Supporting Information

Enzymatic Synthesis of Pharmacologically Relevant Chiral Sulfoxides by Improved *Cb*BVMO Variants

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1. Experimental Procedures

1.1 Materials

All solvents and reagents were purchased commercially and did not undergo further purification. The substrates **1a-5a**, products **1b-5b** and by-products **1c-5c** standards used in this work were kindly provided by Nanjing Osercon Pharmaceutical Co.

1.2 Construction and screening of mutant libraries

Single-point saturation mutant libraries were generated using the polymerase chain reaction (PCR) method, and the primer sequences can be found in **Table S1**. The PCR mixtures (20 μL) consisted of 10 μL of 2× PrimeSTAR Max DNA Premix, 0.1 ng of primer pairs, and 0.1 ng of the recombinant pET-28a plasmid. The PCR amplification was performed under the following conditions: an initial denaturation step at 95 °C for 3 minutes, followed by 25 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 90 seconds. A final extension step was conducted at 72 °C for 10 minutes. Subsequently, the PCR products were purified, digested, and the resulted plasmid was transformed into *E. coli* BL21 (DE3) for expression of the variant proteins.

The CAST library was synthesized utilizing Twist Bioscience technology.[1] The synthetic CAST library was subjected to homologous recombination with the linearized plasmid vector pET-28a, followed by transformation into *E. coli* BL21 (DE3). The homologous recombination process involved the following steps: the linearized plasmid vector was combined with the mutant gene fragment at a molar ratio of 1:2. ClonExpress^{TM} II enzyme was added, and the mixture was incubated in a water bath at 50°C for 15 minutes. The reaction was then promptly transferred to an ice bath, followed by immediate transformation into *E. coli* BL21 (DE3) after a 2-minute interval.

The bacterial colonies were auto picked from agar plates and transferred into the primary 96-deep well plates containing lysogeny broth (LB) medium (200 μL) with kanamycin (50 μg mL^{-1}) by microbial colony picker Qpix 450 (Molecular Devices, LLC., USA). Plates also contained the control wells (*Cb*BVMO$_{V1}$). After incubated at 37 °C, 220 rpm overnight, preculture (20 μL) were auto transferred into the secondary 96-deep well plates containing 600 μL LB medium with kanamycin (50 μg mL^{-1}). After incubated at 37 °C, 220 rpm for 3 h, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture plates (final concentration 0.1 mM) for another 24 h at 16 °C, 220 rpm, for protein expression.

The cell pellets were harvested by centrifuged for 10 min at 3800 (×*g*) and 4 °C. Then, the pellets were resuspended in phosphate buffer (200 μL, 0.1 M, pH 8.0) with lysozyme and DNase to lysis the cell pellets at 25 °C, 220 rpm, for 2 h (an additional 5 h at 37 °C was employed for the thermostability screening). Next, 1 mM substrates lansoprazole sulfide **1 a**, 5 % (*vol/vol*)

methanol as co-solvent and 1 mM NADPH was added to the crude cell lysate (the final volume was 400 μL); the reaction mixtures were shaken at 25 °C, 220 rpm, for 2 h. The reaction was quenched by dilution with 500 μL ethyl acetate and mixed thoroughly to extract the substrate, product, and/or by-product. The quenched mixture was centrifuged at 3800 (×*g*) for 10 min. An equal volume (100 μL) of formic acid (FA) and upper layer organic solution were mixed in 96-well microplates and detected with a microplate reader (SpectraMax M2, Molecular devices, USA).

1.3 Protein expression and purification

*Cb*BVMO$_{V1}$ is a three-point mutant of *Cb*BVMO$_{WT}$ (WP_059411749.1) containing three mutations G266D/L313P/V316A. The expression and preparation of *Cb*BVMO mutants were adopted according to the previous methods.[2] The procedures were as following: *E. coli* BL21 (DE3) cells were cultured in Terrific Broth (TB) medium containing kanamycin and induced by IPTG. Then the cells were harvested by centrifugation and resuspended in buffer A (50-mM Tris-HCl, 500-mM NaCl, 10-mM imidazole, pH 7.5) and disrupted by ultrasonication. After centrifugation for 40 min, the clarified cell-free extracts (CFEs) were loaded onto the His-Trap Ni-nitrilotriacetic acid FF columns which were pre-equilibrated with the binding buffer. The target protein was eluted with buffer B containing 50-mM Tris-HCl and 500-mM NaCl (pH 7.5) accompanied with an increasing gradient of imidazole from 10 to 500 mM. The collected eluents were detected by SDS-PAGE. The fraction containing the purified protein was collected and concentrated by ultrafiltration and exchanged with buffer C (50-mM Tris-HCl, 500-mM NaCl, pH 7.5). The freshly purified enzyme was then used for further measurements.

The construction of co-expression cells containing *Cb*BVMO and *Bst*FDH[3] followed a similar procedure. First, the sequences of *Cb*BVMO mutant and *Bst*FDH were separately amplified using suitable primers. Then, the vector pETDuet-1 underwent homologous recombination with the two amplified gene fragments. Each of the two genes was inserted into separate expression cassettes on the vector. The resulting recombinant plasmids were subsequently introduced into *E. coli* BL21 (DE3) through a transformation process. The PCR and homologous recombination procedures were same as those described above.

1.4 Assay of enzyme activity

The specific activity of *Cb*BVMO and its variants towards **1a** were detected as described in previous work.[2] In short, the reaction mixture contained 0.2 mM substrate, 2 % (*vol/vol*) methanol, 0.2 mM NADPH, and 0.2 μM *Cb*BVMO or variants, mixed in potassium phosphate buffer (100 mM, pH 8.0) and incubated at 3 °C and 1,000 rpm for 10 min. The reaction was quenched by dilution with 500 μL ethyl acetate. Each measurement was performed in triplicate.

The samples were analyzed by HPLC with a mobile phase of n-heptane and ethanol in the ratio

of 7:3 (*vol/vol*). The separation of the samples was carried out on a CHIRALPAK® IA column at 40°C, and the detection of the substrates and products was performed at 300 nm.

