

N-(3-cyclopentyl)propanoyl-L-pipecolyl-L-phenylalanine (3CPPA-Pip-Phe):
^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.88 (1H, d, J = 7.6 Hz), 7.40-7.23 (5H, m), 4.50 (1H, d, J = 12.8 Hz), 4.40 (1H, t, J = 15.4 Hz), 3.12 (2H, t, J = 8.2 Hz), 2.50 (2H, d, J = 6.2 Hz), 2.10-0.86 (19H, m). ESI-MS: *m/z* 401.24 ($[M+H]^+$), 423.23 ($[M+Na]^+$), 445.21 ($[M-H+2Na]^+$), 823.46 ($[2M+Na]^+$), 801.48 ($[2M+H]^+$), 845.44 ($[2M-H+2Na]^+$), 1223.70 ($[3M+Na]^+$), 1245.68 ($[3M-H+2Na]^+$).

N-(3-cyclopentyl)propanoyl-L-pipecolyl-L-cyclohexylalanine (3CPPA-Pip-Cha):
^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.95 (1H, d, J = 7.2 Hz), 4.53 (1H, d, J = 7.6 Hz), 4.27 (1H, t, J = 12.4 Hz), 3.76-0.82 (32H, m). ESI-MS: *m/z* 407.29 ($[M+H]^+$), 429.27 ($[M+Na]^+$), 451.26 ($[M-H+2Na]^+$), 836.56 ($[2M+Na]^+$), 813.57 ($[2M+H]^+$), 1241.84 ($[3M+Na]^+$).

N-caproyl-L-pipecolyl-L-phenylalanine (C6AM-Pip-Phe):
^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.60 (1H, d, J = 7.6 Hz), 7.26-7.20 (5H, m), 4.50 (1H, d, J = 13.6 Hz), 3.85 (1H, t, J = 9.6 Hz), 3.04 (2H, t, J = 13.2 Hz), 2.39 (2H, t, J = 12.0 Hz), 2.1-0.85 (17H, m). ESI-MS: *m/z* 375.23 ($[M+H]^+$), 397.21 ($[M+Na]^+$), 419.20 ($[M-H+2Na]^+$), 771.43 ($[2M+Na]^+$), 749.45 ($[2M+H]^+$), 793.41 ($[2M-H+2Na]^+$), 1145.65 ($[3M+Na]^+$), 1167.63 ($[3M-H+2Na]^+$).

N-(5-methylcaproyl)-L-pipecolyl-L-cyclohexylalanine (5MHA-Pip-Cha):
^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.91 (1H, d, J = 7.6 Hz), 4.51 (1H, d, J = 12.8 Hz), 4.26 (1H, t, J = 13.0 Hz), 3.36 (2H, t, J = 11.2 Hz), 2.33-0.85 (32H, m). ESI-MS: *m/z* 395.29 ($[M+H]^+$), 417.28 ($[M+Na]^+$), 439.26 ($[M-H+2Na]^+$), 811.56 ($[2M+Na]^+$), 789.57 ($[2M+H]^+$), 833.54 ($[2M-H+2Na]^+$), 1205.84 ($[3M+Na]^+$), 1227.83 ($[3M-H+2Na]^+$).

N-enanthoyl-L-pipecolyl-L-phenylalanine (C7AM-Pip-Phe):
^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.92 (1H, d, J = 8.0 Hz), 7.26-7.17 (5H, m), 4.20 (1H, d, J = 12.8 Hz), 3.52 (1H, d, J = 13.2 Hz), 3.03 (2H, t, J = 10.4Hz), 2.35-0.85 (27H, m). ESI-MS: *m/z* 389.24 ($[M+H]^+$), 411.23 ($[M+Na]^+$), 433.21 ($[M-H+2Na]^+$), 799.46 ($[2M+Na]^+$), 777.48 ($[2M+H]^+$), 821.44 ($[2M-H+2Na]^+$), 1187.70 ($[3M+Na]^+$), 1209.68 ($[3M-H+2Na]^+$).

General procedure for the preparation of combinatorial libraries of 400 Z-dipeptides by split-mix solid phase synthesis

As described in Figure S1, 20 combinatorial libraries of Z-dipeptides, which were used in step one of screening, were prepared by split-mix solid phase synthesis.[6] 20 canonical amino acids were coupled to Cl-Trt(2-Cl) resin (50 mg, 1.60 mmol g^{-1}) in 20 reaction vessels individually as Yaa. After the coupling reaction, all the resin in 20 reaction vessels was combined into one reaction vessel. The mixed resin was shaken for 1 hour in dichloromethane (30 mL). After mixing, the resin was divided into 20 portions. After deprotection of Fmoc-protecting group, 20 canonical amino acids were coupled to resin in 20 reaction vessels individually as Xaa. Deprotection of Fmoc-protecting group, coupling of Z-protecting group by Z-Cl, cleavage of molecules from the resin, and removal of protecting groups on the amino acid residues were executed according to the protocols described above. 20 combinatorial libraries containing 20 molecules each were used for screening without further purification (Figure S2).

Figure S1. Scheme of synthesis of 400 Z-dipeptides.

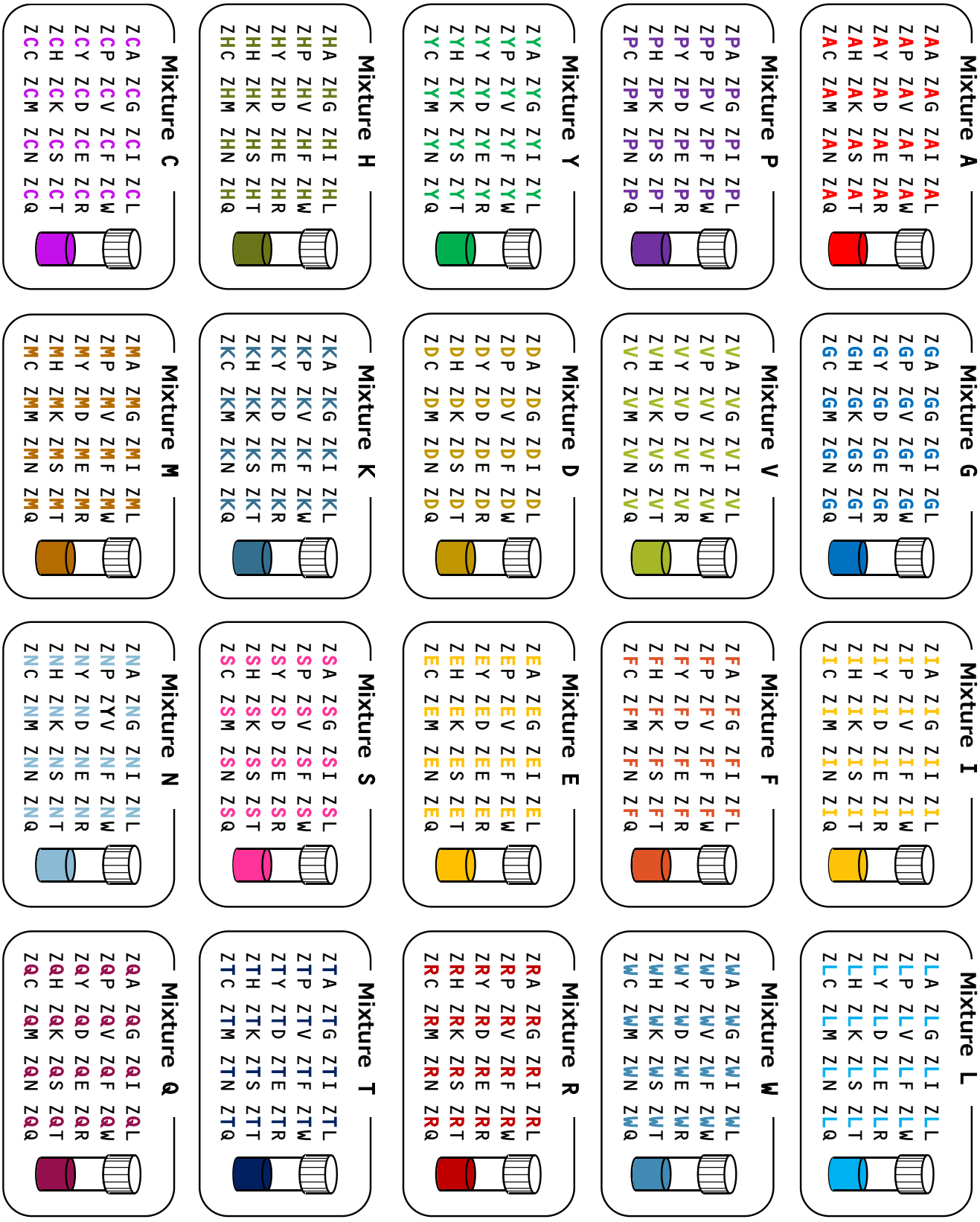

Figure S2. Combinatorial libraries of 400 Z-dipeptides.

