Supporting Information

Tuning peroxidase activity of artificial P450 peroxygenase by engineering redox-sensitive residues

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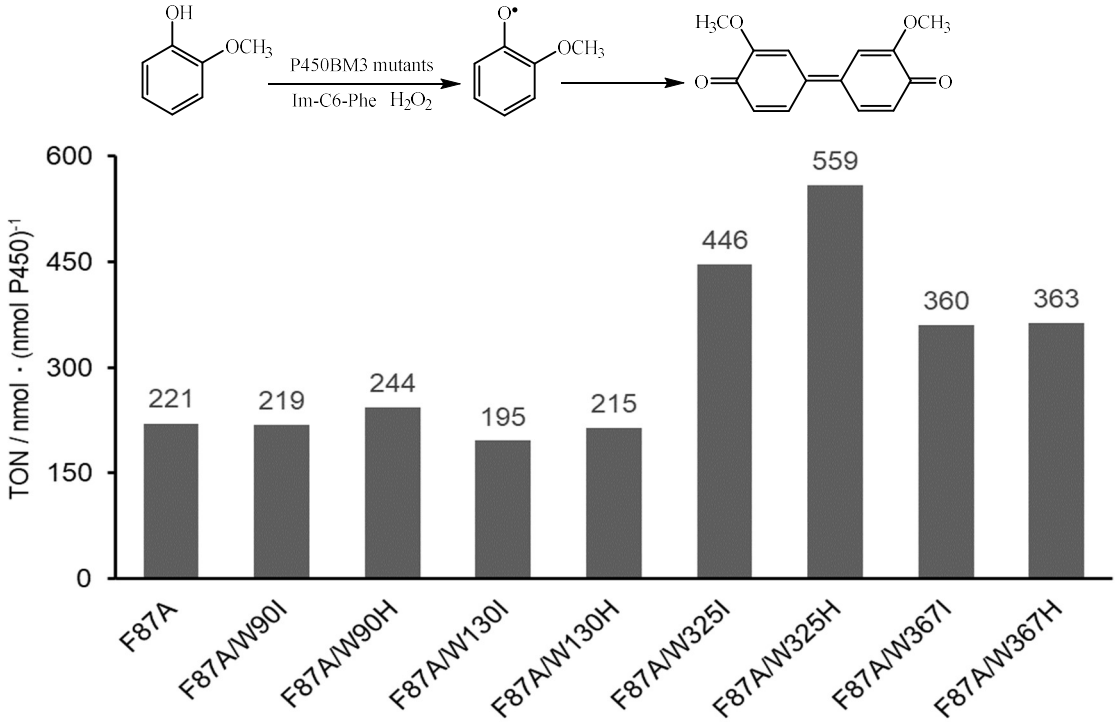


Figure S1 Relative peroxidase activity for guaiacol oxidation by the F87A/ monotryptophan mutations of redox-sensitive residues in the presence of Im-C6-Phe. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

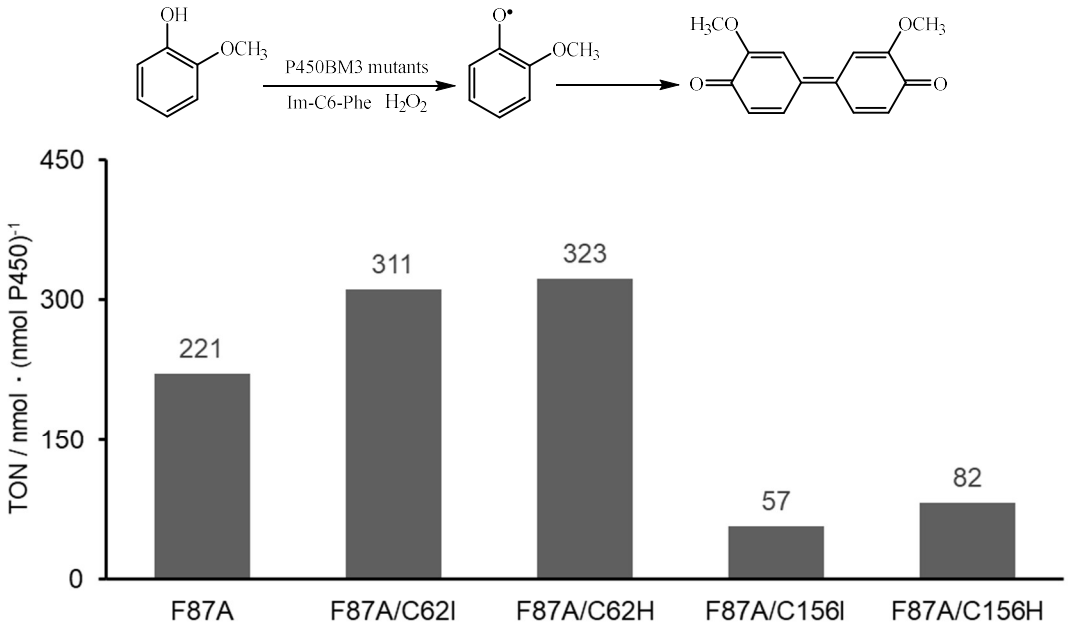


Figure S2 Relative peroxidase activity for guaiacol oxidation by the F87A/ monocysteine mutations of redox-sensitive residues in the presence of Im-C6-Phe. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

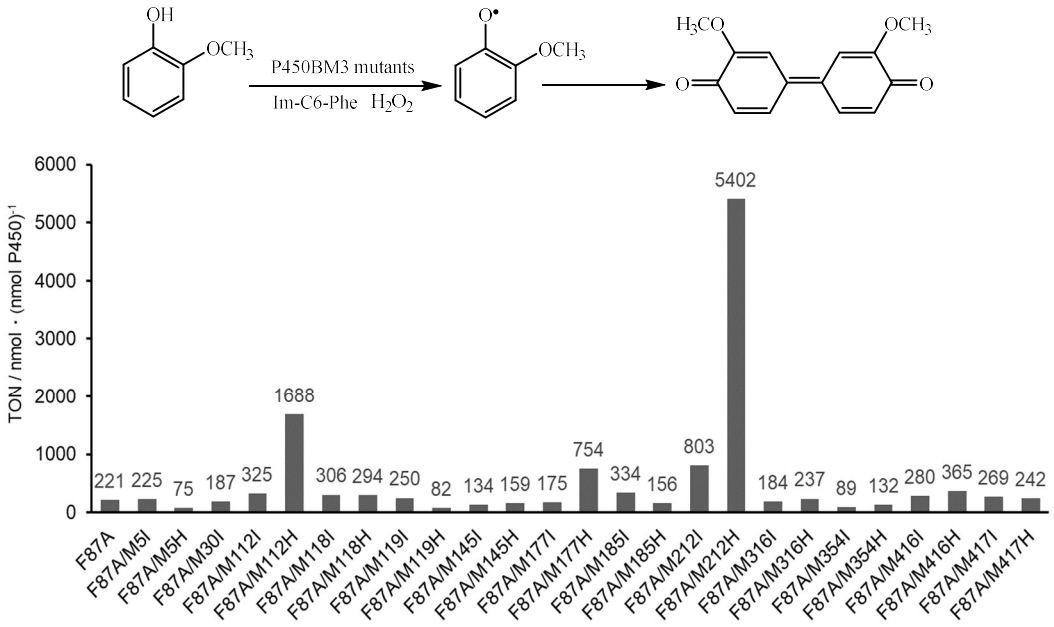


Figure S3 Relative peroxidase activity for guaiacol oxidation by the F87A/ monomethionine mutations of redox-sensitive residues in the presence of Im-C6-Phe. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

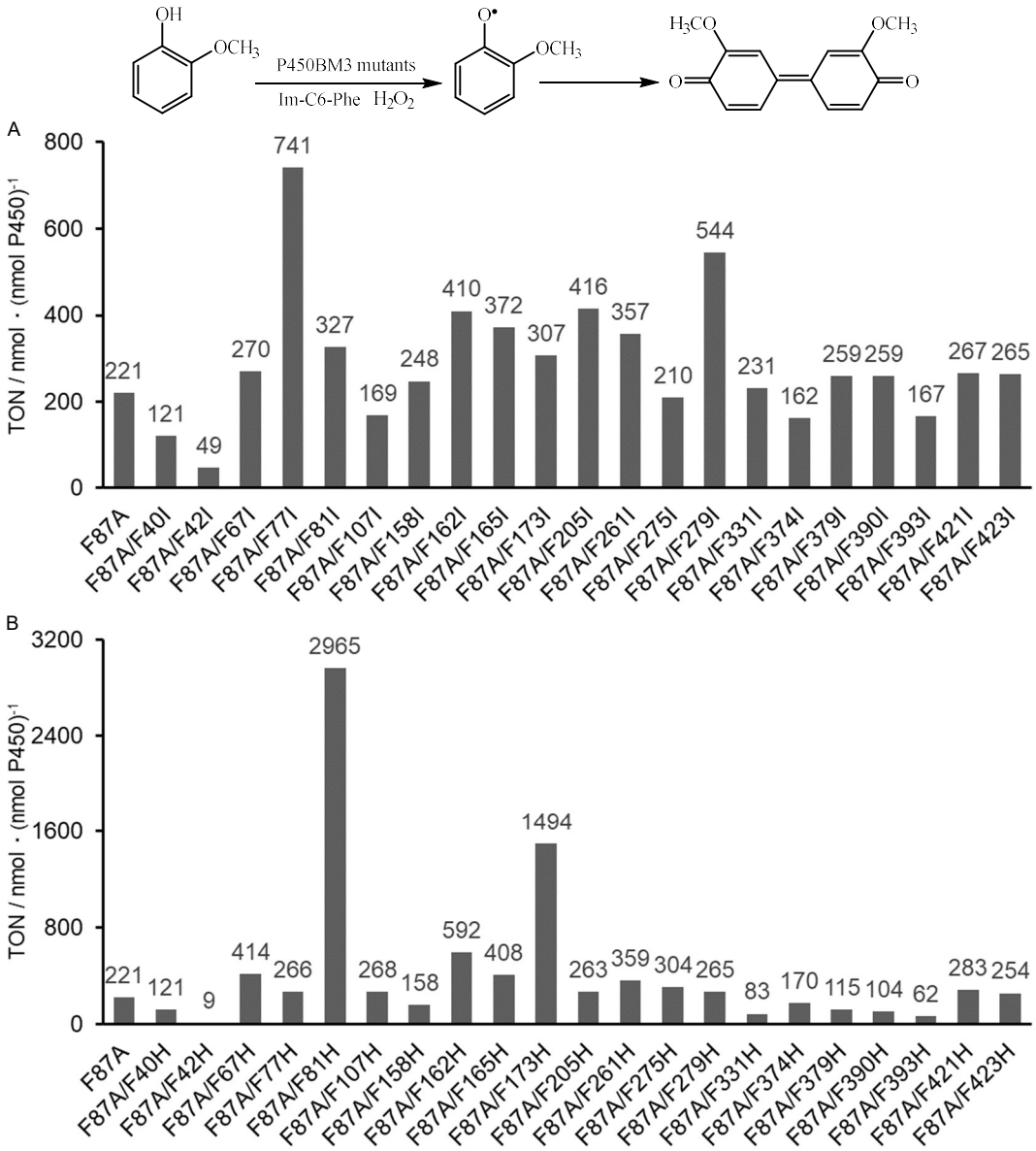


Figure S4 Relative peroxidase activity for guaiacol oxidation by the F87A/ monophenylalanine mutations of redox-sensitive residues in the presence of Im-C6-Phe. (A) F mutates to I (B) F mutates to H. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