1.5 Characterization of the thermal stability of the enzyme

To assess the thermal stability of the *Cb*BVMO mutant, we employed the concept of dissolution temperature. The procedure involved dilution of the pure enzyme protein to a concentration of 0.5 mg/mL using Buffer C, which was originally used in the purification process. Subsequently, 20 μL of the diluted pure enzyme solution was dispensed into a fluorescence quantitative polymerase chain reaction (qPCR) tube. This solution was then subjected to monitoring using a SYBR green fluorescence emission filter, with the temperature adjusted incrementally from 25 °C to 95 °C at intervals of 0.5 °C. At each temperature, a 10-second incubation period was enforced to acquire stable fluorescence signals. The apparent melting point temperature of the protein was determined based on the maximum value of the first-order derivative derived from fitting the flavin adenine dinucleotide (FAD) fluorescence.[4]

1.6 Enzymatic synthesis of (*R*)-lansoprazole and (*R*)-rabeprazole

The reaction mixture (10 mL) was prepared by dissolving 30 or 50 mmol/L substrate **1a or 3a**, 0.1 mol/L sodium formate, 100 or 150 g/L fresh-cell lysates of co-expression cells, 5 % methanol (*vol/vol*), and 0.4 mM $NADP^+$ in a potassium phosphate buffer (100 mM, pH 9.0). The two-phase reaction system also contains different volume of toluene which depends on the ratio of the two phases. The reaction was carried out at 30 °C and 180 rpm for an appropriate time. Samples were intermittently removed and extracted for analysis o by HPLC as described in 1.4 .

1.7 Structural model obtaining and molecular docking

The 3D model of *Cb*$BVMO_{V1}$ was obtained by AlphaFold 2 software.[5] Molecular docking was performed with AutoDock Vina using the default program parameters.[6] The C4a atom of FAD was chosen as the center of the grid box, and the size of the grid box was set as 15 Å in each dimension. The docking results were selected according to their binding affinities and molecule conformations. Both model acquisition and molecular docking were performed using the default parameters of AlphaFold 2 and AutoDock Vina.

2. Sequence

Table S1 Primers used in the NNK saturation mutant library.

Primers	Sequences (5'—3')
D98-NNK-F	GAGCATCGCGGCGGGT**NNK**GCGATCCTGGAGTAC
D98-NNK-R	CTCCAGGATCGC**MNN**ACCCGCCGCGATGCTCTTATC
Y161-NNK-F	TTCTGTACCTGTGCAGCGGT**NNK**TATGATTATGCGGATGGTTA
Y161-NNK-R	TAACCATCCGCATAATCATA**MNN**ACCGCTGCACAGGTACAGAA
Y162-NNK-F	TGTACCTGTGCAGCGGTTAC**NNK**GATTATGCGGATGGTTATAT
Y162-NNK-R	ATATAACCATCCGCATAATC**MNN**GTAACCGCTGCACAGGTACA
A251-NNK-F	GGCTGCGTCGTAAGCTGCCG**NNK**GGTATGGCGCACCGTGTGAC
A251-NNK-R	GTCACACGGTGCGCCATACC**MNN**CGGCAGCTTACGACGCAGCC
G252-NNK-F	TGCGTCGTAAGCTGCCGGCG**NNK**ATGGCGCACCGTGTGACCCG
G252-NNK-R	CGGGTCACACGGTGCGCCAT**MNN**CGCCGGCAGCTTACGACGCA
M253-NNK-F	GTCGTAAGCTGCCGGCGGGT**NNK**GCGCACCGTGTGACCCGTTG
M253-NNK-R	CAACGGGTCACACGGTGCGC**MNN**ACCCGCCGGCAGCTTACGAC
R256-NNK-F	TGCCGGCGGGTATGGCGCAC**NNK**GTGACCCGTTGGAAAAACGT
R256-NNK-R	ACGTTTTTCCAACGGGTCAC**MNN**GTGCGCCATACCCGCCGGCA
V263-NNK-F	GTGTGACCCGTTGGAAAAAC**NNK**CTGTTCGATATGTACTTTTA
V263-NNK-R	TAAAAGTACATATCGAACAG**MNN**GTTTTTCCAACGGGTCACAC
L264-NNK-F	TGACCCGTTGGAAAAACGTT**NNK**TTCGATATGTACTTTTATCA
L264-NNK-R	TGATAAAAGTACATATCGAA**MNN**AACGTTTTTCCAACGGGTCA
F265-NNK-F	CCCGTTGGAAAAACGTTCTG**NNK**GATATGTACTTTTATCACCT
F265-NNK-R	AGGTGATAAAAGTACATATC**MNN**CAGAACGTTTTTCCAACGGG
F269-NNK-F	ACGTTCTGTTCGATATGTAC**NNK**TATCACCTGGCGCGTCGTAA
F269-NNK-R	TTACGACGCGCCAGGTGATA**MNN**GTACATATCGAACAGAACGT

L272-NNK-F	TCGATATGTACTTTTATCAC**NNK**GCGCGTCGTAAGCCGGAGCT
L272-NNK-R	AGCTCCGGCTTACGACGCGC**MNN**GTGATAAAAGTACATATCGA
D310-NNK-F	GCCCGGCGTATAAACCGTGG**NNK**CAACGTCCGTGCCTGGTGCC
D310-NNK-R	GGCACCAGGCACGGACGTTG**MNN**CCACGGTTTATACGCCGGGC
Q311-NNK-F	CGGCGTATAAACCGTGGGAT**NNK**CGTCCGTGCCTGGTGCCGGA
Q311-NNK-R	TCCGGCACCAGGCACGGACG**MNN**ATCCCACGGTTTATACGCCG
R312-NNK-F	CGTATAAACCGTGGGATCAA**NNK**CCGTGCCTGGTGCCGGATAG
R312-NNK-R	CTATCCGGCACCAGGCACGG**MNN**TTGATCCCACGGTTTATACG
C314-NNK-F	AACCGTGGGATCAACGTCCG**NNK**CTGGTGCCGGATAGCGACCT
C314-NNK-R	AGGTCGCTATCCGGCACCAG**MNN**CGGACGTTGATCCCACGGTT
L315-NNK-F	CGTGGGATCAACGTCCGTGC**NNK**GTGCCGGATAGCGACCTGTT
L315-NNK-R	AACAGGTCGCTATCCGGCAC**MNN**GCACGGACGTTGATCCCACG
A316-NNK-F	GGGATCAACGTCCGTGCCTG**NNK**CCGGATAGCGACCTGTTCAA
A316-NNK-R	TTGAACAGGTCGCTATCCGG**MNN**CAGGCACGGACGTTGATCCC
P317-NNK-F	ATCAACGTCCGTGCCTGGTG**NNK**GATAGCGACCTGTTCAAAAG
P317-NNK-R	CTTTTGAACAGGTCGCTATC**MNN**CACCAGGCACGGACGTTGAT
K367-NNK-F	CCGCGACCGGTCTGCAGCTG**NNK**GTGGCGGGTGGCATGCGTAT
K367-NNK-R	ATACGCATGCCACCCGCCAC**MNN**CAGCTGCAGACCGGTCGCGG
N408-NNK-F	CGGTGGCGATGGGTTACGTT**NNK**GCGAGCTGGACCCTGAAAGC
N408-NNK-R	GCTTTCAGGGTCCAGCTCGC**MNN**AACGTAACCCATCGCCACCG
A409-NNK-F	TGGCGATGGGTTACGTTAAC**NNK**AGCTGGACCCTGAAAGCGGA
A409-NNK-R	TCCGCTTTCAGGGTCCAGCT**MNN**GTTAACGTAACCCATCGCCA
S410-NNK-F	CGATGGGTTACGTTAACGCG**NNK**TGGACCCTGAAAGCGGAGCT
S410-NNK-R	AGCTCCGCTTTCAGGGTCCA**MNN**CGCGTTAACGTAACCCATCG