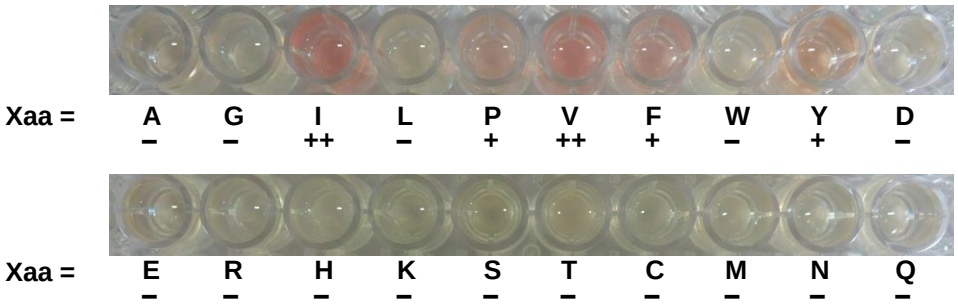

Figure S3. Pictures of colored well plates during the 1st step of screening. When Xaa = isoleucine (I), proline (P), valine (V), phenylalanine (F) or tyrosine (Y), decoy activity was observed.

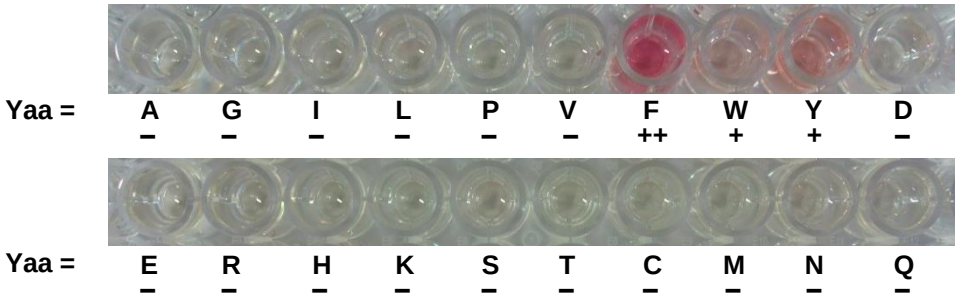

Figure S4. Pictures of colored well plates during the 2nd step of screening. When Yaa = phenylalanine, tryptophan or tyrosine, decoy activity was observed.

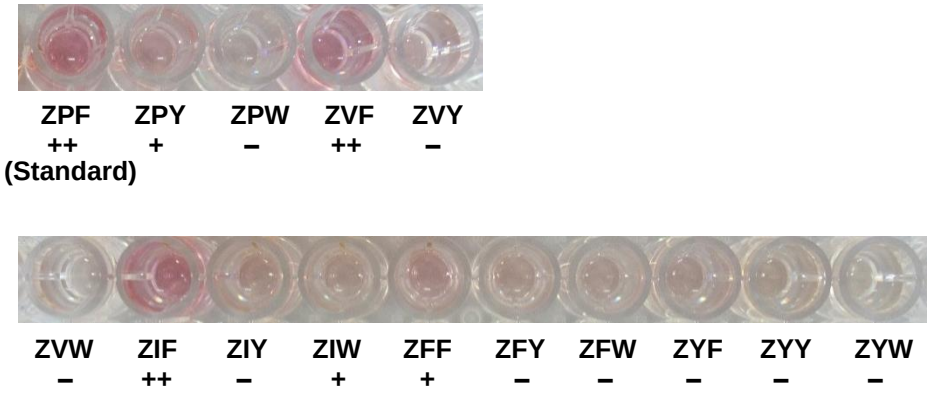

Figure S5. Pictures of colored well plates during the 3rd step of screening. When the combination of Xaa-Yaa = isoleucine-phenylalanine, proline-phenylalanine, or valine-phenylalanine, high decoy activity was observed.

Table S1. Absorbance of each well at 509 nm during the 3rd step of screening.

Decoy molecules	Absorbance at 509 nm	Decoy molecules	Absorbance at 509 nm
Z-Pro-Phe (Standard)	0.3908	Z-Val-Tyr	0.1828
Z-Pro-Tyr	0.2173	Z-Val-Trp	0.1034
Z-Pro-Trp	0.1062	Z-Ile-Phe	0.3493
Z-Val-Phe	0.3467	Z-Ile-Tyr	0.1600
		Z-Ile-Trp	0.1446
		Z-Phe-Phe	0.1967
		Z-Phe-Tyr	0.1653
		Z-Phe-Trp	0.1625
		Z-Tyr-Phe	0.1557
		Z-Tyr-Tyr	0.1227
		Z-Tyr-Trp	0.0911

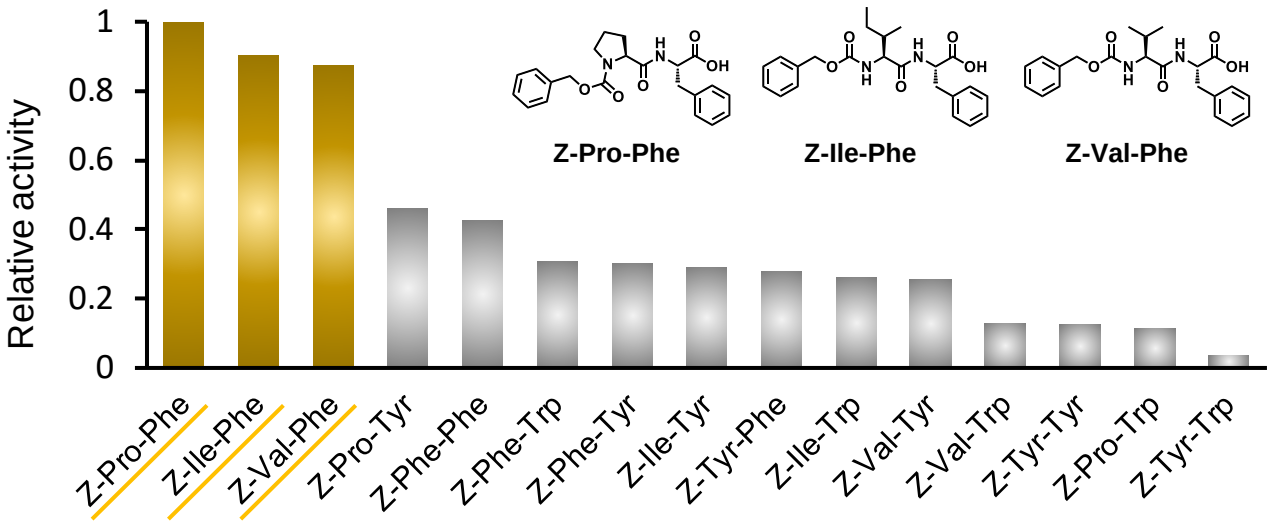

Figure S6. Relative activity of Z-dipeptides calculated from absorbance at 509 nm during the 3rd step of screening. The activity of Z-Pro-Phe is set to 1.

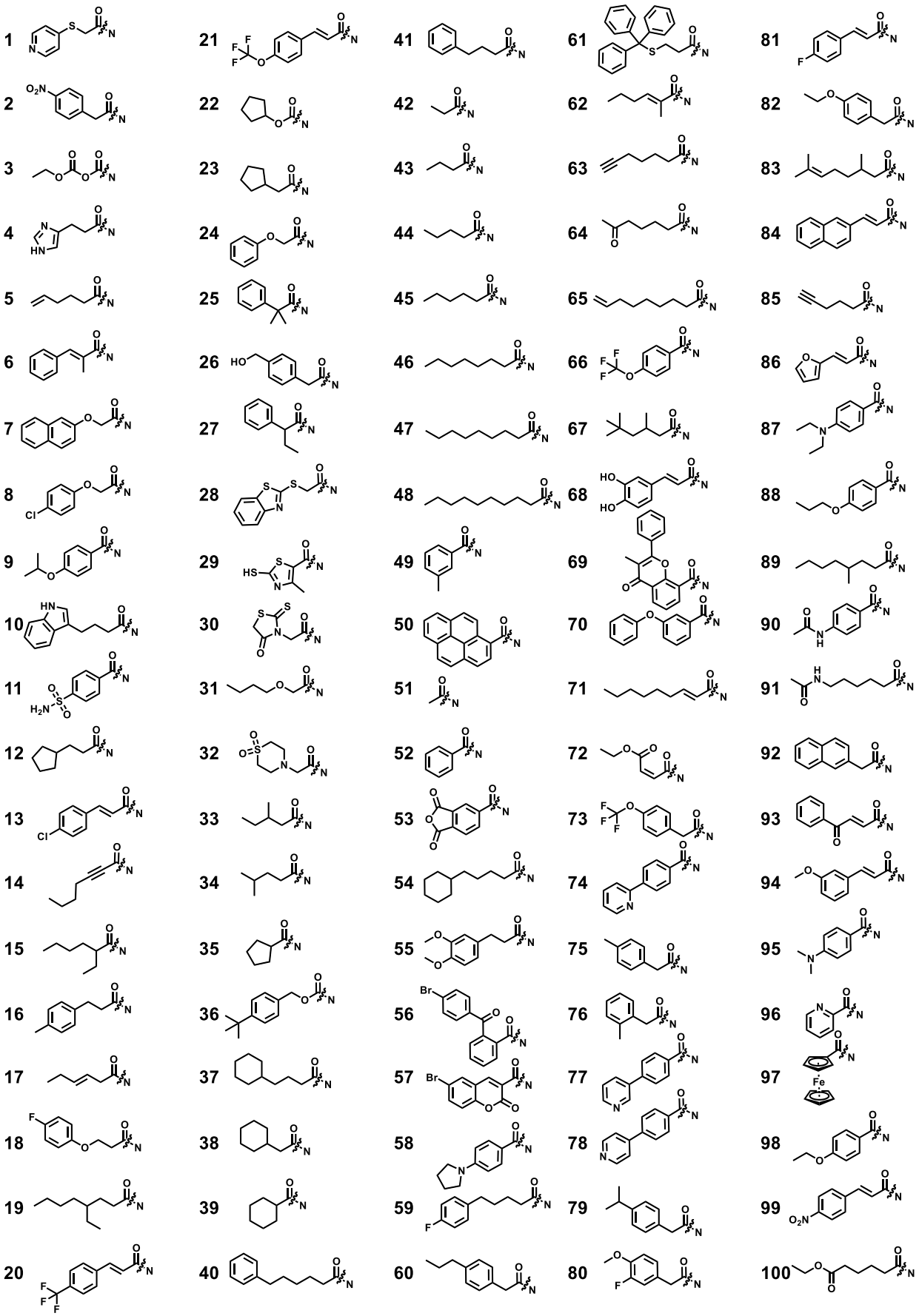

Figure S7. The chemical structures of *N*-substituents employed for the 4th step of screening.