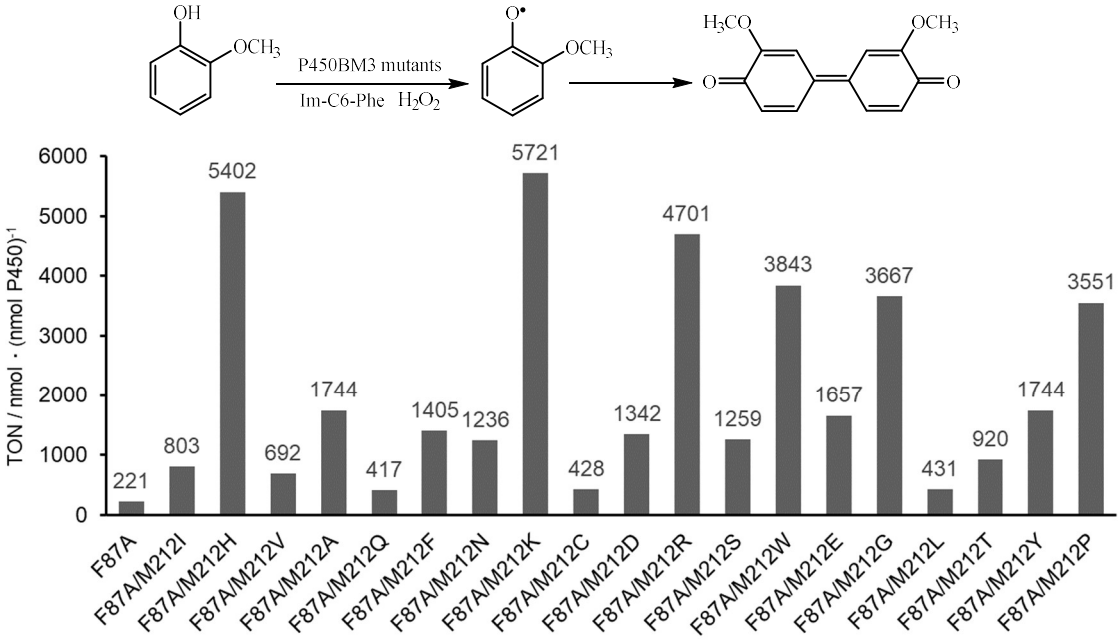


Figure S5 Relative peroxidase activity for guaiacol oxidation by the site-directed saturation mutagenesis of M212 in the presence of Im-C6-Phe. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

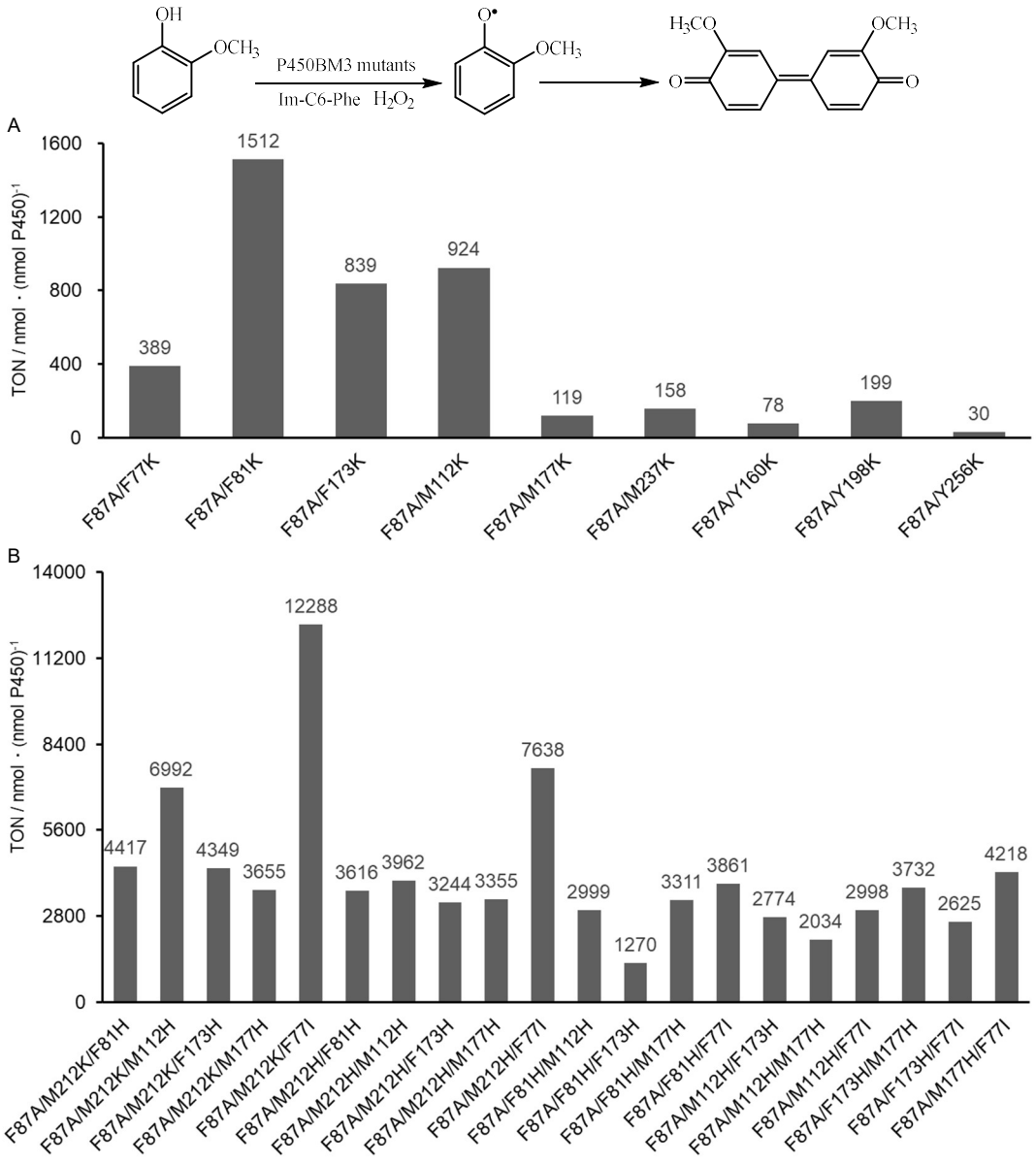


Figure S6 Engineering P450BM3 for one-electron oxidation of guaiacol in the presence of Im-C6-Phe. (A) lysine mutation of other identified sites (B) a series of double mutants based on F87A by combinatorial mutation. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

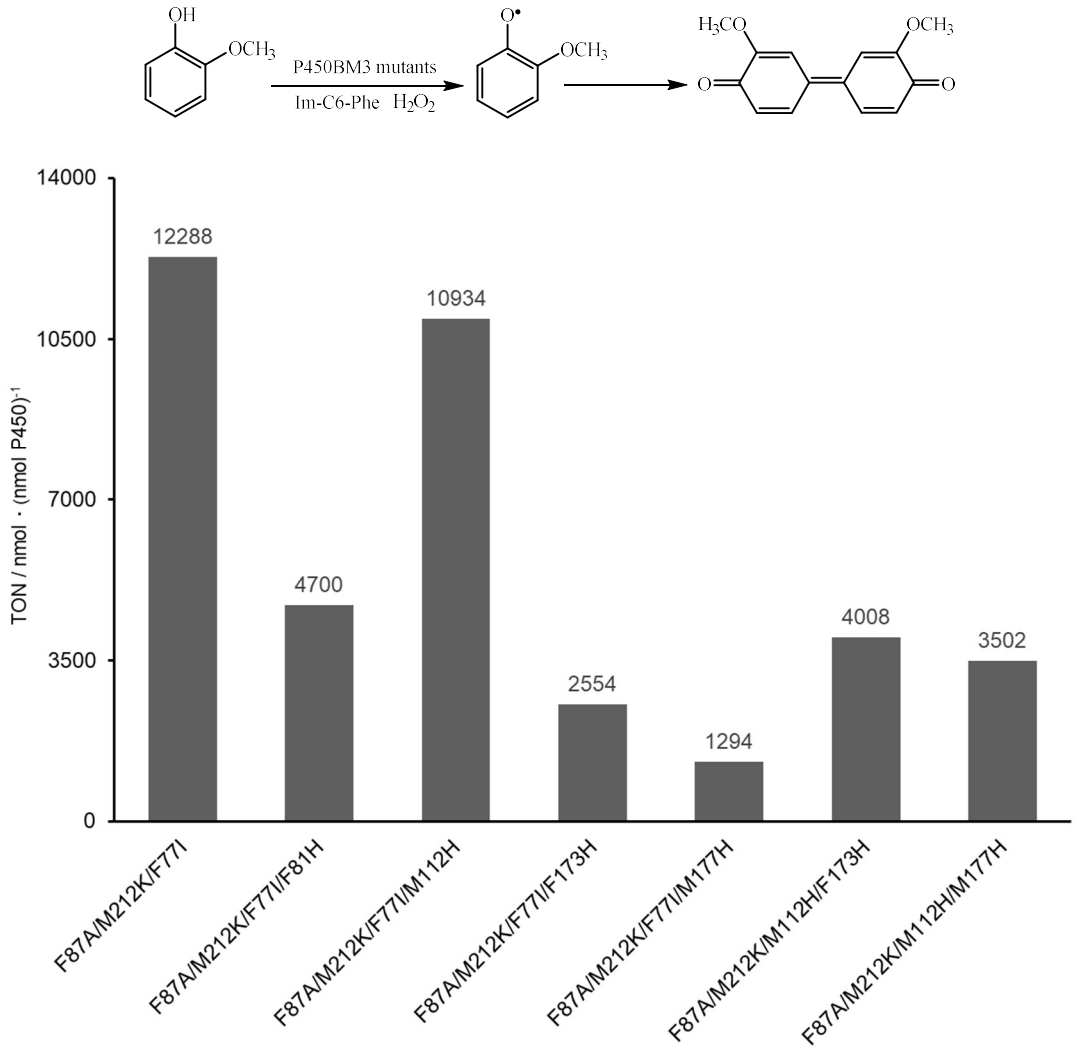


Figure S7 Engineering P450BM3 for one-electron oxidation of guaiacol in the presence of Im-C6-Phe. F87A/M212K/F77I incorporates other beneficial single mutants (F81H, F173H, M177H, and M112H). Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