W411-NNK-F	TGGGTTACGTTAACGCGAGC**NNK**ACCCTGAAAGCGGAGCTGAG
W411-NNK-R	CTCAGCTCCGCTTTCAGGGT**MNN**GCTCGCGTTAACGTAACCCA
Q469-NNK-F	CGAGCGGTATTCTGCCGCGT**NNK**GGTAGCAAGCGTCCGTGGCG
Q469-NNK-R	CGCCACGGACGCTTGCTACC**MNN**ACGCGGCAGAATACCGCTCG
W475-NNK-F	GTCAAGGTAGCAAGCGTCCG**NNK**CGTGTGCACCAGAACTACCT
W475-NNK-R	AGGTAGTTCTGGTGCACACG**MNN**CGGACGCTTGCTACCTTGAC
R476-NNK-F	AAGGTAGCAAGCGTCCGTGG**NNK**GTGCACCAGAACTACCTGTT
R476-NNK-R	AACAGGTAGTTCTGGTGCAC**MNN**CCACGGACGCTTGCTACCTT
F483-NNK-F	GTGTGCACCAGAACTACCTG**NNK**GACCTGATGGCGCTGAAGTT
F483-NNK-R	AACTTCAGCGCCATCAGGTC**MNN**CAGGTAGTTCTGGTGCACAC
D484-NNK-F	TGCACCAGAACTACCTGTTC**NNK**CTGATGGCGCTGAAGTTTGG
D484-NNK-R	CCAAACTTCAGCGCCATCAG**MNN**GAACAGGTAGTTCTGGTGCA
L485-NNK-F	ACCAGAACTACCTGTTCGAC**NNK**ATGGCGCTGAAGTTTGGTAA
L485-NNK-R	TTACCAAACTTCAGCGCCAT**MNN**GTCGAACAGGTAGTTCTGGT
M486-NNK-F	AGAACTACCTGTTCGACCTG**NNK**GCGCTGAAGTTTGGTAAAGT
M486-NNK-R	ACTTTACCAAACTTCAGCGC**MNN**CAGGTCGAACAGGTAGTTCT
A487-NNK-F	ACTACCTGTTCGACCTGATG**NNK**CTGAAGTTTGGTAAAGTTGA
A487-NNK-R	TCAACTTTACCAAACTTCAG**MNN**CATCAGGTCGAACAGGTAGT
L488-NNK-F	ACCTGTTCGACCTGATGGCG**NNK**AAGTTTGGTAAAGTTGAGGA
L488-NNK-R	TCCTCAACTTTACCAAACTT**MNN**CGCCATCAGGTCGAACAGGT

Table S2 Primers for co-expression vector construction.

Entry	Primer name	Sequences
1	V3-MCS1-F	AGGAGATATACCATGGGCAGCAGCCATCATCATC
2	V3-MCS1-R	GCATTATGCGGCCGCTTAGCCGTGCGCGCTTTC
3	V3-MCS2-F	GAAGGAGATATACATATGGGCAGCAGCCATCATC
4	V3-MCS2-R	CTTTACCAGACTCGAGTTAGCCGTGCGCGCTTTC
5	FDH-MCS1-F	GGAGATATACCATGGCGACCGTGCTGTG
6	FDH-MCS1-R	GCATTATGCGGCCGCTCAGTGGTGGTGGTGGTG
7	FDH-MCS2-F	GAAGGAGATATACATATGGCGACCGTGCTGTGC
8	FDH-MCS2-R	CTTTACCAGACTCGAGTCAGTGGTGGTGGTG
9	pETDuet-MCS1-F	CCATGGTATATCTCCTTCTTAAAG
10	pETDuet-MCS1-R	GCGGCCGCATAATGC
11	pETDuet-MCS2-F	CATATGTATATCTCCTTCTTATACTTAAC
12	pETDuet-MCS2-R	CTCGAGTCTGGTAAAGAAAC
13	FDH-TAA-F	GAGCTATCGTCTGACC**TAA**CTCGAGCACCACCAC
14	FDH-TAA-R	GTGGTGGTGCTCGAG**TTA**GGTCAGACGATAGCTC

Table S3 Combinatorial library synthesized by Twist.

Variant	1	2	3	4	5	6	7	8	9	10
Position	252	253	256	269	272	316	485	486	487	488
AA	G	M	R	F	L	A	L	M	A	L
Codon	GGT	ATG	CGT	TTT	CTG	ATG	CTG	ATG	GCG	CTG
A						50			33	
C										
D						50			34	
E					50				33	
F				50			34			
G	33									
H										
I										
K										
L	34				50		33	50		50
M		25	33					50		
N										
P	33	25								
Q		25								
R			33							
S				50						
T			34							50
V		25								
W							33			
Y										
Variant AA's	3	4	3	2	2	2	3	2	3	2
Total %	100	100	100	100	100	100	100	100	100	100