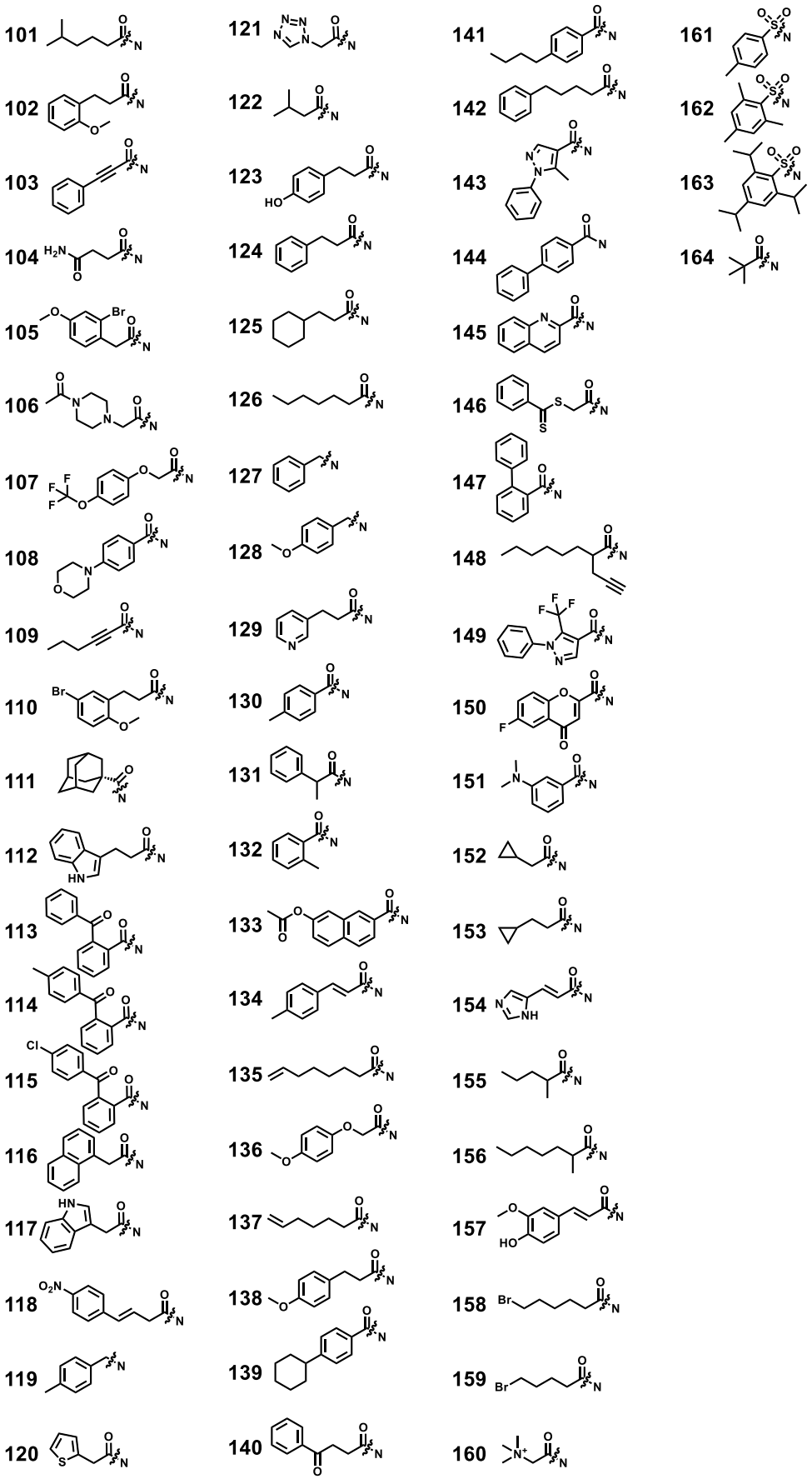

Figure S7. (continued).

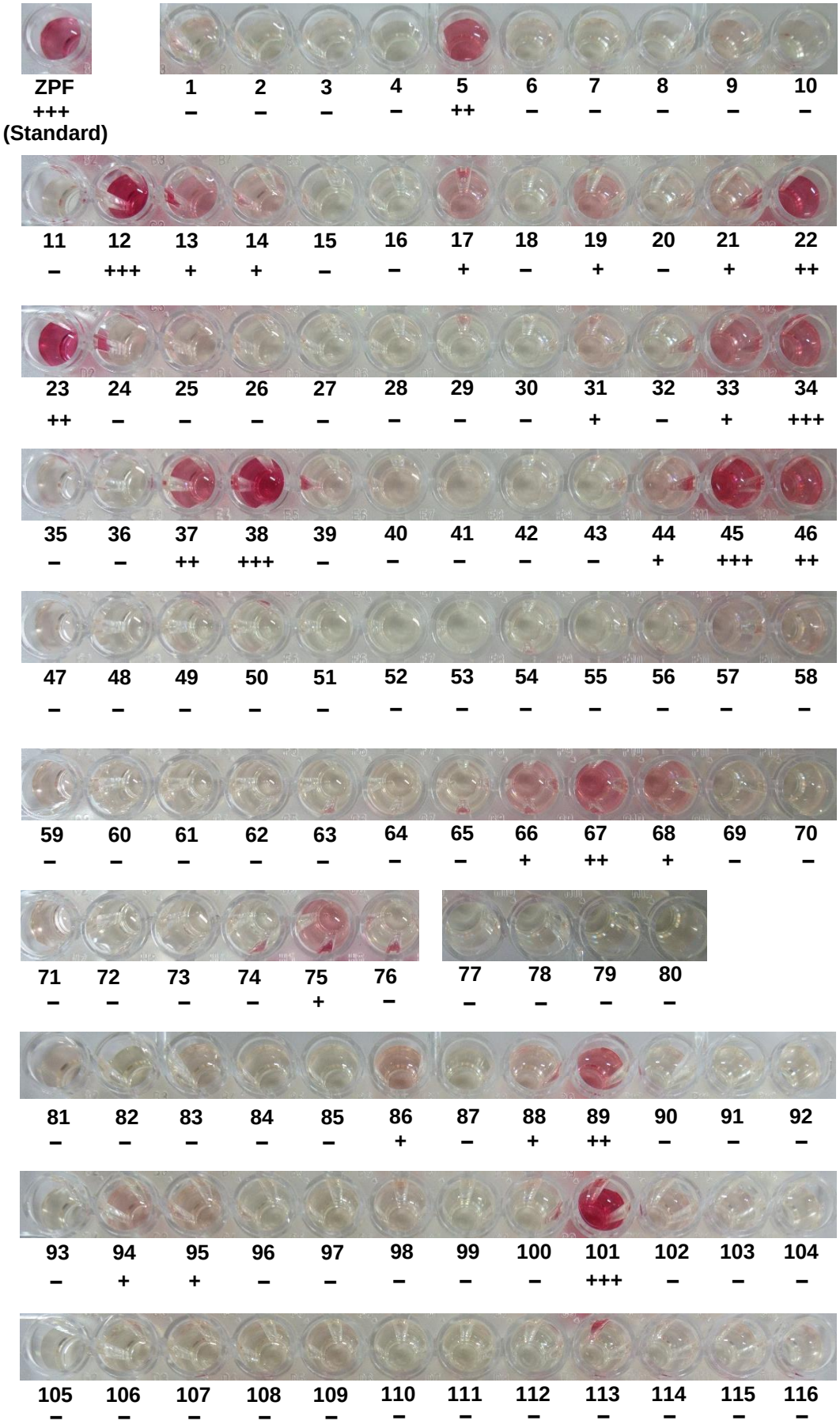

Figure S8. Pictures of colored well plates during the 4th step of screening and chemical structures of effective *N*-substituents