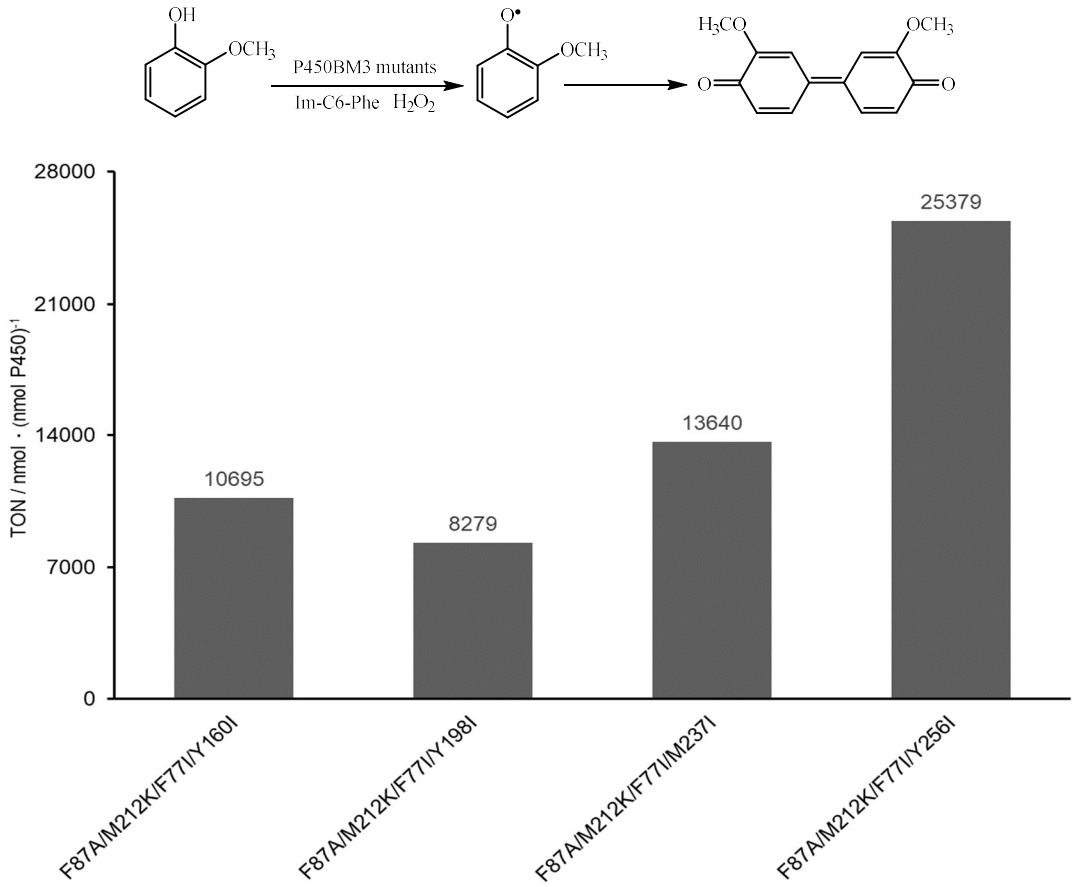


Figure S8 Engineering P450BM3 for one-electron oxidation of guaiacol in the presence of Im-C6-Phe. F87A/M212K/F77I incorporates other beneficial single mutants (Y160I, Y198I, M237I, and Y256I). Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

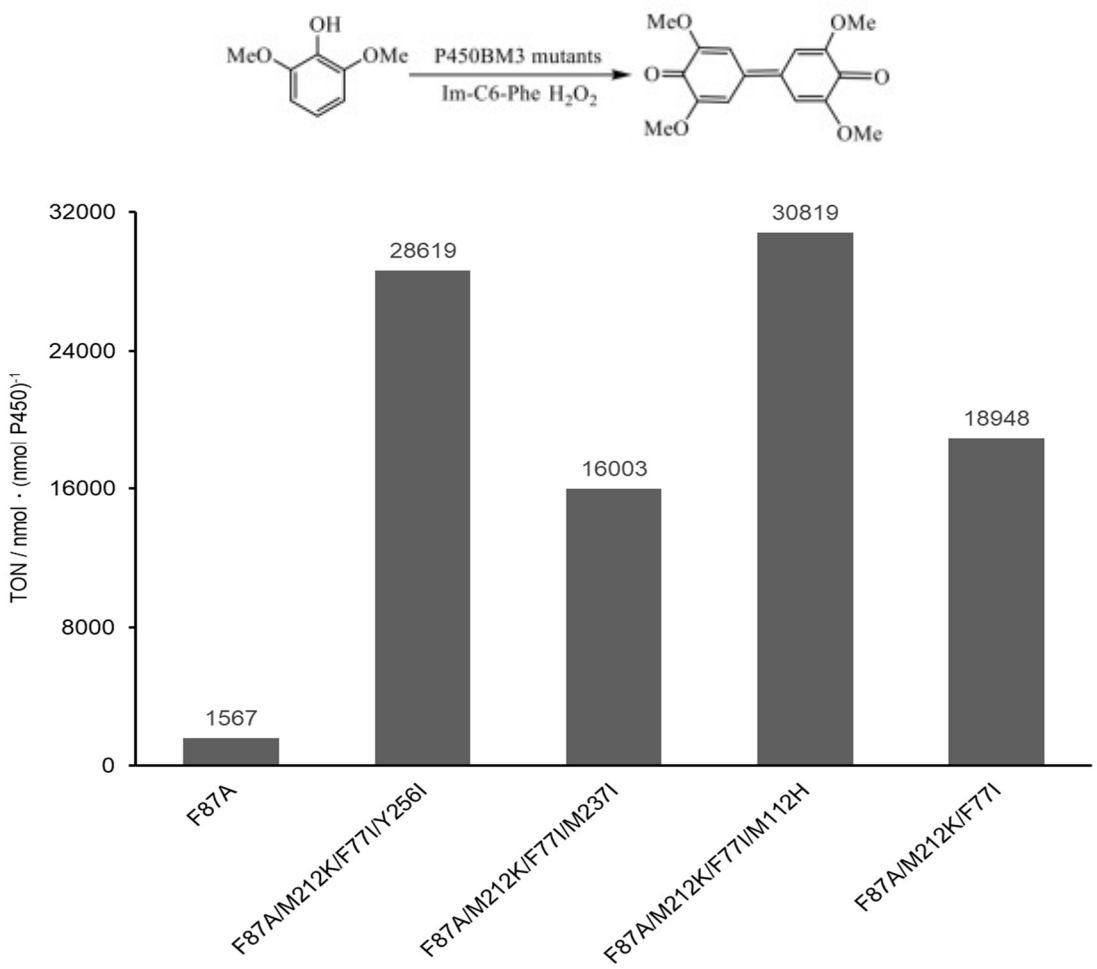


Figure S9 One-electron oxidation of DMP by selected P450 variants in the presence of Im-C6-Phe. Reaction conditions: P450BM3 variants (10-20 nM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), substrates DMP (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

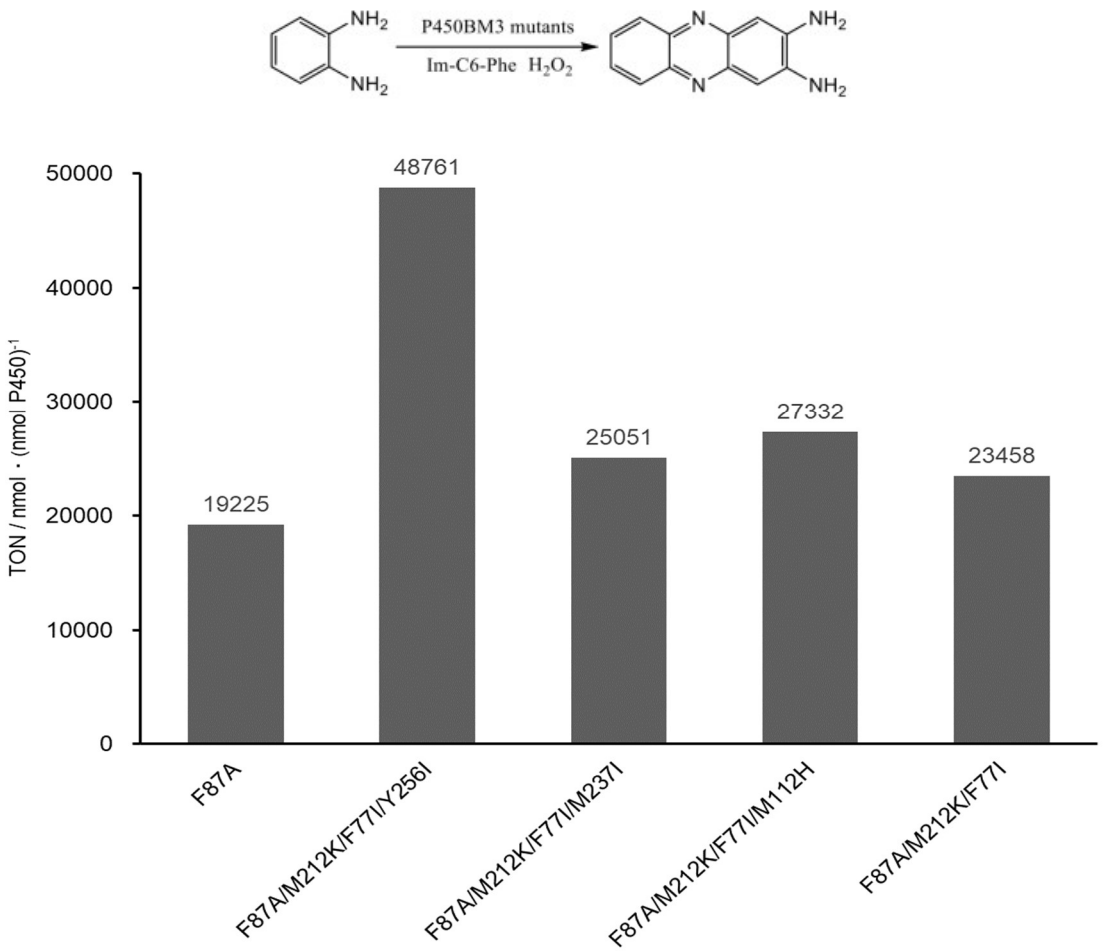


Figure S10 One-electron oxidation of OPD by selected P450 variants in the presence of Im-C6-Phe. Reaction conditions: P450BM3 variants (10-20 nM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), substrates OPD (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