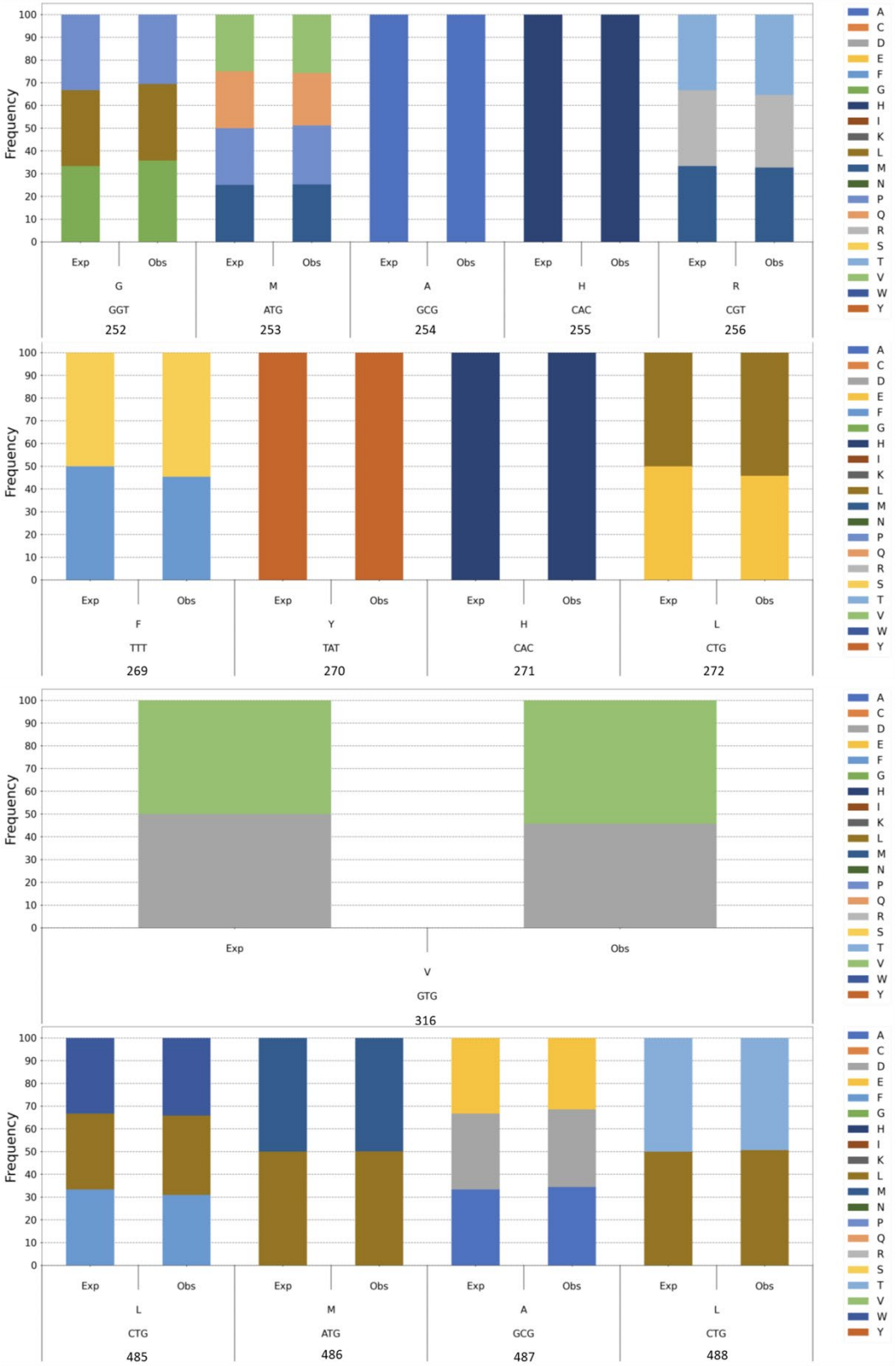


Figure S1. Distribution of mutant amino acids in Twist library. These results were calculated from NGS results. Exp: expected result; Obs: observed results.

3. Result

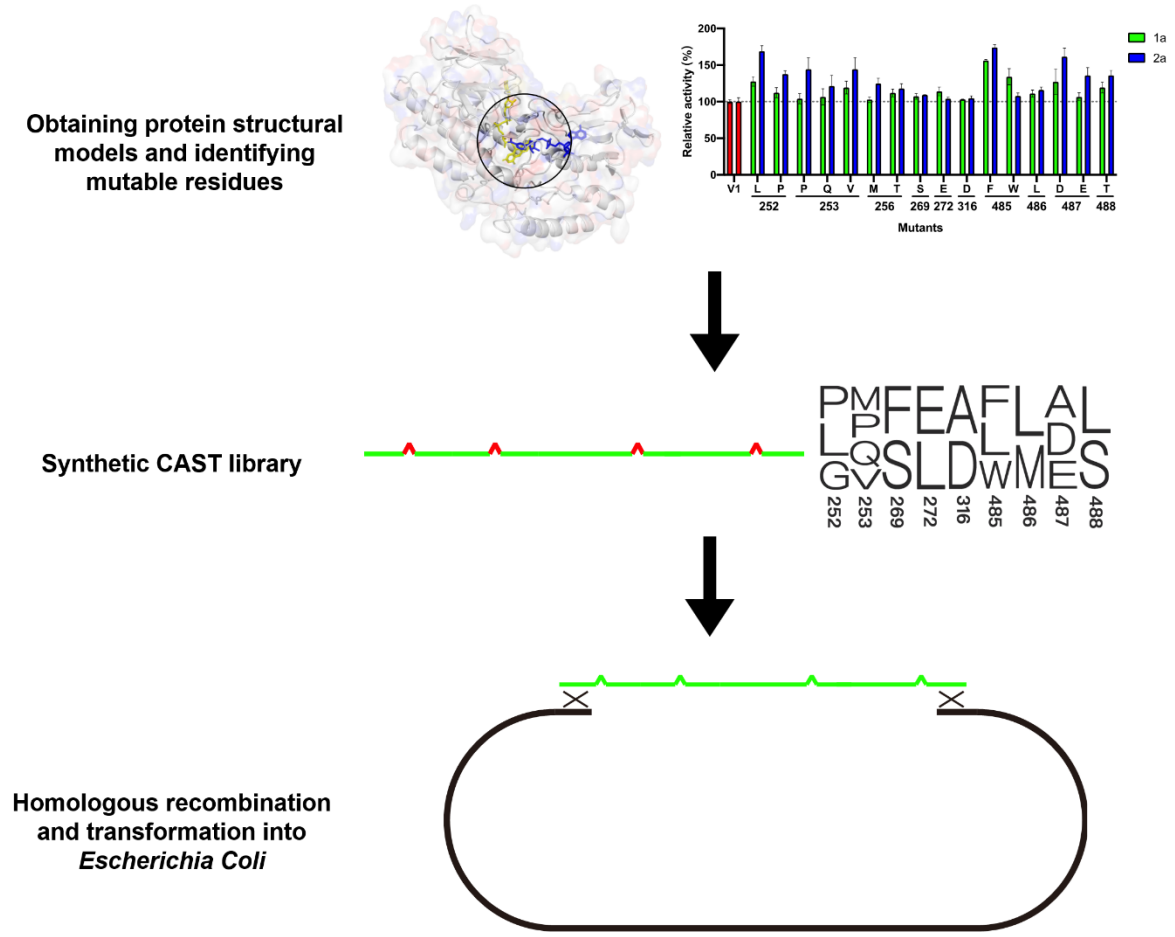


Figure S2. Synthetic CAST Library construction.