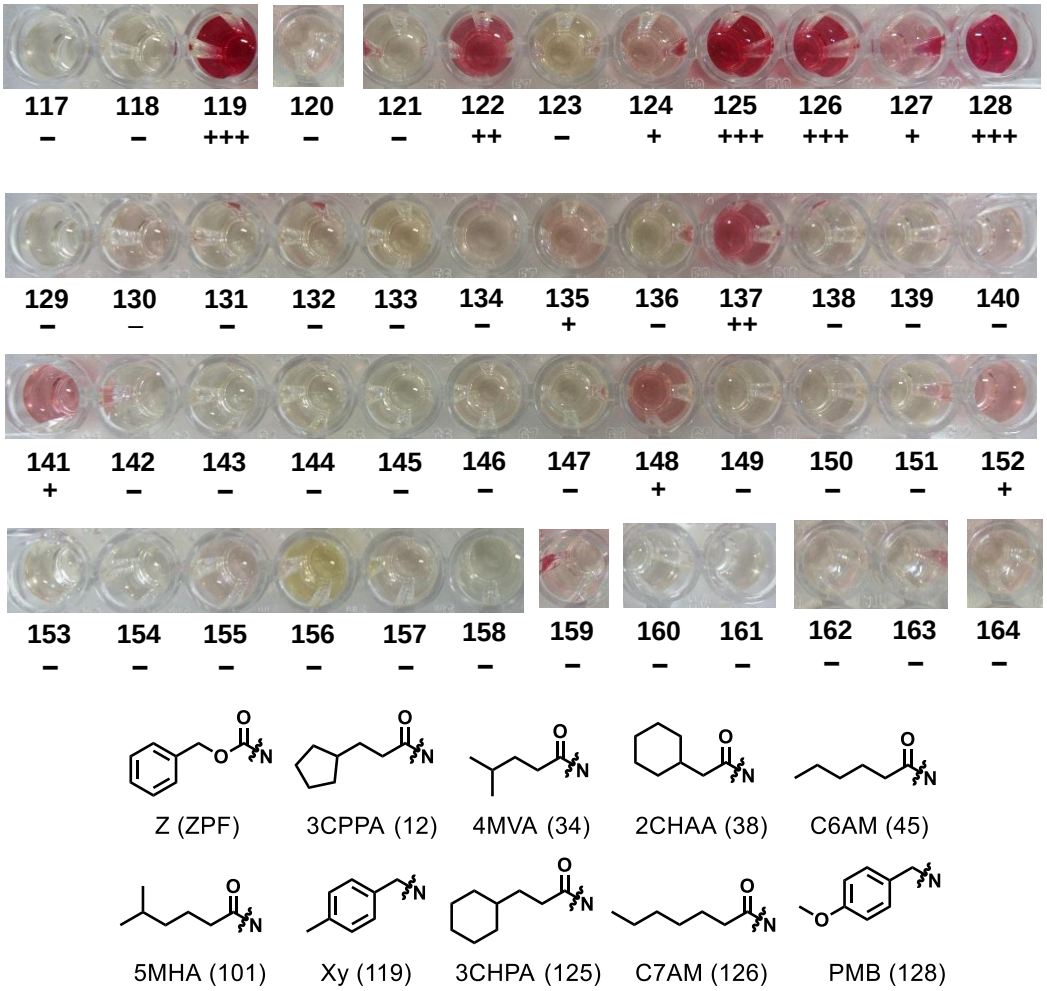

Figure S8. (continued).

Table S2. Absorbance of each well at 509 nm during the 4th step of screening.

Top hits (+++ in FigureS8) are highlighted in pink.

Decoys	Absorbance at 509 nm	Decoys	Absorbance at 509 nm	Decoys	Absorbance at 509 nm	Decoys	Absorbance at 509 nm
1	0.0825	42	0.1158	83	0.1165	124	0.2522
2	0.0814	43	0.0832	84	0.1145	125	1.1966
3	0.0847	44	0.1995	85	0.0965	126	0.9439
4	0.0921	45	0.8135	86	0.2197	127	0.3208
5	0.6129	46	0.5196	87	0.1063	128	1.3022
6	0.0867	47	0.1232	88	0.1643	129	0.1102
7	0.0905	48	0.1036	89	0.5138	130	0.1544
8	0.0818	49	0.0812	90	0.1092	131	0.0978

9	0.1203	50	0.0717	91	0.0829	132	0.1223
10	0.0972	51	0.0801	92	0.0889	133	0.1575
11	0.0843	52	0.0846	93	0.0843	134	0.1496
12	1.1624	53	0.0883	94	0.2246	135	0.2017
13	0.3807	54	0.0849	95	0.1654	136	0.1316
14	0.1899	55	0.0983	96	0.1073	137	0.6078
15	0.0863	56	0.089	97	0.1048	138	0.098
16	0.0761	57	0.1362	98	0.1368	139	0.1172
17	0.2713	58	0.1302	99	0.1111	140	0.1507
18	0.1118	59	0.1502	100	0.1215	141	0.4217
19	0.2957	60	0.1177	101	1.0857	142	0.0951
20	0.0944	61	0.1153	102	0.1348	143	0.0918
21	0.1826	62	0.1049	103	0.1177	144	0.0861
22	0.7000	63	0.1053	104	0.0941	145	0.0841
23	0.4201	64	0.1068	105	0.0895	146	0.1119
24	0.1368	65	0.1093	106	0.0834	147	0.089
25	0.1244	66	0.2951	107	0.0913	148	0.4021
26	0.1159	67	0.4999	108	0.0887	149	0.1229
27	0.0741	68	0.3268	109	0.1143	150	0.1117
28	0.1124	69	0.1056	110	0.0987	151	0.1042
29	0.0698	70	0.0969	111	0.0883	152	0.3411
30	0.0733	71	0.1317	112	0.0645	153	0.0864
31	0.1667	72	0.0915	113	0.09	154	0.1128
32	0.0822	73	0.109	114	0.1036	155	0.157
33	0.3865	74	0.0779	115	0.1028	156	0.1588
34	1.0031	75	0.3342	116	0.1119	157	0.1204
35	0.0976	76	0.0908	117	0.1133	158	0.0814
36	0.0977	77	0.0839	118	0.1165	159	0.0789
37	0.6229	78	0.0917	119	1.6871	160	0.1212
38	1.1201	79	0.089	120	0.1566	161	0.1064

39	0.1392	80	0.0812	121	0.1505	162	0.2622
40	0.1525	81	0.1299	122	0.7727	163	0.0827
41	0.1158	82	0.0944	123	0.151	164	0.0995
						ZPF	1.2596

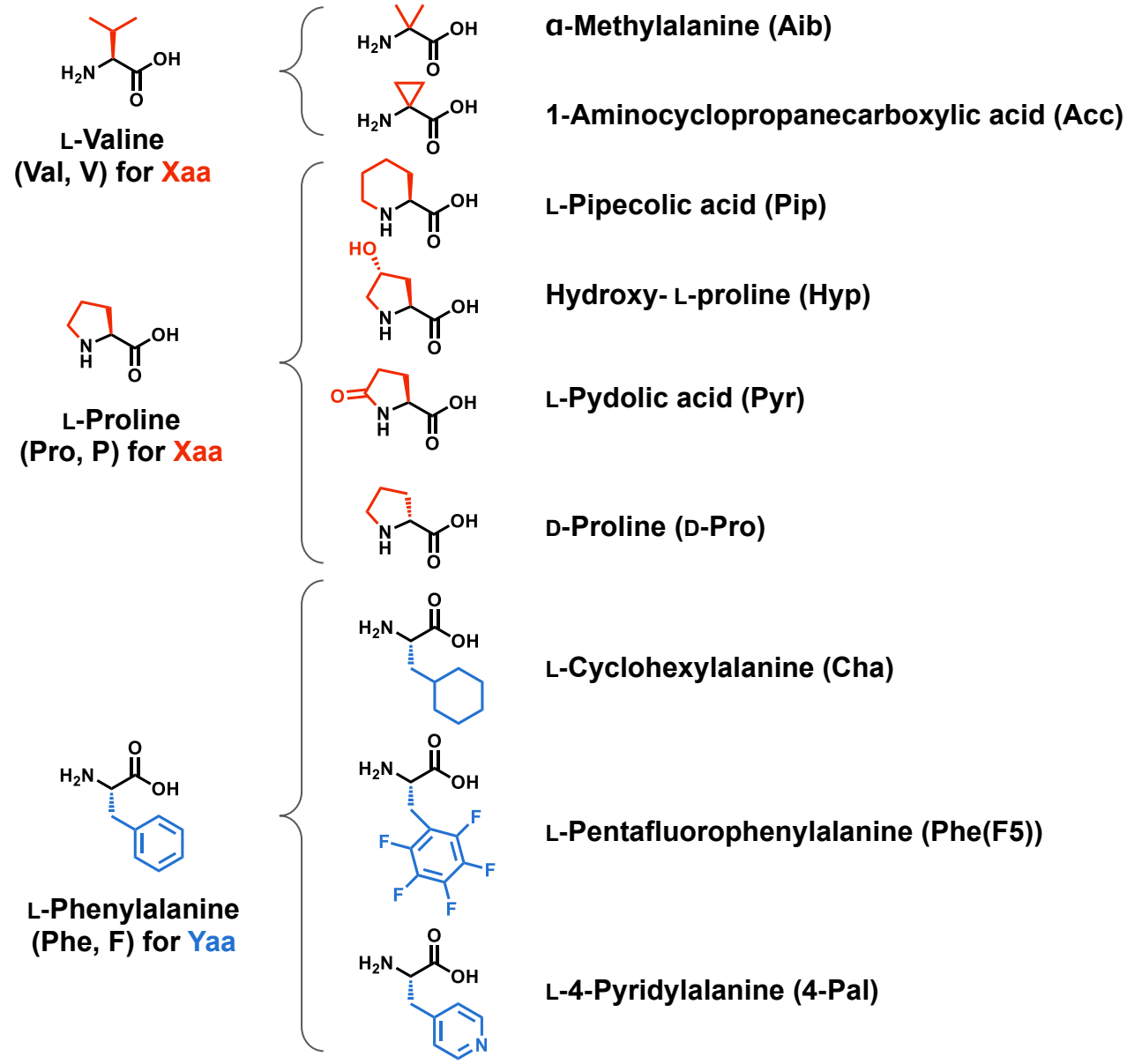

Figure S9. The chemical structures of non-canonical amino acids employed during the 5th step of screening, and correspondence between effective canonical amino acids and employed non-canonical amino acids.