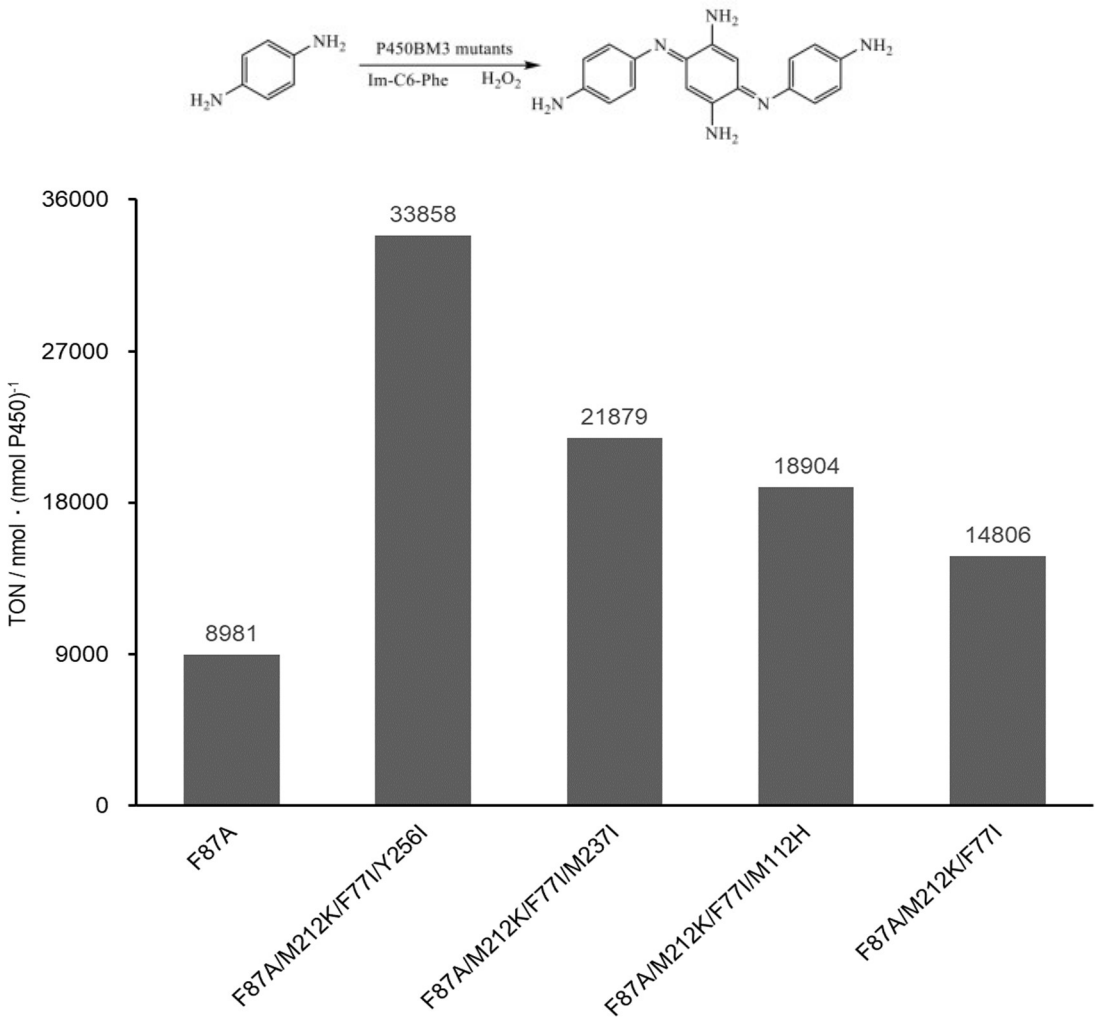


Figure S11 One-electron oxidation of PPD by selected P450 variants in the presence of Im-C6-Phe. Reaction conditions: P450BM3 variants (5-10 nM), H_2O_2 (20 mM), Im-C6-Phe (0.5 mM), substrates PPD (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

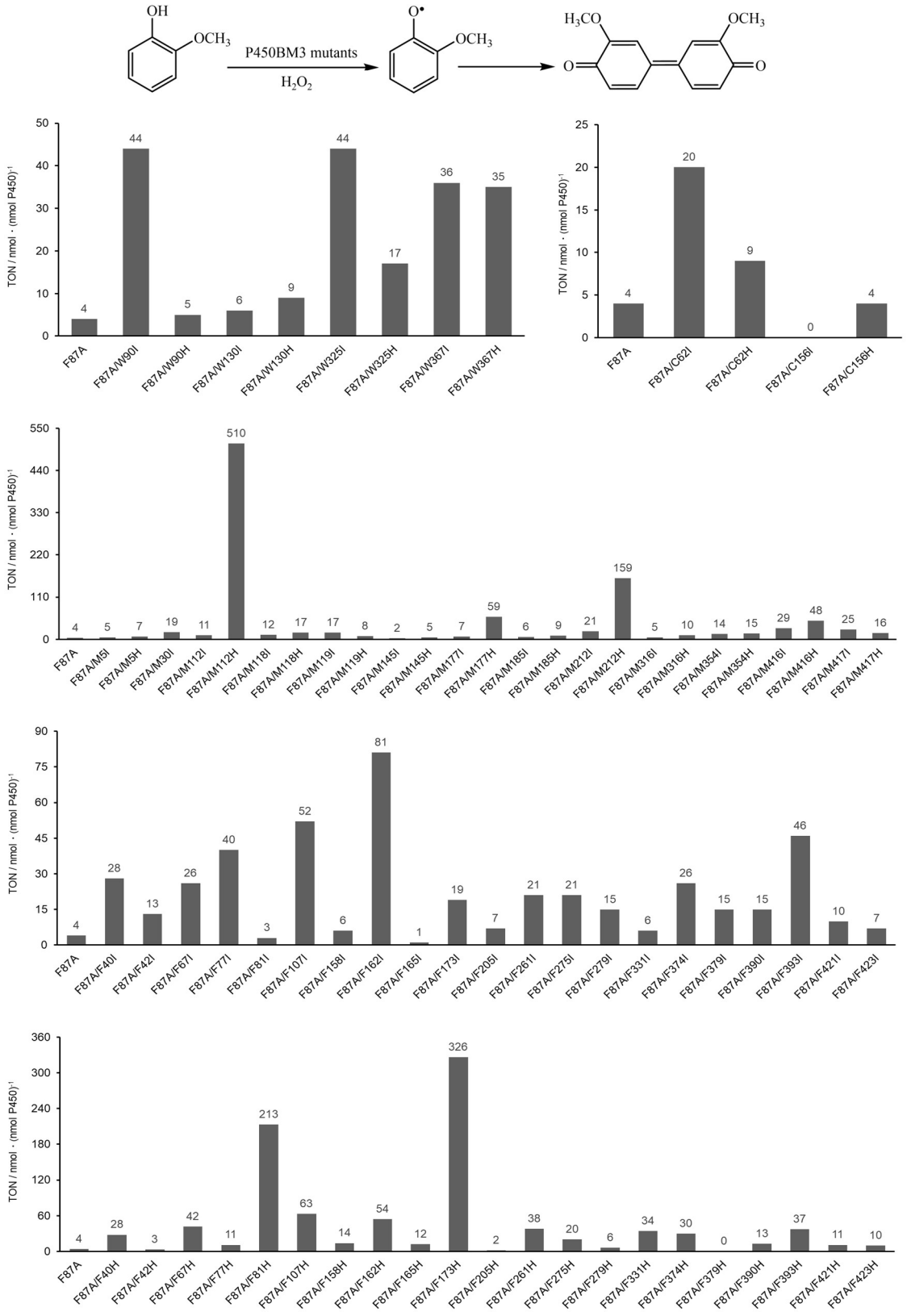


Figure S12 Relative peroxidase activity for guaiacol oxidation by the single-site mutations of redox-sensitive residues in the absence of DFSM. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

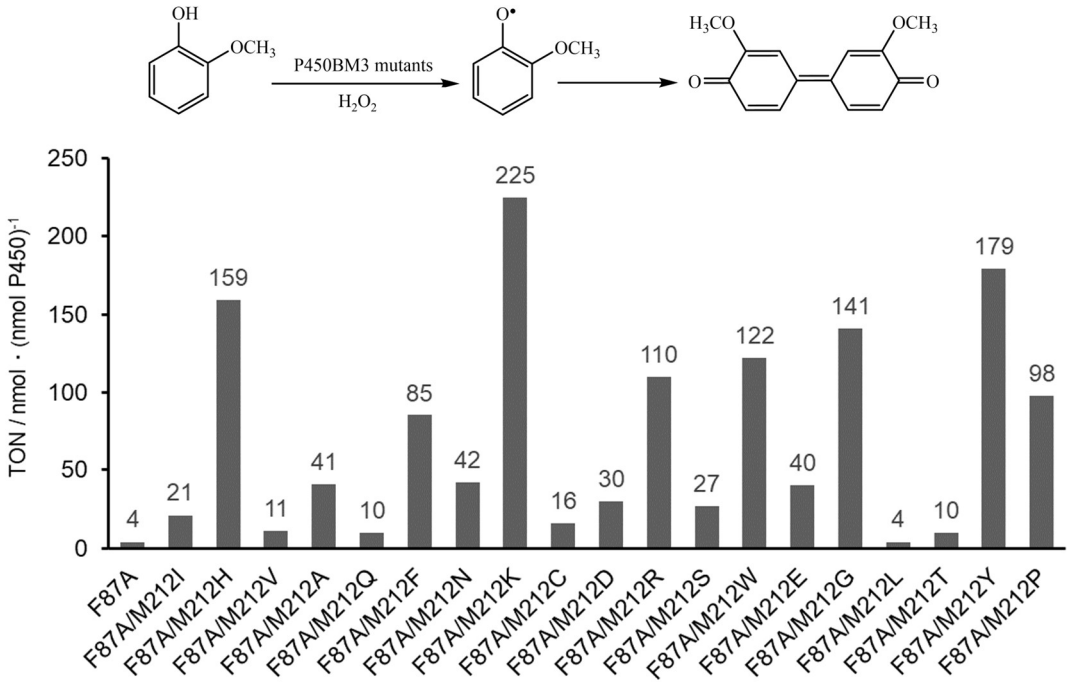


Figure S13 Relative peroxidase activity for guaiacol oxidation by the site-directed saturation mutagenesis of M212 in the absence of Im-C6-Phe. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