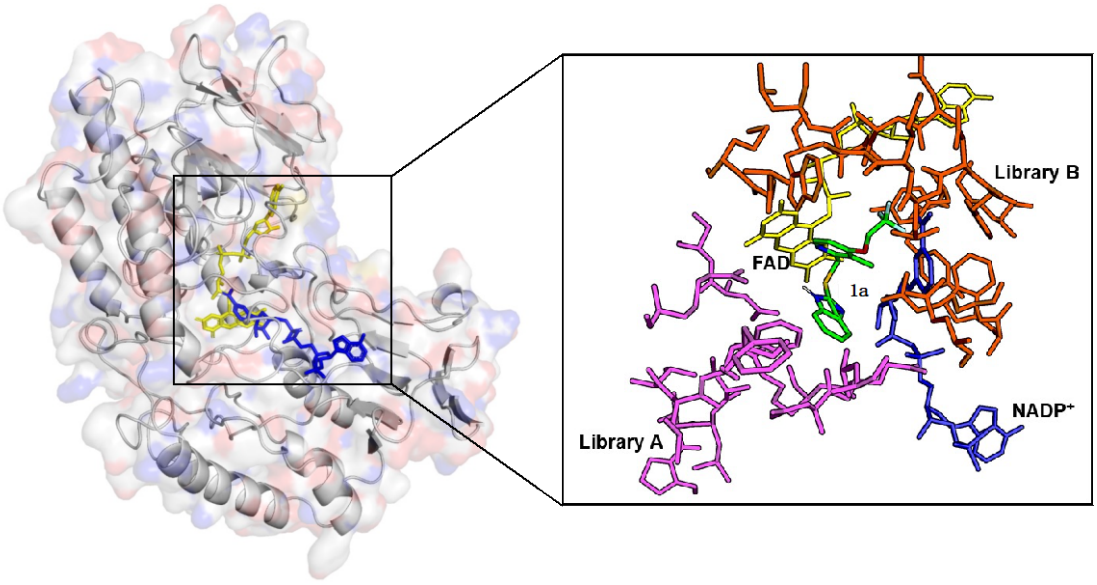


Figure S3. The overall structure model of *Cb*BVMOv1 (left) with amino acid residues in the 6 Å range around the substrate **1a** (right). The *Cb*BVMOv1 protein structure was predicted by AlphaFold 2, FAD (yellow), $NADP^+$ (blue) and substrate **1a** (green, right) were docked by the AutoDock Vina. The 32 amino acid sites in the 6 Å range around the substrate were selected for mutagenesis experiments.

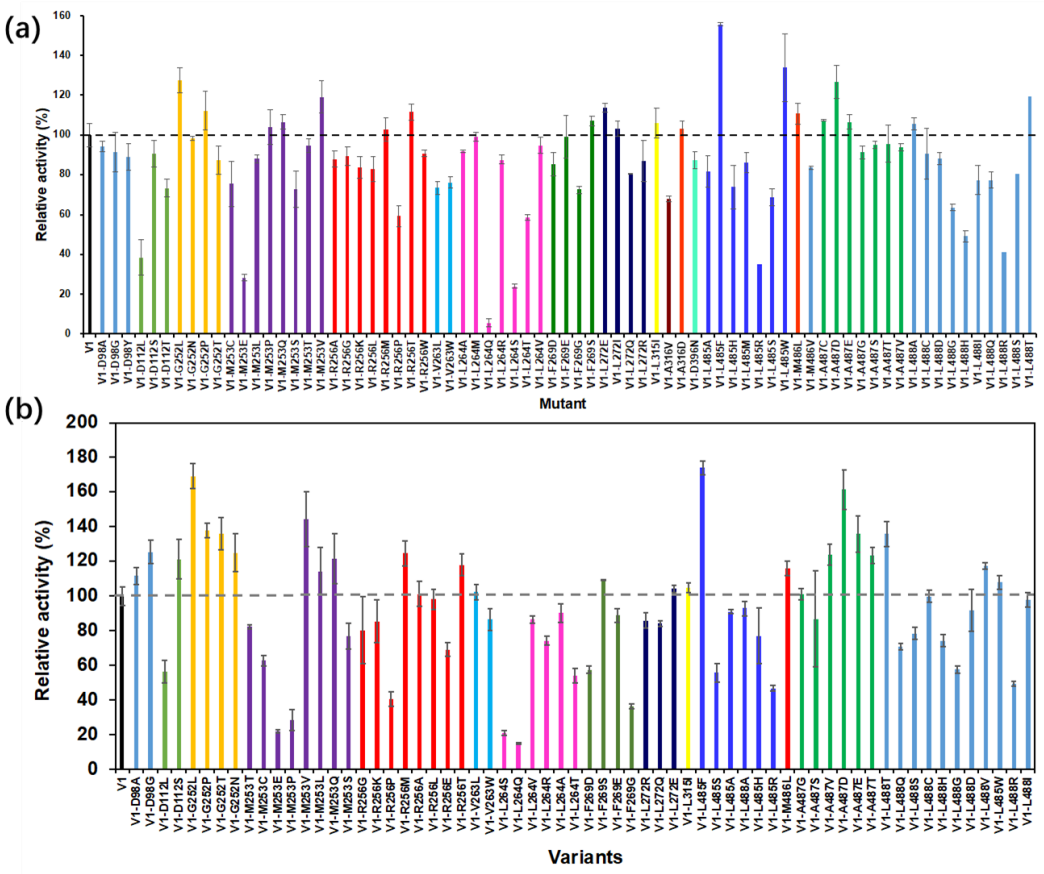


Figure S4. Activity determination of the rescreened variants towards **1a** (A) and **2a** (B). The specific activity assay mixture, containing 0.2 mM 1a or 2a, 0.2 mM NADPH, 2% methanol and KPB (100 mM, pH 8.0) and 0.35 μM purified variants, was shaken at 30 ºC and 1000 rpm for 10 min. All experiments were done in triplicates and the average values were adopted, and result was analyzed by HPLC.