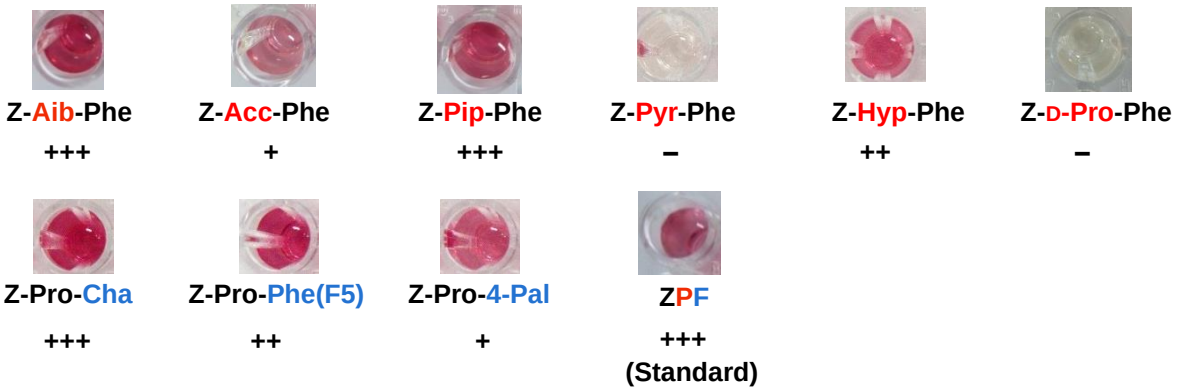

Figure S10. Pictures of colored well plates during the 5th step of screening.

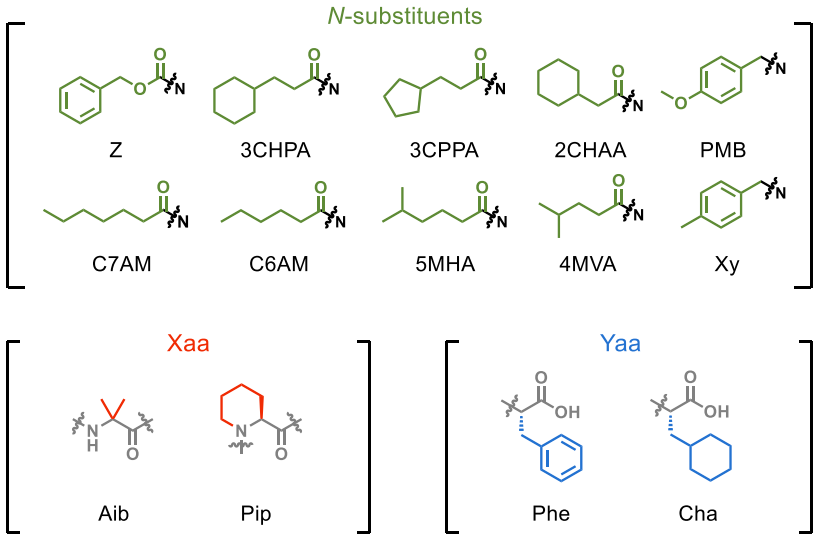

Figure S11. Substructures employed during the 6th step of screening.

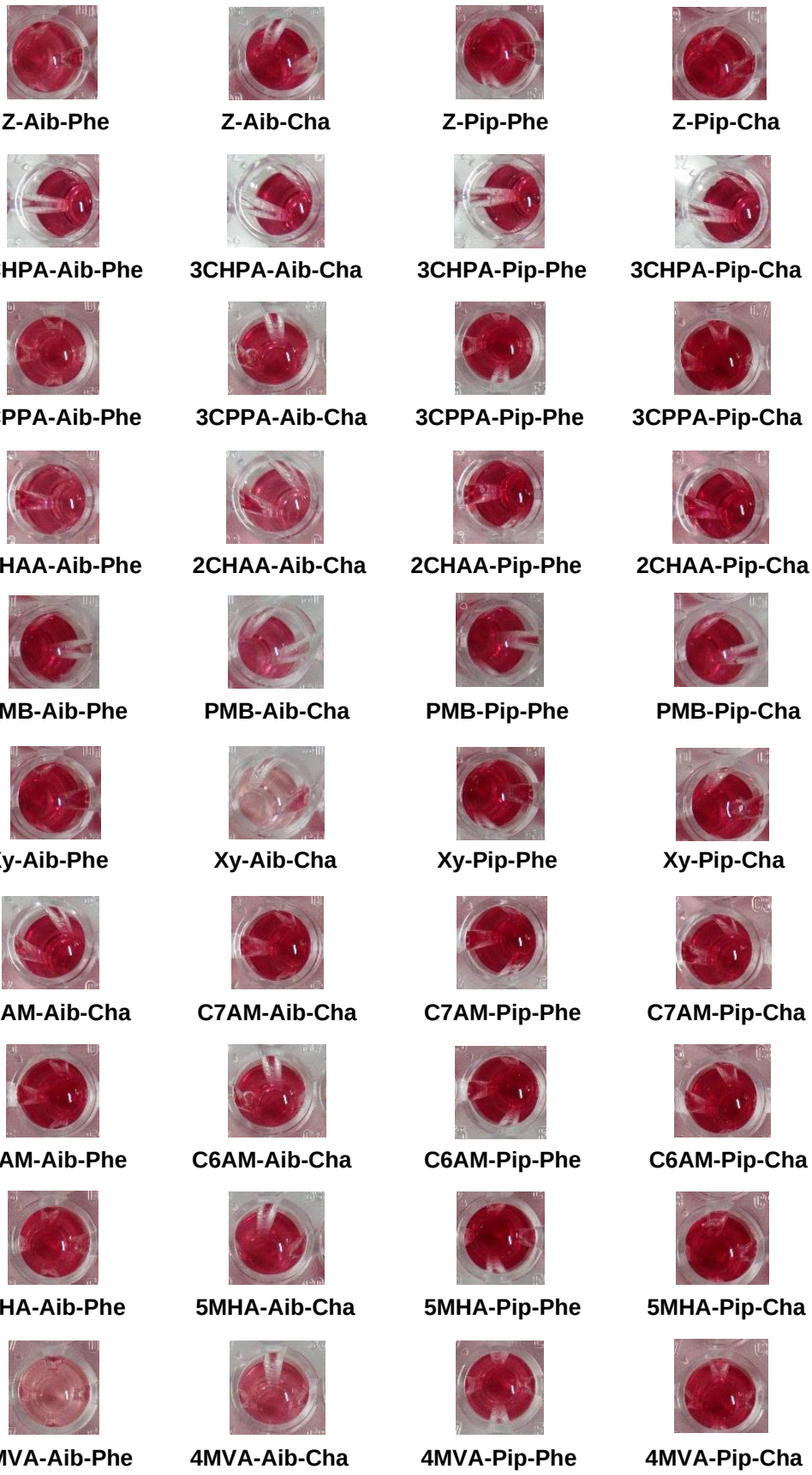

Figure S12. Pictures of colored well plates during the 6th step of screening.