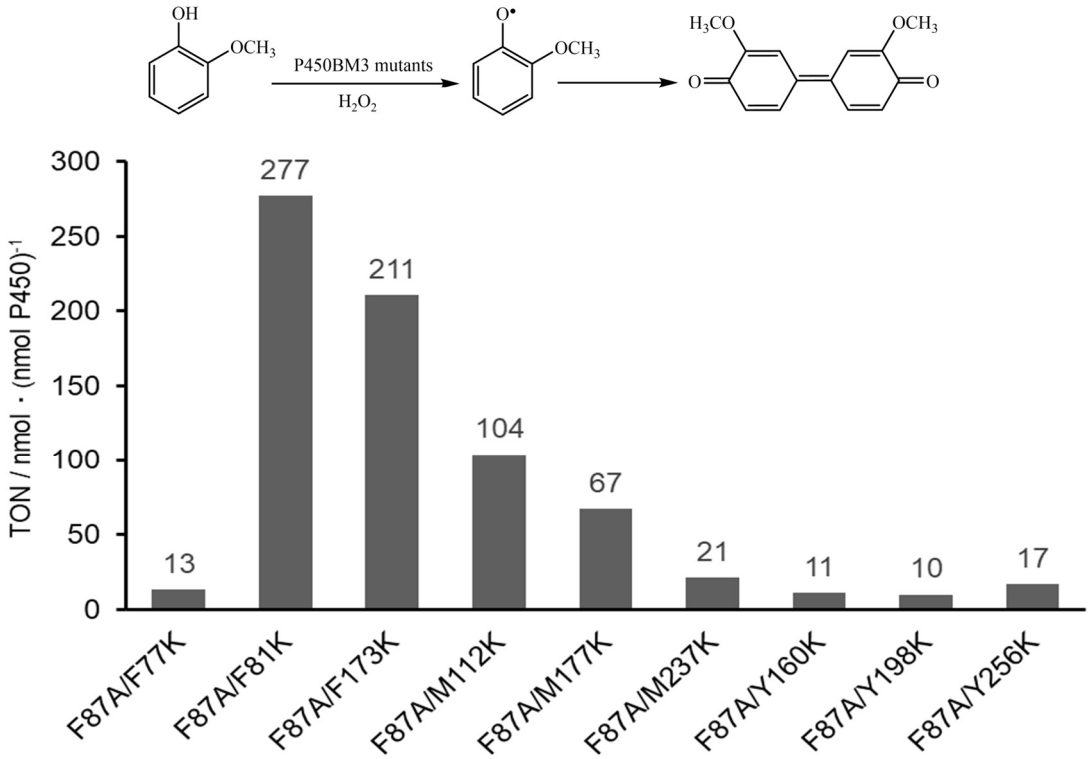


Figure S14 Relative peroxidase activity for guaiacol oxidation by the lysine mutation of identified redox-sensitive residues in the absence of DFSM. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

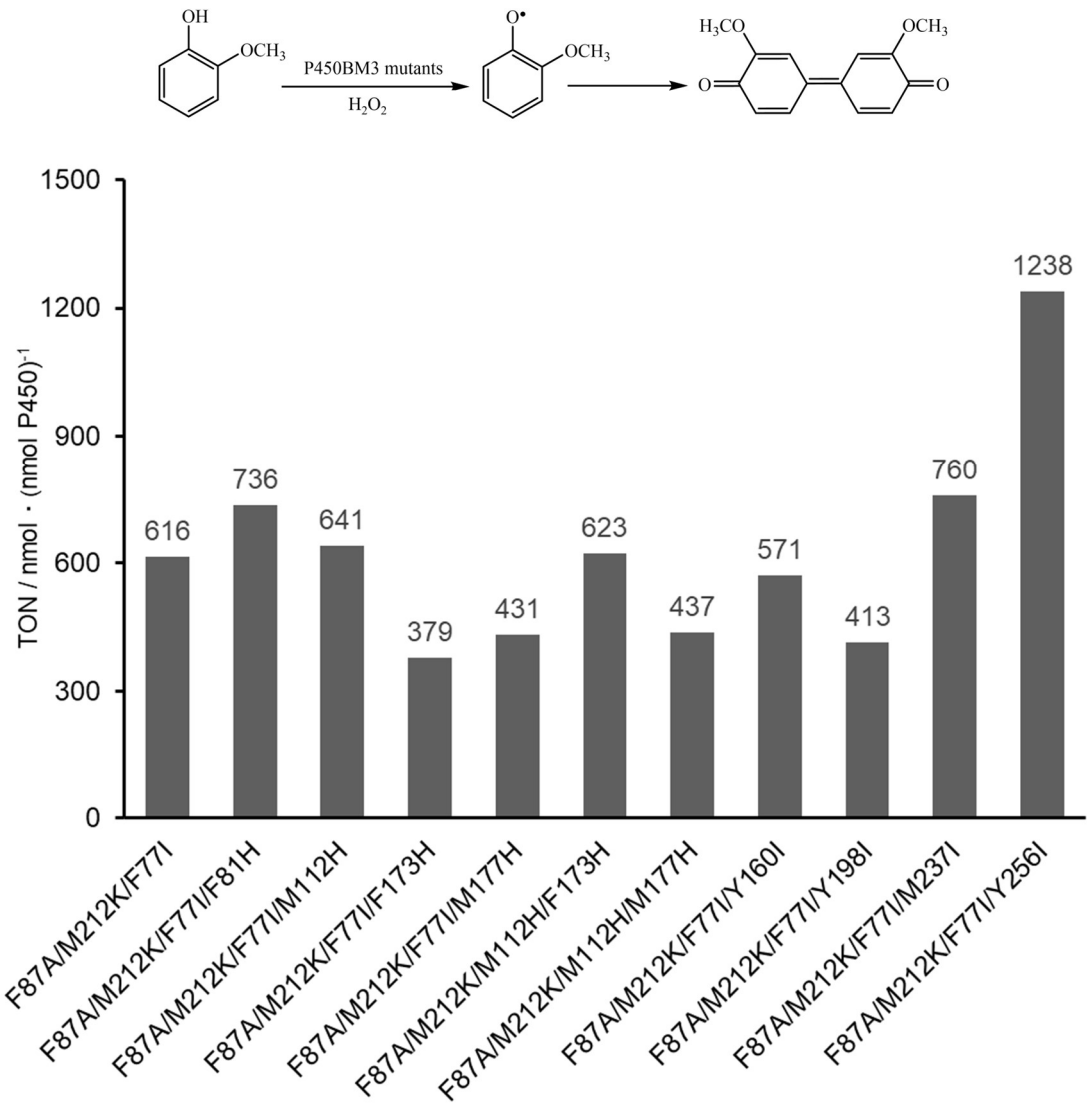


Figure S15 Engineering P450BM3 for one-electron oxidation of guaiacol in the absence of Im-C6-Phe. F87A/M212K/F77I incorporates other beneficial single mutants. Reaction conditions: P450BM3 variants (0.01-0.5 μM), H_2O_2 (20 mM), guaiacol (4 mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.

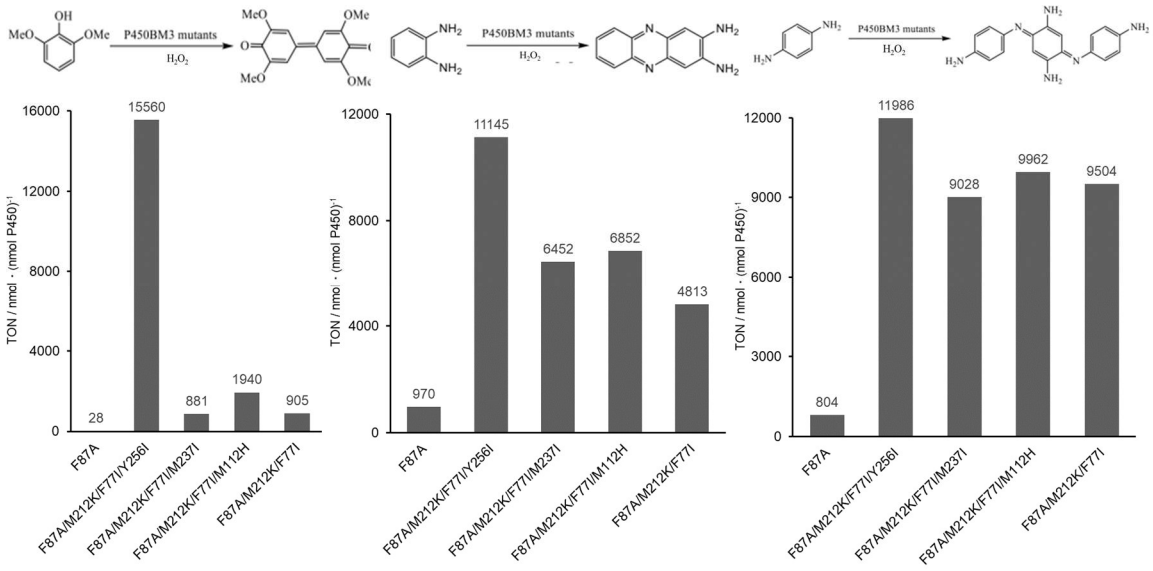
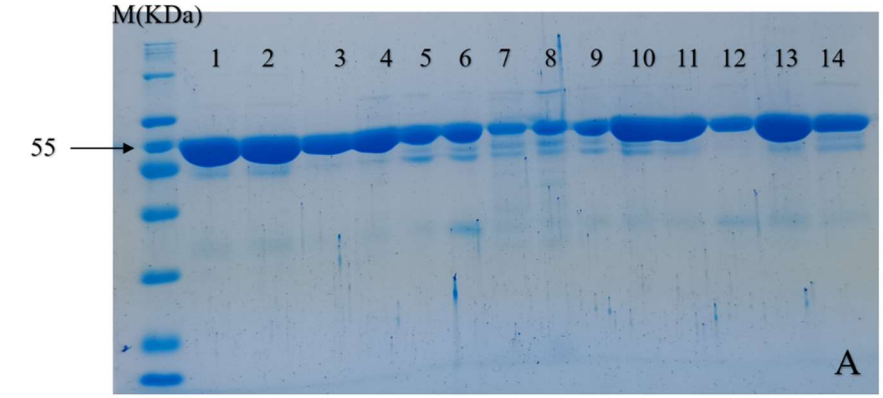