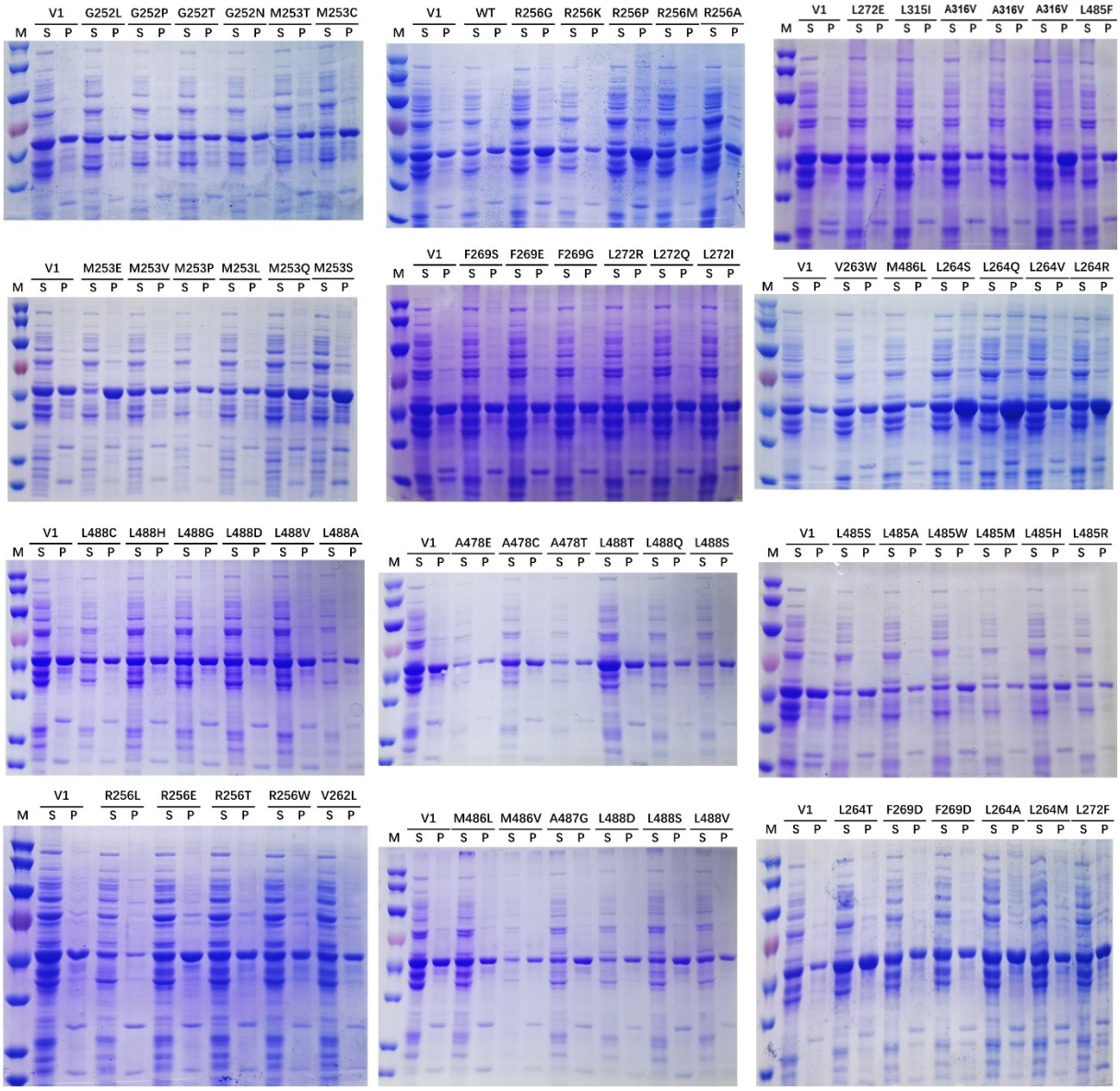


Figure S5. Protein expression of rescreened variants in *E. coli* BL21 (DE3) cells. V1:*Cb*BVMO$_{G266D/L313P/V316A}$; S: Supernatant; P: Precipitation.

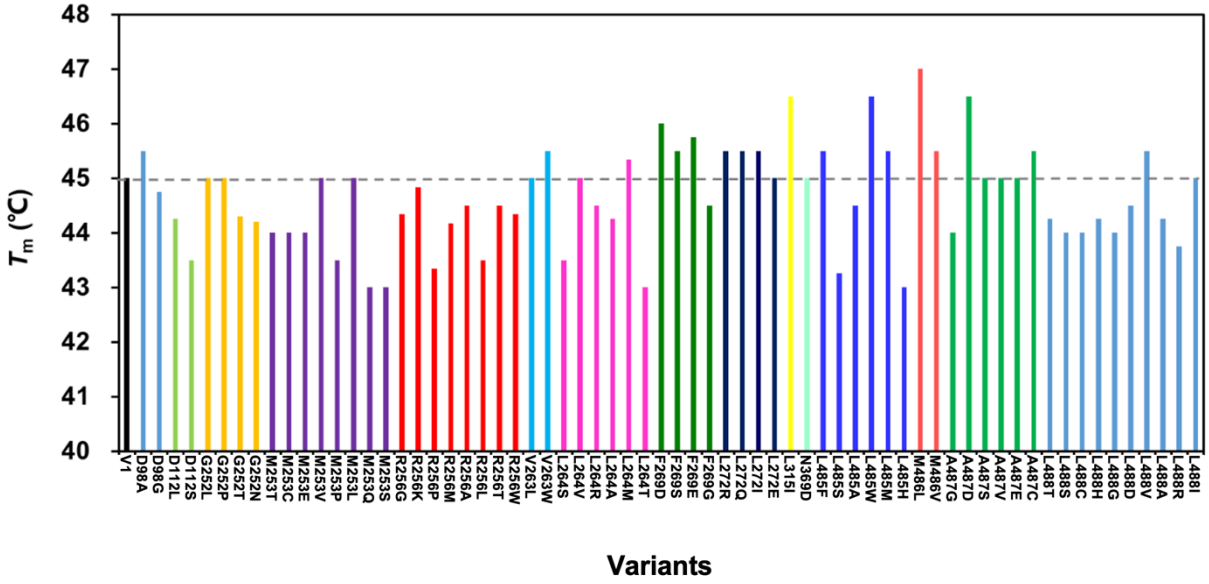


Figure S6. Determination the melting point temperature of the rescreened variants.