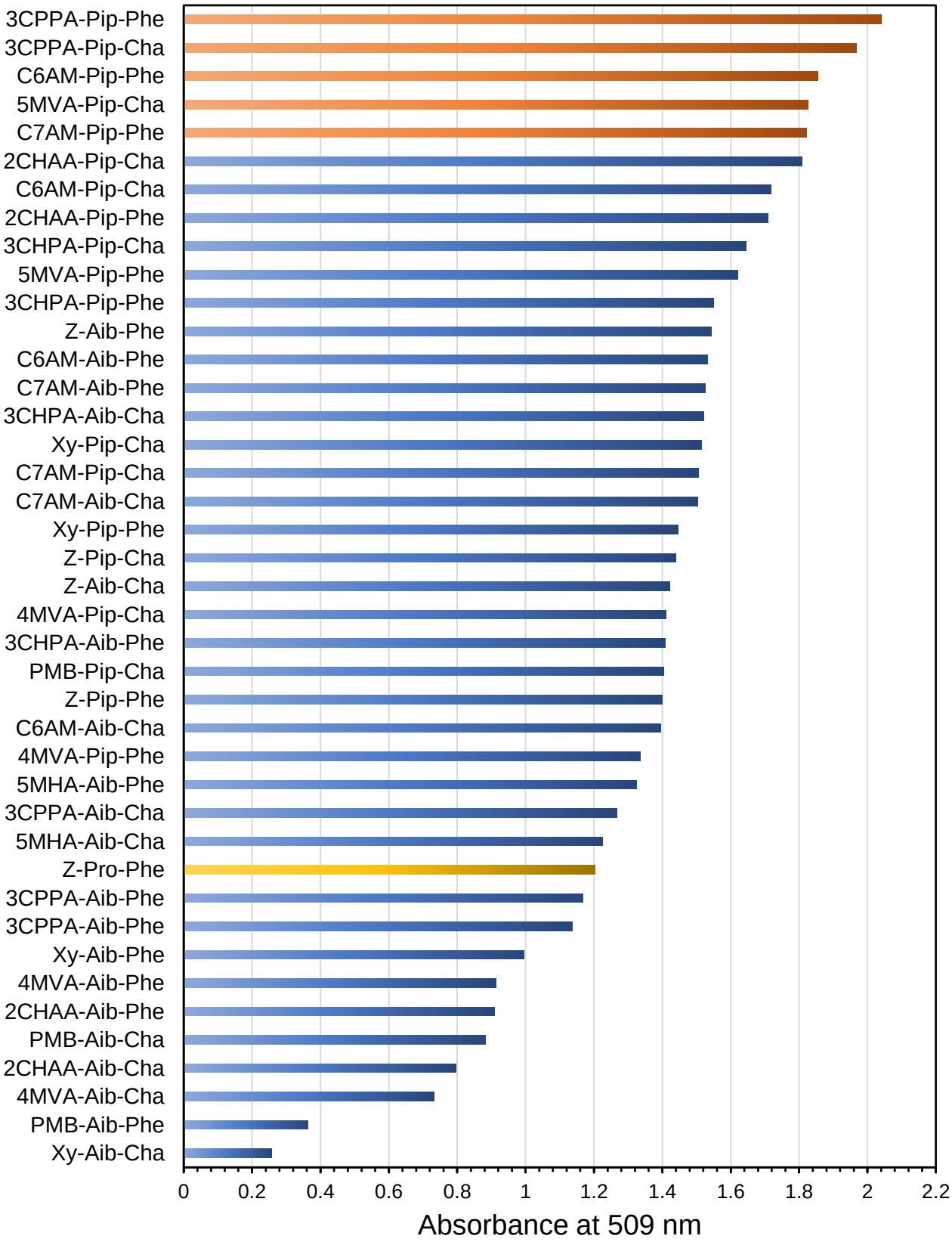

Figure S13. Absorbance of reaction mixtures at 509 nm during the 6th step of screening.

Hydroxylation reactions of non-native substrates

Hydroxylation of benzene

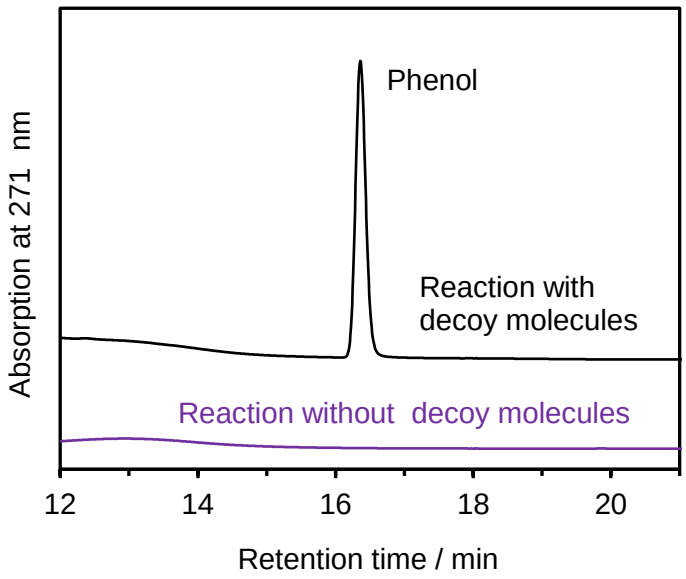

Figure S14. HPLC analysis of the reaction mixture of benzene hydroxylation catalyzed by P450BM3 in the presence (black line) and absence (purple line) of Z-Pro-Phe as a decoy molecule monitored at 271 nm.

Hydroxylation of anisole and toluene for TOF estimation

Hydroxylation of anisole and toluene by P450BM3 was performed according to the reported procedure.[4] Hydroxylation reaction was carried out in 1 mL of 20 mM Tris-HCl (pH = 7.4) buffer containing 100 mM KCl at 25 °C for 10 min in the presence of 0.25 μM P450BM3, 10 mM anisole or toluene, 5 mM NADPH, and 100 μM 3CPPA-Pip-Phe as a decoy molecule in a glass vial. 3CPPA-Pip-Phe was dissolved in DMSO and added to the reaction mixture. Reaction mixture was stirred vigorously. After a 5 min reaction, a solution of hydrochloric acid (1 M) was added to the reaction mixture to quench the reaction and the mixture was then neutralized with a solution of NaOH (1 M). The resulting solution was filtered and analyzed by reversed-phase HPLC. The HPLC analytical conditions were as follows: flow rate of 0.5 mL min^{-1}, acetonitrile/water ratio of 1/1, column temperature of 40 °C, and monitored absorption wavelength at 272 nm or 276 nm for anisole hydroxylation analysis and toluene hydroxylation analysis, respectively. Guaiacol (anisole hydroxylated product) and *o*-cresol (toluene hydroxylated product) were identified using authentic samples. Reaction was performed at least in triplicate. The NADPH consumption was estimated as follows: a 30 μL of reaction mixture after 10 min reaction was diluted 20 times and the absorbance of NADPH at 340 nm was monitored. The concentration of NADPH was calculated using a molar extinction coefficient of 6220 M^{-1} cm^{-1}.

Hydroxylation of cyclohexane for TOF estimation

The reaction was carried out in the same manner as for the hydroxylation of benzene. 200 μL of the reaction mixture was mixed with dichloromethane (200 μL) and *n*-pentanol (5 μL, 20 mM DMSO solution, internal standard). The organic phase was separated. The obtained solution was directly analyzed by GC-MS. The GC-MS analytical conditions were as follows: column temperature 100 °C (3 min hold); 20 °C min^{-1}; 220 °C (6 min hold), injection temperature: 250 °C, interface temperature: 200 °C, ion source temperature: 200 °C, carrier gas: helium. Cyclohexanol was identified using authentic samples. Reaction was performed at least in triplicate. The NADPH consumption was estimated as follows: a 30 μL of reaction mixture after 5 min reaction was diluted 20 times and the absorbance of NADPH at 340 nm was monitored. The concentration of the NADPH was calculated using a molar extinction coefficient of 6220 M^{-1} cm^{-1}.

Hydroxylation of propane for TOF estimation

The reaction was carried out in the similar manner to that for the hydroxylation of benzene, but gas-saturated buffer solution (propane/oxygen: 80/20, v/v) was used for propane hydroxylation. In addition, a propane and oxygen gas balloon (v/v = 80/20) was connected to glass vial to supply propane and oxygen gas. 200 μL of the reaction mixture was mixed with dichloromethane (200 μL) and 3-pentanol (5 μL, 20 mM DMSO solution, internal standard). The organic phase was separated. The obtained solution was directly analyzed by GC-MS. The GC-MS analytical conditions were as follows: column temperature 40 °C (2 min hold); 20 °C min^{-1}; 200 °C (5 min hold), injection temperature: 240 °C, interface temperature: 200 °C, ion source temperature: 200 °C, carrier gas: helium. 2-propanol was identified using authentic samples. Reaction was performed at least in triplicate. The NADPH consumption was estimated as follows; a 30 μL of reaction mixture after 5 min reaction was diluted 20 times and the absorbance of NADPH at 340 nm was monitored. The concentration of the NADPH was calculated using a molar extinction coefficient of 6220 M^{-1} cm^{-1}.

Table S3. Date collection and refinement statics of decoy-bound P450BM3.

	3CPPA-Pip-Phe bound form	3CHPA-Pro-Phe bound form	C7AM-Pro-Phe bound form
PDB code	6L1B	6K3Q	6L1A
Data collection			
Wavelength	1.000	1.000	1.000
Space group	$P\,2_1$	$P\,2_1\,2_1\,2_1$	$P\,2_1\,2_1\,2_1$
Cell dimensions			
a, b, c (Å)	58.85, 148.30, 63.69	58.95, 148.48, 64.48	58.83, 128.43, 148.89
α, β, γ (°)	90.00, 98.61, 90.00	90.00, 99.31, 90.00	90.00, 90.00, 90.00
Resolution (Å)	48.00-1.74 (1.77-1.74)	48.31-2.06 (2.11-2.06)	48.62-1.84 (1.87-1.84)
No. of total observed reflections	841936	471093	1347367
No. of unique reflections	109197	67423	98678
$CC_{1/2}$	0.999 (0.517)	0.997 (0.610)	0.999 (0.543)
R_{meas}	0.126 (1.704)	0.168 (1.720)	0.152 (2.505)
R_{pim}	0.045 (0.609)	0.063 (0.644)	0.041 (0.660)
$< I/\sigma(I) >$	13.0 (1.4)	8.0 (1.3)	13.4 (1.3)
Completeness (%)	99.1 (98.1)	99.6 (94.7)	100.0 (100.0)
Multiplicity	7.7 (7.7)	7.0 (6.9)	13.7 (14.3)
Refinement statistics			
Resolution range (Å)	48.00-1.74	48.31-2.06	48.62-1.84
No. of monomer/asymmetric unit	2	2	2
R_{work}/R_{free} (%)	17.18/20.29	21.01/26.51	19.14/22.58
RMSD bond length (Å)	0.0104	0.0067	0.0097
RMSD bond angles (Å)	1.6382	1.4721	1.6379
No. of atoms	8540	7825	8271
Average B-factor (Å^2)	24.71	38.81	31.44