Figure S16 One-electron oxidation of DMP, OPD, and PPD by selected P450 variants in the absence of DFSM. Reaction conditions: P450BM3 variants (5-20 nM), H2O2 (20 mM), substrates DMP (4mM), OPD (4mM), PPD (4mM) in 0.05 M pH 7.0 phosphate buffer at 25 ºC.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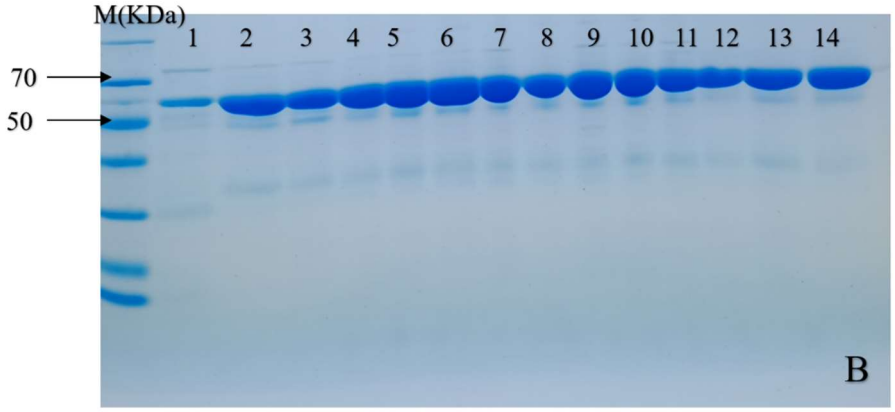
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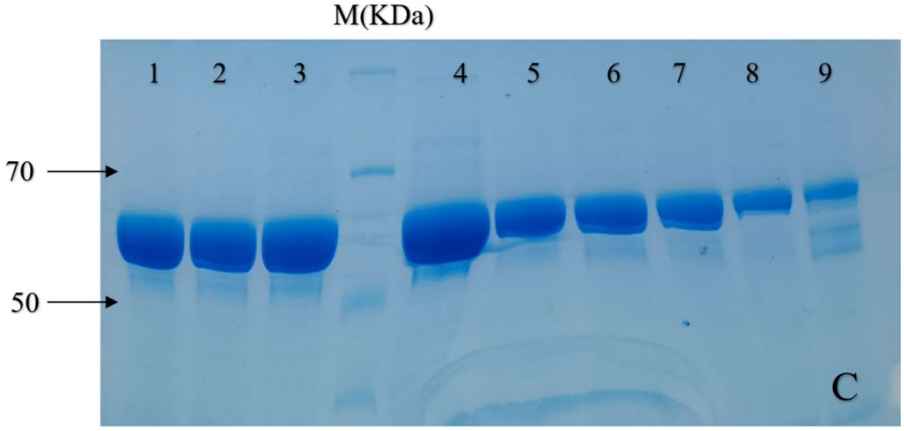
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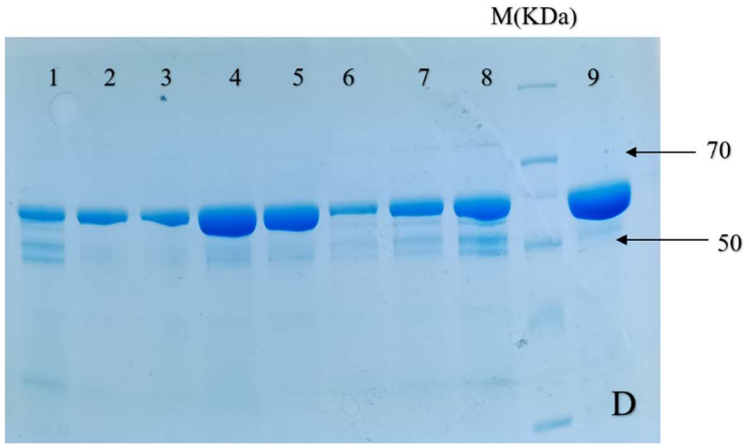
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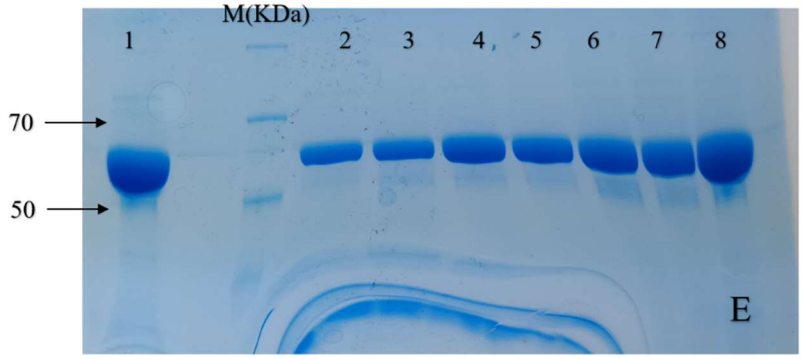
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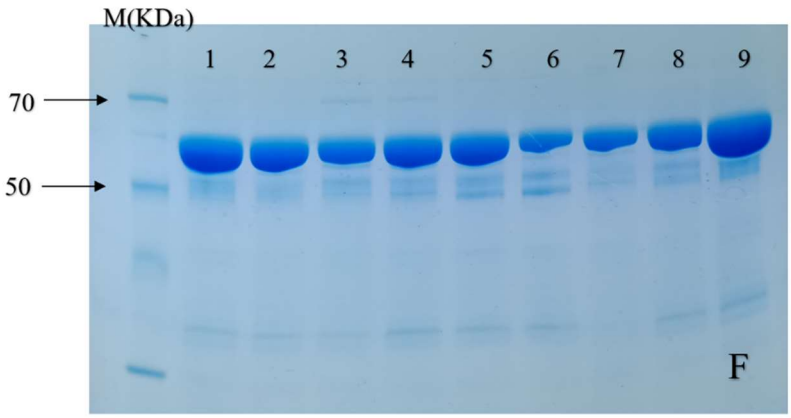
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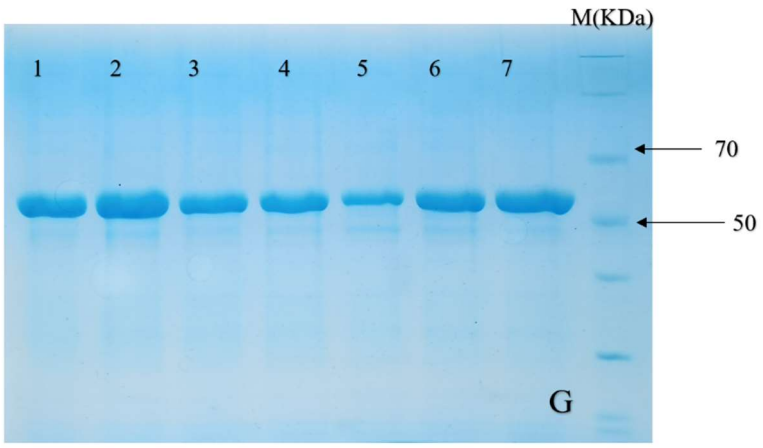
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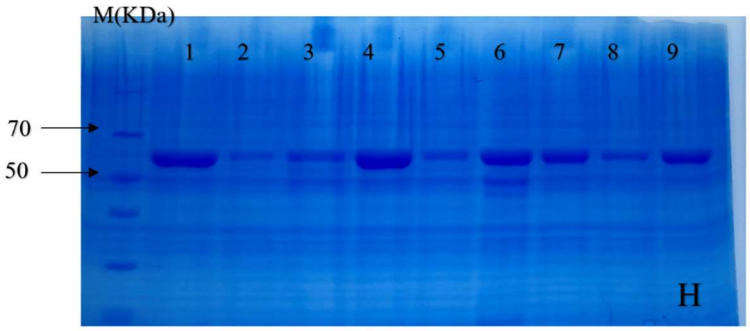
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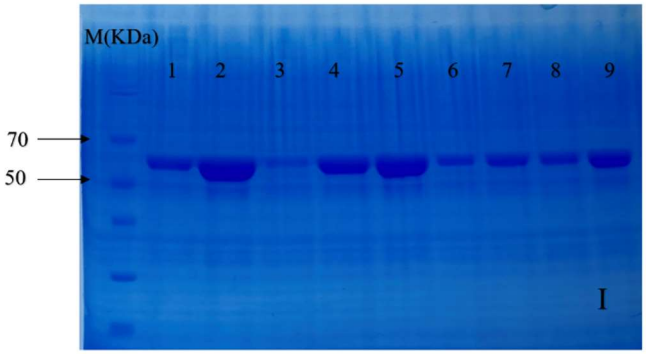
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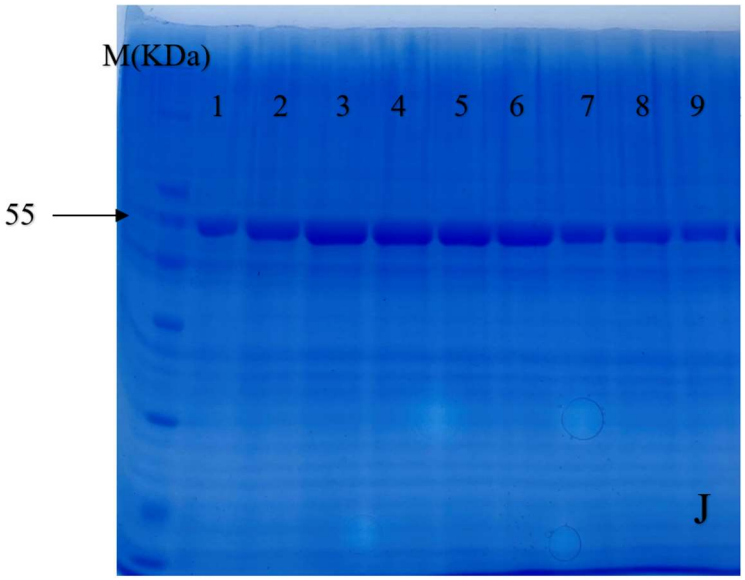
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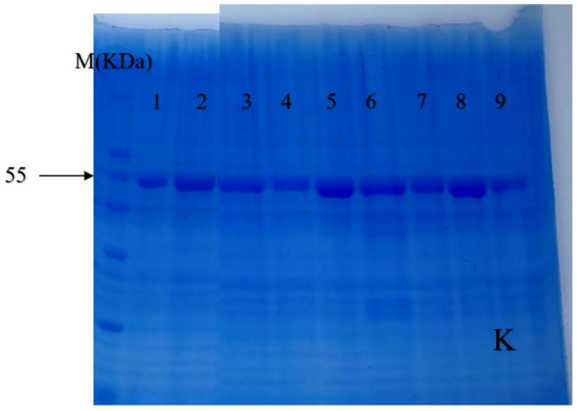
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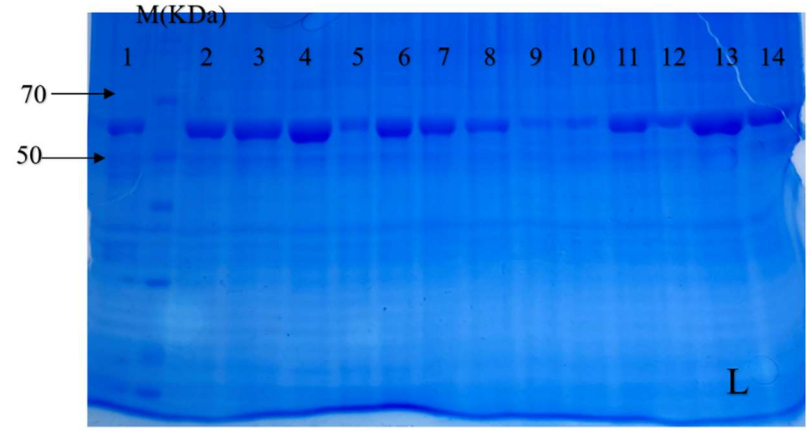
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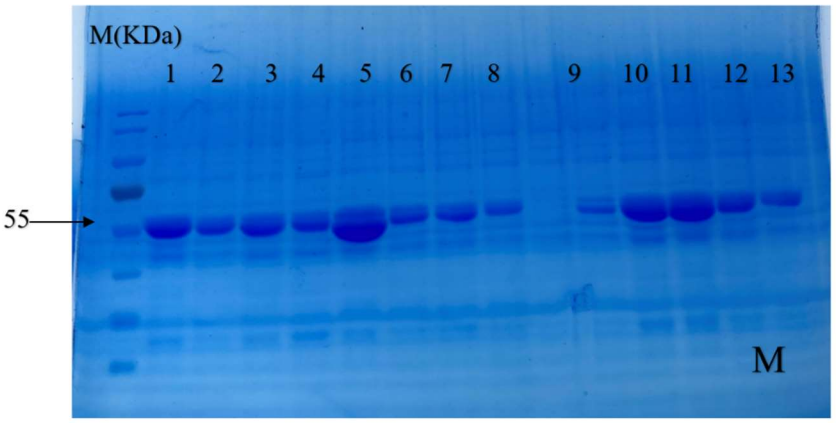
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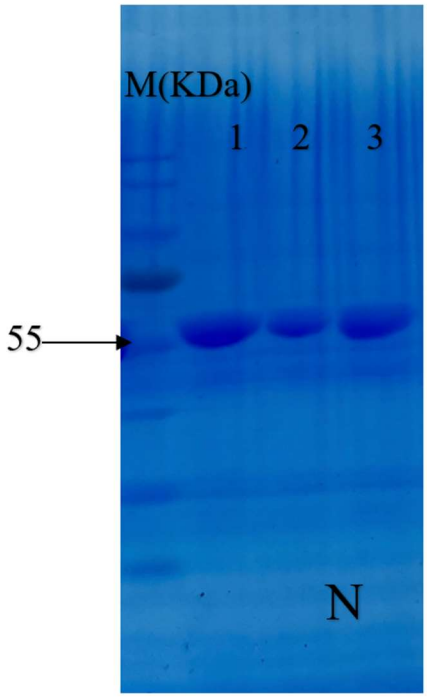
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Figure S17 SDS-PAGE of P450BM3 mutants. Lane M: molecular mass standards; (A) Lane 1-14: F87A/W90I、F87A/W90H、F87A/W130I、F87A/W130H、F87A/W325I、F87A/W325H、F87A/W367I、F87A/W367H、F87A/C62I、F87A/C62H、F87A/C156I、F87A/C156H、F87A/M5I、F87A/M5H; (B) Lane 1-14:F87A/M30I、F87A/M30H、F87A/M112I、F87A/M112H、F87A/M118I、F87A/M118H、F87A/M119I、F87A/M119H、F87A/M145I、F87A/M145H、F87A/M177I、F87A/M177H、F87A/M185I、F87A/M185H; (C) Lane 1-9:F87A/M212I、F87A/M212H、F87A/M316I、F87A/M316H、F87A/M354I、F87A/M354H、F87A/M416I、F87A/M416H、F87A/M417I; (D) Lane 1-9:F87A/M417H、F87A/F40I、F87A/F40H、F87A/F42I、F87A/F42H、F87A/F67I、F87A/F67H、F87A/F77I、F87A/F77H; (E) Lane 1-8:F87A/F81I、F87A/F81H、F87A/F107I、F87A/F107H、F87A/F158I、F87A/F158H、F87A/F162I、F87A/F162H; (F) Lane 1-9:F87A/F165I、F87A/F165H、F87A/F173I、F87A/F173H、F87A/F205I、F87A/F205H、F87A/F261I、F87A/F261H、F87A/F275I; (G) Lane 1-7:F87A/F275H、F87A/F279I、F87A/F279H、F87A/F331I、F87A/F331H、F87A/F374I、F87A/F374H; (H) Lane 1-9: F87A/F379I、F87A/F379H、F87A/F390I、F87A/F390H、F87A/F393I、F87A/F393H、F87A/F421I、F87A/F421H、F87A/F423I; (I) Lane 1-9: F87A/F423H、F87A/M212A、F87A/ M212Y、F87A/ M212P、F87A/M212V、F87A/M212Q、F87A/M212F、F87A/M212N、F87A/M212K; (J) Lane 1-10: F87A/M212C、F87A/M212D、F87A/ M212R、F87A/ M212S、F87A/M212W、F87A/M212E、F87A/M212G、F87A/M212L、F87A/M212T; (K) Lane 1-9: F87A/F81K、F87A/M112K、F87A/ F173K、F87A/ F77K、F87A/M177K、F87A/Y160K、F87A/Y198K、F87A/Y256K、F87A/M237K; (L) Lane 1-14: F87A/M212K/F81H、F87A/M212K/M112H、F87A/M212K/F173H、F87A/ M212K/M177H、F87A/M212K/F77I、F87A/M212H/M112H、F87A/M212H/F81H、F87A/ M212H/F173H、F87A/M212H/M177H 、F87A/M212H/F77I、F87A/F81H/M112H、F87A/ F81H/F173H、F87A/F81H/M177H、F87A/F81H/F77I; (M) Lane 1-13: F87A/M112H/F173H、F87A/M112H/M177H、F87A/M112H/F77I、F87A/ F173H/M177H、F87A/F173H/F77I、F87A/M177H/F77I、F87A/M212K/F77I/F81H、F87A/M212K/F77I/M112H、F87A/M212K/F77I/F173H 、F87A/M212K/F77I/M177H、F87A/M212K/M112H/F173H、F87A/M212K/M112H/M177H、F87A/M212K/F77I/Y160I; (N) Lane 1-3: F87A/M212K/F77I/Y198I、F87A/M212K/F77I/Y256I、F87A/M212K/F77I/ M237I.