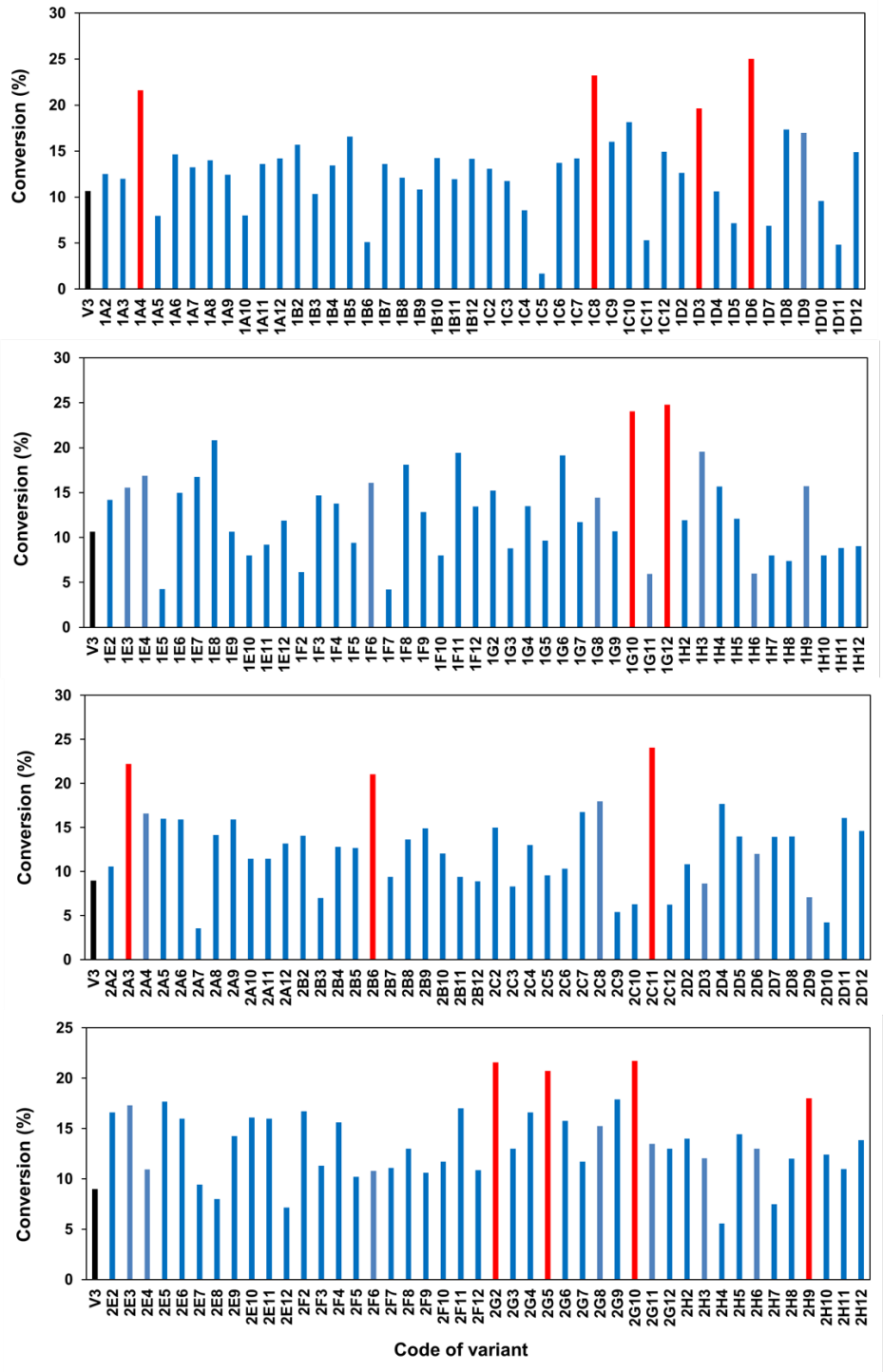


Figure S7. Rescreening results of Twist library. The 400 μL reaction mixture, containing 1.0 mM lansoprazole sulfide, 1.0 mM NADPH, 10% methanol, and cell lysate in KPB (100 mM, pH 8.0), was shaken at 30ºC and 800 rpm for 2 h. The reaction was quenched by adding 500 μL ethyl acetate and analyzed by HPLC. All experiments were done in triplicates and the average values were adopted.