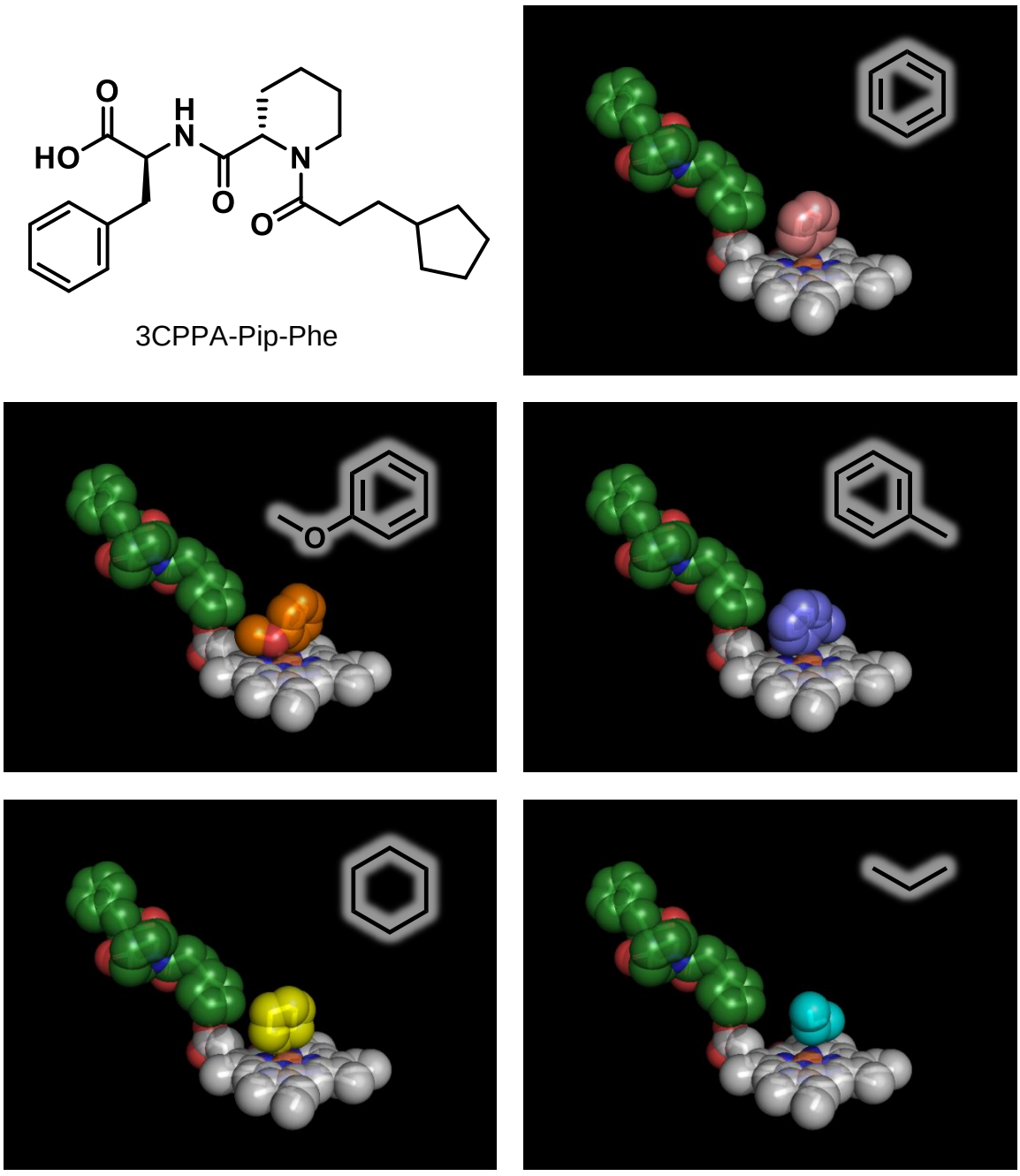

Figure S15. Plausible structures of 3CPPA-Pip-Phe bound form of P450BM3 with benzene, anisole, toluene, cyclohexane, and propane calculated by AutoDock Vina[6] using the crystal structure of 3CPPA-Pip-Phe-bound P450BM3 as a rigid receptor for the docking of substrates.

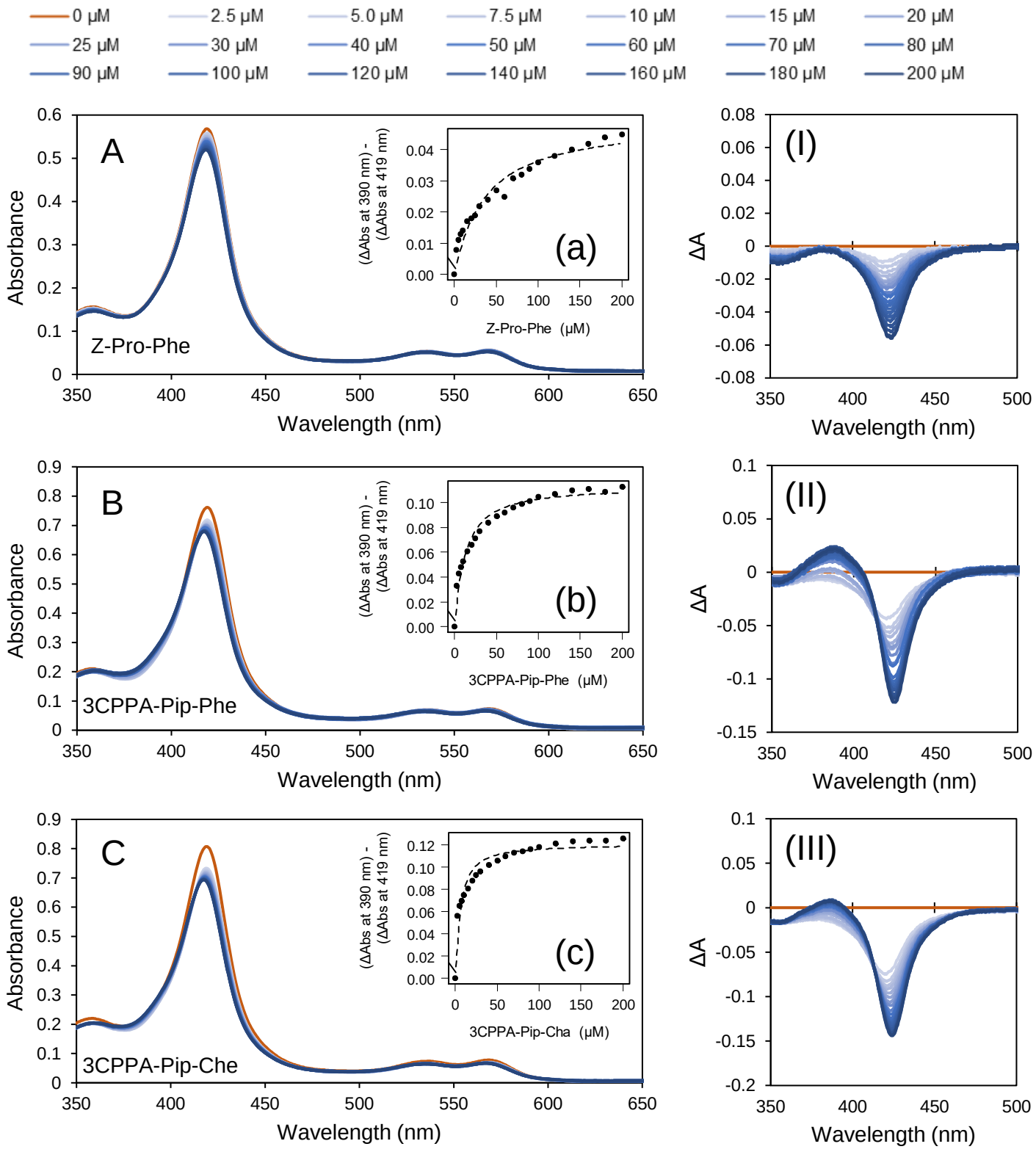

Figure S16. Spectral changes of P450BM3 (4 μM) induced by (A) Z-Pro-Phe, (B) 3CPPA-Pip-Phe, (C) 3CPPA-Pip-Cha, (D) C6AM-Pip-Phe, (E) 5MHA-Pip-Cha, and (F) C7AM-Pip-Phe in a buffer consisting of 20 mM Tris HCl (pH 7.4) and 100 mM KCl at 25 °C. (I) to (VI) depict absorbance changes observed during titration of P450BM3 with each decoy molecule. Inset graphs (a) to (f) depict fitted plots of absorbance changes against the concentration of decoy molecules. Dashed lines represent fitted curves. Titration of P450BM3 was performed according to the reported procedure.[2][4]