Table S1 Primers used in reverse PCR method

primer	sequence
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W90H-F	5’-AGCATCACGCATGAAAAAAACTGG-3’
W90-R	5’-TGTCGCTAACCCGTCTCCTGC-3’
W130I-F	5’-ATCGAGCGTCTAAATGCAGATGA-3’
W130H-F	5’-CATGAGCGTCTAAATGCAGATGA-3’
W130-R	5’-CTTTTGAACAAGCTGCACGGC-3’
W325I-F	5’-ATTCCAACTGCTCCTGCGTT-3’
W325H-F	5’-CATCCAACTGCTCCTGCGTT-3’
W325-R	5’-TAAGCGCAGCGCTTCGTTTAAG-3’
W367I-F	5’-ATTGGAGACGATGTGGAAGAGT-3’
W367H-F	5’-CATGGAGACGATGTGGAAGAGT-3’
W367-R	5’-AATTGGTTTATCACGGTGAAGCTG-3’
C62I-F	5’-GCAATCGATGAATCACGCTTTG-3’
C62H-F	5’-GCACATGATGAATCACGCTTTG-3’
C62-R	5’-TTCTTTAATCAGACGCTGGCTTG-3’
C156I-F	5’-ATCGGCTTTAACTACCGCTTT-3’
C156H-F	5’-CATGGCTTTAACTACCGCTTT-3’
C156-R	5’-CAGACCAATT GTATCAAGCGT-3’
M5I-F	5’-ATTCCTCAGCCAAAAACGTTTGG-3’
M5H -F	5’-CATCCTCAGCCAAAAACGTTTGG-3’
M5-R	5’-TTCTTTGATTGTCATGTTCTCTGCC-3’

M145I-F	5’-GACATTACACGTTTAACGCTTG-3’
M145H-F	5’-GACCATACACGTTTAACGCTTG-3’
M145-R	5’-TTCCGGTACTTCAATATGCTC-3’
M185I-F	5’-ATTAACAAGCTGCAGCGAGC-3’
M185H-F	5’-CATAACAAGCTGCAGCGAGC-3’
M185-R	5’-TGCTTCATCCAGTGCACG-3’
M30I-F	5’-GATTAAGATTGCGGATGAGTTAGG-3’
M30H-F	5’-GCATAAGATTGCGGATGAGTTAGG-3’
M30-R	5’-AAAGCTTGAACCGGTTTACT-3’
M112I-F	5’-GGCAATTAAAGGCTATCATGCG-3’
M112H-F	5’-GGCACATAAAGGCTATCATGCG-3’
M112K-F	5’-GGCAAAGAAAGGCTATCATGCG-3’
M112-R	5’-TGCTGACTGAAGCTTGG-3’
M177I-F	5’-ATTGTCCGTGCACTGGATG-3’
M177H-F	5’-CATGTCCGTGCACTGGATG-3’
M177K-F	5’-AAAGTCCGTGCACTGGATG-3’
M177-R	5’-ACTTGTGATAAATGGATGAGGC-3’
M212I-F	5’-GTGATTAACGACCTAGTAGA-3’
M212I-F	5’-GTGCATAACGACCTAGTAGA-3’
M212-R	5’-CTTGATATCTTCTTGAAACTGGC-3’
M212A-F	5’-GTGGCGAACGACCTAGTAGA-3’
M212R-F	5’-GTGCGTAACGACCTAGTAGA-3’
M212N-F	5’-GTGAATAACGACCTAGTAGA-3’

M212D-F	5’-GTGGCTAACGACCTAGTAGA-3’
M212C-F	5’-GTGTGCAACGACCTAGTAGA-3’
M212E-F	5’-GTGGAAAACGACCTAGTAGA-3’
M212Q-F	5’-GTGCAGAACGACCTAGTAGA-3’
M212G-F	5’-GTGGGCAACGACCTAGTAGA-3’
M212L-F	5’-GTGCTGAACGACCTAGTAGA-3’
M212K-F	5’-GTGAAAAACGACCTAGTAGA-3’
M212F-F	5’-GTGTTTAACGACCTAGTAGA-3’
M212S-F	5’-GTGAGCAACGACCTAGTAGA-3’
M212T-F	5’-GTGACCAACGACCTAGTAGA-3’
M212W-F	5’-GTGTGGAACGACCTAGTAGA-3’
M212Y-F	5’-GTGTATAACGACCTAGTAGA-3’
M212V-F	5’-GTGGTGAACGACCTAGTAGA-3’
M212P-F	5’-GTGCCGAACGACCTAGTAGA-3’
M118I-F	5’-GCGATTATGGTCGATATCGCCG-3’
M118H-F	5’-GCGCATATGGTCGATATCGCCG-3’
M118-R	5’-ATGATAGCCTTTCATTGCCTGC-3’
M119I-F	5’-GCGATGATTGTCGATATCGCCG-3’
M119H-F	5’-GCGATGCATGTCGATATCGCCG-3’
M119-R	5’-ATGATAGCCTTTCATTGCCTGC-3’
M316I-F	5’-ATTGTCTTAAACGAAGCGC-3’
M316H-F	5’-CATGTCTTAAACGAAGCGC-3’
M316-R	5’-GCCGACATATTTAAGCTGTTTGAC-3’

M354I-F	5’-ATTGTTCTGATTCCTCAGCTTCACC-3’
M354H-F	5’-CATGTTCTGATTCCTCAGCTTCACC-3’
M354-R	5’-TAGTTCGTCGCCTTTTTC-3’
M416I-F	5’-ACGCTGGTACTTGGTATTATGCTA-3’
M416H-F	5’-ACGCTGGTACTTGGTCATATGCTA-3’
M416-R	5’-TGCTTCATGAAGAGCGAACTGC-3’
M417I-F	5’-GGTATGATTCTAAAACAC-3’
M417H-F	5’-GGTATGCATCTAAAACAC-3’
M417-R	5’-AAGTACCAGCGTTGCTT-3’
F40I-F	5’-ATCATTAAATTCGAGGCGCC-3’
F40H-F	5’-ATCCATAAATTCGAGGCGCC-3’
F40-R	5’-TTCTCCTAATTCATCCGC-3’
F42I-F	5’-AAAATTGAGGCGCCTGGTCGTG-3’
F42H-F	5’-AAACATGAGGCGCCTGGTCGTG-3’
F42-R	5’-GAAGATTTCTCCTAATTCATCCGC-3’
F67I-F	5’-GAGTCACGCATTGATAAGAAC-3’
F67H-F	5’-GAGTCACGCCATGATAAGAAC-3’
F67-R	5’-ATCGCATGCTTCTTTAATTAGACGC-3’
F77I-F	5’-ATTGTACGTGATTTTGCAGGAGACG-3’
F77H-F	5’-CATGTACGTGATTTTGCAGGAGACG-3’
F77K-F	5’-AAAGTACGTGATTTTGCAGGAGACG-3’
F77-R	5’-TTTAAGCGCTTGACTTAAG-3’
F81I-F	5’-GATATTGCAGGAGACGGGTTA-3’

F81H-F	5’-GATCATGCAGGAGACGGGTTA-3’
F81K-F	5’-GATAAAGCAGGAGACGGGTTA-3’
F81-R	5’-ACGTACGAATTTAAGCGC-3’
F107I-F	5’-ATTAGTCAGCAGGCAATG-3’
F107H-F	5’-CATAGTCAGCAGGCAATG-3’
F107-R	5’-GCTTGGAAGTAAGATATTATGCGC-3’
F158I-F	5’-GGCATTAACTATCGCTTTAACAGC-3’
F158H-F	5’-GGCCATAACTATCGCTTTAACAGC-3’
F158-R	5’-GCAAAGACCAATTGTATCAAGCG-3’
F162I-F	5’-CGCATTAACAGCTTTTACCG-3’
F162H-F	5’-CGCCATAACAGCTTTTACCG-3’
F162-R	5’-ATAGTTAAAGCCGCAAAGACC-3’
F165I-F	5’-AGCATTTACCGAGATCAGCC-3’
F165H-F	5’-AGCCATTACCGAGATCAGCC-3’
F165-R	5’-GTTAAAGCGATAGTTAAAGCCGC-3’
F173I-F	5’-CCAATTATTACAAGTATGGTCCG-3’
F173H-F	5’-CCACATATTACAAGTATGGTCCG-3’
F173K-F	5’-CCAAAAATTACAAGTATGGTCCG-3’
F173-R	5’-TGGATGAGGCTGATCTCGGTA-3’
F205I-F	5’-CGCCAGATTCAAGAAGATATC-3’
F205H-F	5’-CGCCAGCATCAAGAAGATATC-3’
F205-R	5’-CTTGTTTTCATCATAAGCTGGG-3’
F261I-F	5’-ATTACAATTTTAATTGCGGGACACG-3’

F261H-F	5’-ATTACACATTTAATTGCGGGACACG-3’
F261-R	5’-AATTTGATAGCGAATGCTCTCG-3’
F279I-F	5’-ATTCTGGTGAAAAATCCAC-3’
F279H-F	5’-CATCTGGTGAAAAATCCAC-3’
F279-R	5’-ATACAGCGCGAATGAT-3’
F331I-F	5’-GCGATTTCCCTATATGCA-3’
F331H-F	5’-GCGCATTCCCTATATGCA-3’
F331-R	5’-AGGAGCAGTTGGCCATAAG-3’
F374I-F	5’-GAAGAGATTCGTCCAGAGC-3’
F374H-F	5’-GAAGAGCATCGTCCAGAGC-3’
F374-R	5’-CACATCGTCTCCCCAAAT-3’
F379I-F	5’-CGTATTGAAAATCCAAGTGCG-3’
F379H-F	5’-CGTCATGAAAATCCAAGTGCG-3’
F379-R	5’-CTCTGGACGGAACTCTTC-3’
F390I-F	5’-GCGATTAAACCGTTTGGA-3’
F390H-F	5’-GCGCATAAACCGTTTGGA-3’
F390-R	5’-ATGCTGCGGAATCGCACTT-3’
F393I-F	5’-ATTGGAAACGGTCAGCG-3’
F393H-F	5’-CATGGAAACGGTCAGCG-3’
F393-R	5’-CGGTTTAAACGCATGCTGC-3’
F421I-F	5’-CACATTGACTTCGAAG-3’
F421H-F	5’-CACCATGACTTTGAAG-3’
F421-R	5’-TTTTAGCATCATACCAAGTACCAGCG-3’

F423I-F	5’-GACATTGAAGATCATACAAACTACGAGC-3’
F423H-F	5’-GACCATGAAGATCATACAAACTACGAGC-3’
F423-R	5’-GAAGTGTTTTAGCATCATACC-3’

Table 2 Primers used in In-Fusion Cloning

primer	sequence
M112H-F	5’-GCAGGCACATAAAGGCTATCATGCGATGATGG-3’
M112H-R	5’-AGCCTTTATGTGCCTGCTGACTGAAGCTTGGA-3’
Y160I-F	5’-CGGCTTTAACATTCGCTTTAACAGCTTTTACCGAGA-3’
Y160I-R	5’-AGCGAATGTTAAAGCCGCAAAGACCAATTGTA-3’
M177H-F	5’- ACAAGTCATGTCCGTGCACTGGATGAAGCAA-3’
M177H-R	5’-CACGGACATGACTTGTAATAAATGGATGAGGCTGAT-3’
M212K-F	5’-CAAGGTGAAAAACGACCTAGTAGATAAAATTATTGCAGA-3’
M212K-R	5’-GGTCGTTTTTCACCTTGATATCTTCTTGAAACTGGC -3’
F77I-F	5’- GCGCTTAAAATTGTACGTGATTTTGCAGGAGACG -3’
F77I-R	5’- CGTACAATTTTAAGCGCTTGACTTAAGTTTTTATC -3’
F81H-F	5’- ACGTGATCATGCAGGAGACGGGTTATTTACAAG -3’
F81H-R	5’- ACGTGATCATGCAGGAGACGGGTTATTTACAAG -3’
F173H-F	5'- CAGCCTCATCCACATATTACAAGTATGGTCCGTGCACTG -3'
F173H-R	5'- ATATGTGGATGAGGCTGATCTCGGTAAAAGCT -3'
Y256I-F	5'- CGAGAACATTCGCATTCAAATTATTACATTCTTAATTGCGGG-3'
Y256K-F	5'- CGAGAACATTCGCAAACAAATTATTACATTCTTAATTGCGGG-3'
Y256-R	5'- GAATGCGAATGTTCTCGTCATCAAGCGGCTCA-3'
M237K-F	5'-ACGCATAAACTAAACGGAAAAGATCCAGAAACG -3'
M237K-R	5'- CCGTTTAGTTTATGCGTTAATAAATCATCGCTTTG -3'
Y160K-F	5'-CGGCTTTAACAAACGCTTTAACAGCTTTTACCGAGA -3'
Y160K-R	5'- AGCGTTTGTTAAAGCCGCAAAGACCAATTGTA -3'
Y198K-F	5'-GACCCAGCTAAAGATGAAAACAAGCGCCAGTTTC -3'
Y198K-R	5'- TCATCTTTAGCTGGGTCGTCTGGATTTGCTCG -3'