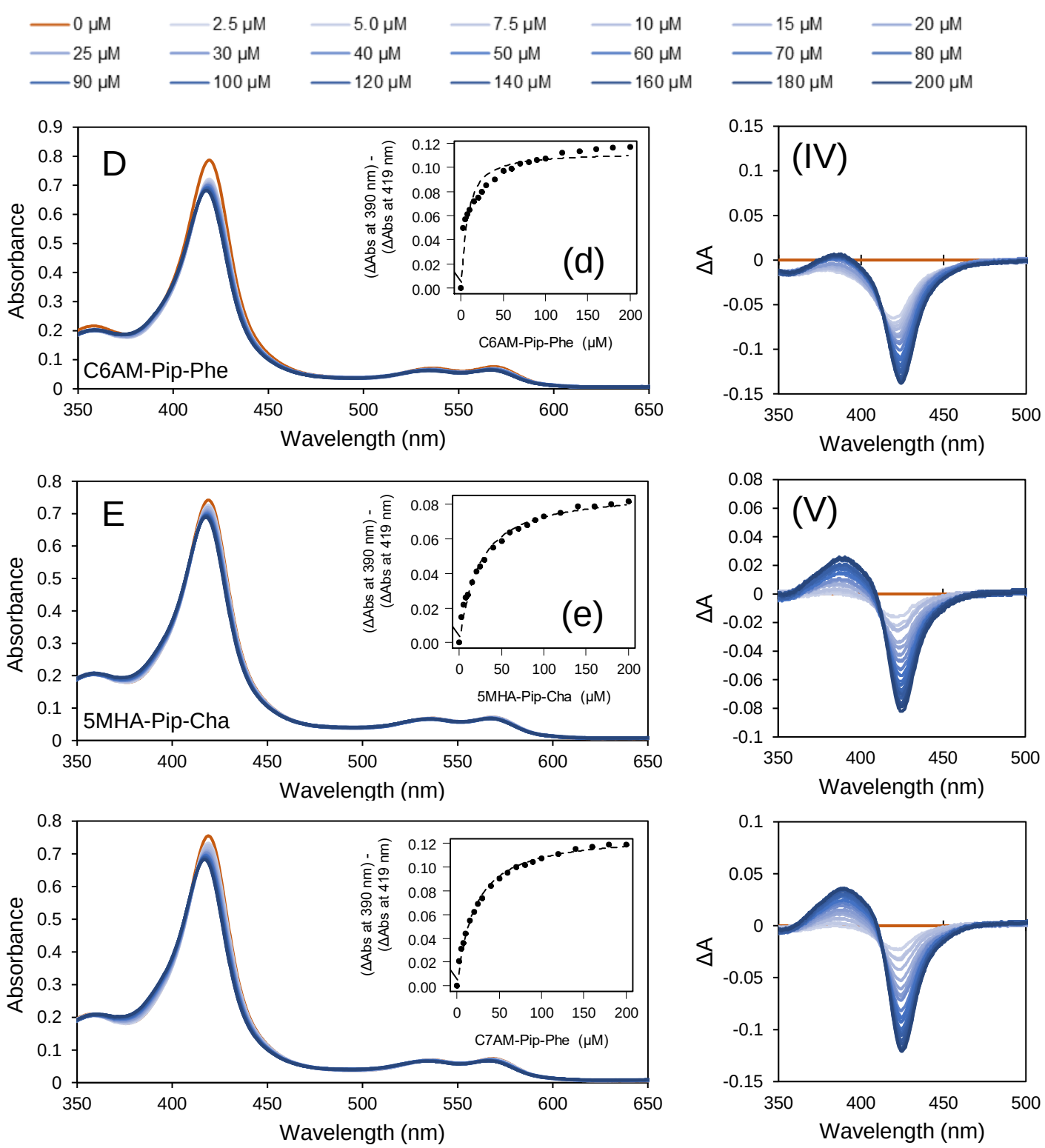
0 μM
2.5 μM
5.0 μM
7.5 μM
10 μM
15 μM
20 μM
25 μM
30 μM
40 μM
50 μM
60 μM
70 μM
80 μM
90 μM
100 μM
120 μM
140 μM
160 μM
180 μM
200 μM
D
C6AM-Pip-Phe
Absorbance
Wavelength (nm)
(ΔAbs at 390 nm) - (ΔAbs at 419 nm)
(d)
C6AM-Pip-Phe (μM)
(IV)
ΔA
Wavelength (nm)
E
5MHA-Pip-Cha
Absorbance
Wavelength (nm)
(ΔAbs at 390 nm) - (ΔAbs at 419 nm)
(e)
5MHA-Pip-Cha (μM)
(V)
ΔA
Wavelength (nm)
Absorbance
Wavelength (nm)
(ΔAbs at 390 nm) - (ΔAbs at 419 nm)
C7AM-Pip-Phe (μM)
ΔA
Wavelength (nm)

Table S4. Dissociation constants of decoy molecules bound to P450BM3.

Decoy Molecule	K_d [μM]
Z-Pro-Phe	34.3
3CPPA-Pip-Phe	10.3
3CPPA-Pip-Cha	4.2
C6AM-Pip-Phe	5.5
5MHA-Pip-Cha	20.4
C7AM-Pip-Phe	18.3

Dissociation constants (K_d) were determined by fitting the plots to the following equation.

$$\Delta A_{390} - \Delta A_{419} = \Delta A_{max} \frac{([E] + [S] + K_d) - \sqrt{([E] + [S] + K_d)^2 - 4 \times [E][S]}}{2[E]}$$

ΔA_{max} is the maximum change in absorbance at infinite decoy molecule concentration. [E] is the total concentration of cytochrome P450BM3. [S] is the concentration of decoy molecule. Dissociation constants (K_d) were determined from titration curves by fitting with the above tight binding equation.

Table S5. TOF and coupling efficiency of ethane hydroxylation catalyzed by wild-type P450BM3 in the presence of decoy molecules. [a]

Decoy Molecule	TOF [min^{-1} $P450BM3^{-1}$][b]	Coupling efficiency [%][c]
PFC9-Phe[d]	28.2 ± 1.8	1.4
Z-Pro-Phe	23.4 ± 2.6	4.5
3CPPA-Pip-Phe	29.8 ± 1.0	1.5
3CPPA-Pip-Cha	27.5 ± 0.9	1.6
C6AM-Pip-Phe	53.9 ± 2.6	3.9
5MHA-Pip-Cha	24.5 ± 1.0	1.4
C7AM-Pip-Phe	82.7 ± 1.1	6.5

[a] Reaction conditions: P450BM3 (0.2 μM), decoy molecule (20 μM), NADPH (5 mM), ethane-pressure (5 MPa), ethane-saturated Tris-HCl buffer (20 mM Tris-HCl, 100 mM KCl, pH 7.4) at room temperature for 10 min. [b] The uncertainty is given as the standard deviation of at least three measurements. [c] ([Product]/NADPH consumption] × 100. [d] Previously reported top decoy molecule for ethane hydroxylation.[7]

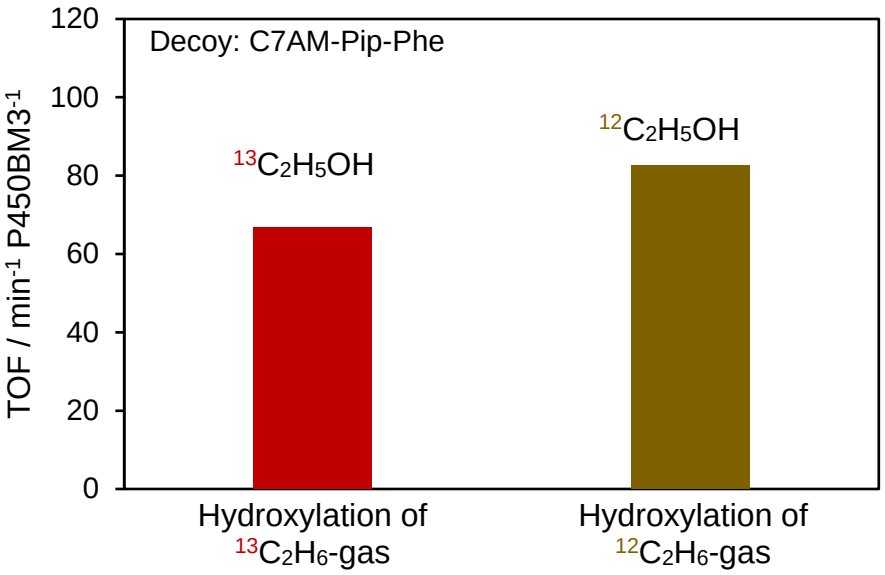

Figure S17. TOF of ethane hydroxylation with C7AM-Pip-Phe as a decoy molecule at 5 MPa-gas (left: $^{13}C_2H_6$, right: $^{12}C_2H_6$). In the left graph, GC-MS signal at MW. 48 was assigned to $^{13}C_2H_5OH$. In the right graph, GC-MS signal at MW. 46 was assigned to $^{12}C_2H_5OH$. The TOF of $^{13}C_2H_5OH$ was slightly smaller when compared to that of $^{12}C_2H_5OH$, which is due to the inhibition by impurities in the gas used for the experiments ($^{12}C_2H_5OH$: 99.99 % purity, $^{13}C_2H_5OH$: 99 atom% purity). Reaction conditions: P450BM3 (0.2 μM), decoy molecule (20 μM), NADPH, (5 mM), gas ($^{13}C_2H_6$ or $^{12}C_2H_6$)-saturated Tris-HCl buffer (20 mM Tris-HCl, 100 mM KCl, pH 7.4), gas ($^{13}C_2H_6$ or $^{12}C_2H_6$)-pressure (5 MPa), at room temperature for 10 min. Intensity of $^{12}C_2H_5OH$ was used as the standard for both experiments.

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