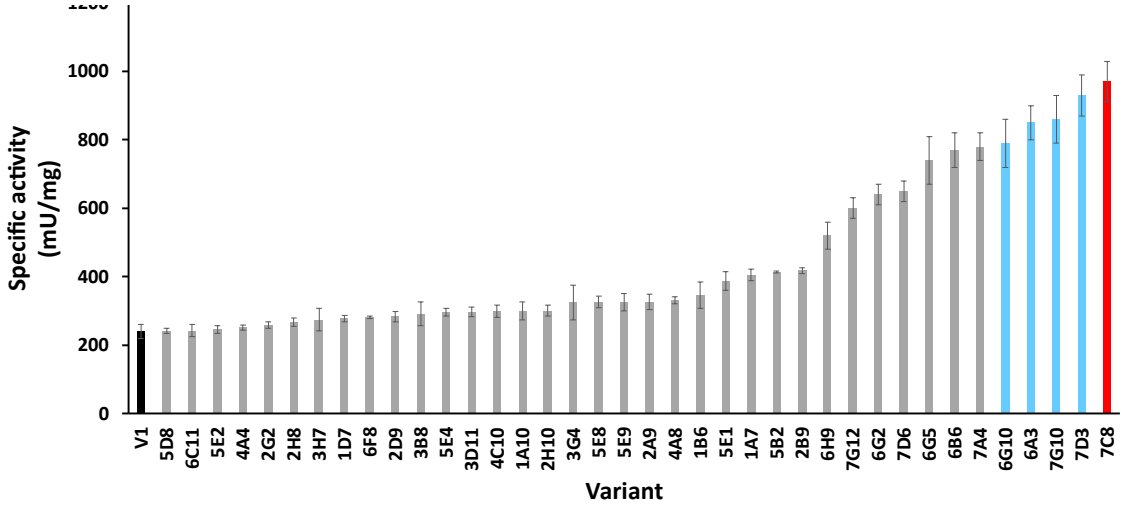
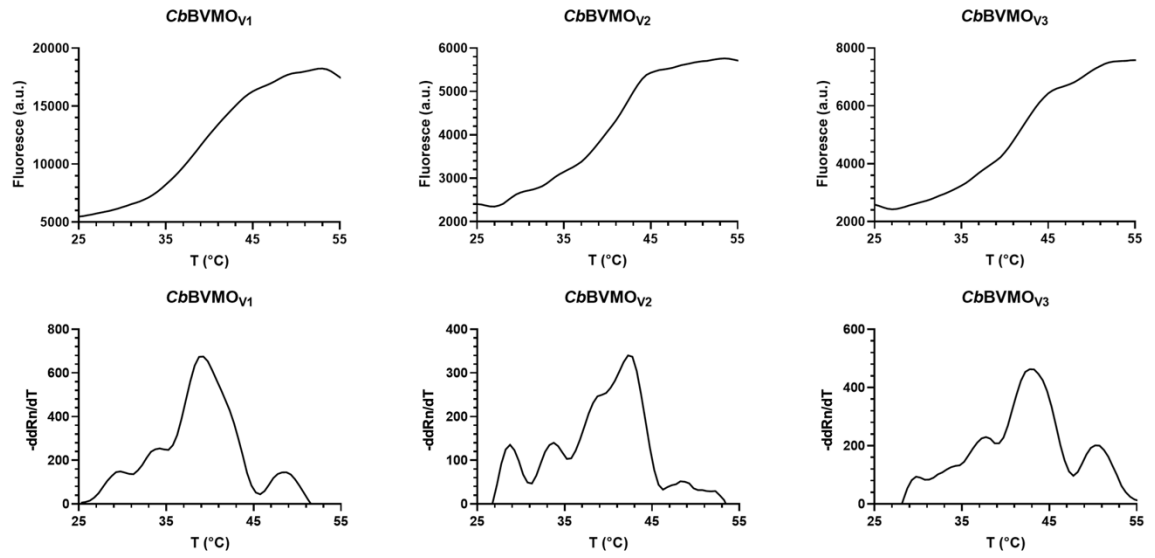


Figure S8. A total of 38 combinatorial mutants with increased activity towards **1a** were obtained. Five mutants with the higher activity are indicated in a different colour, with the best mutant, *Cb*BVMO$_{V2}$, in red and the remaining four in sky blue. The specific activity was analyzed by HPLC.



	*Cb*BVMO$_{V1}$	*Cb*BVMO$_{V2}$	*Cb*BVMO$_{V3}$
T_m (°C)	39.3	41.8	43.5

Figure S9. The stability assessment of *Cb*BVMO$_{V1}$, *Cb*BVMO$_{V2}$, and *Cb*BVMO$_{V3}$ mutants was conducted. Solubilization profiles were established through the utilization of a quantitative polymerase chain reaction (qPCR) instrument, with fluorescence being recorded using SYBR green fluorescence emission filters. This process involved the continuous monitoring of fluorescence variations at 0.5 °C intervals, spanning a temperature range from 25-95 °C.

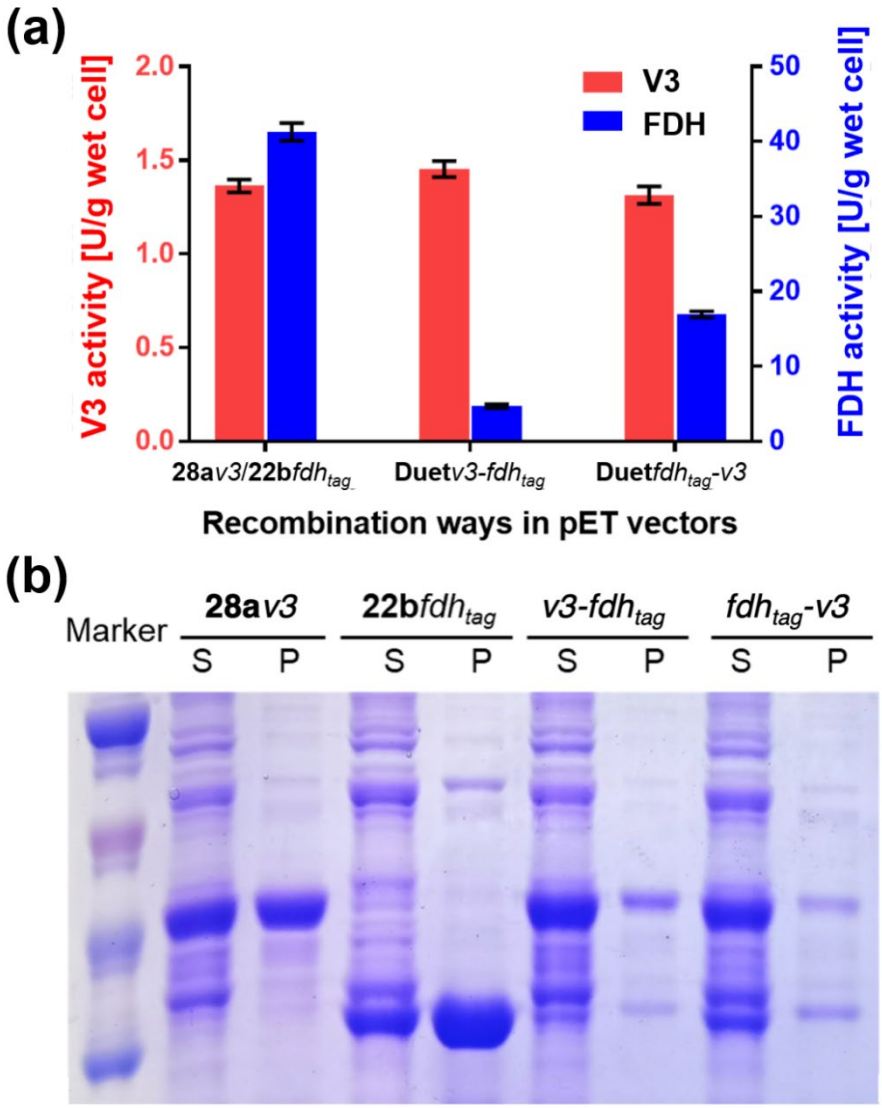


Figure S10. The effect of the sequence order of *Cb*BVMO$_{V3}$ and FDH encoding genes on co-expression. (a) Crude enzyme activity of *Cb*BVMO$_{V3}$ and FDH; (B) Protein expression of *Cb*BVMO$_{V3}$ and FDH.

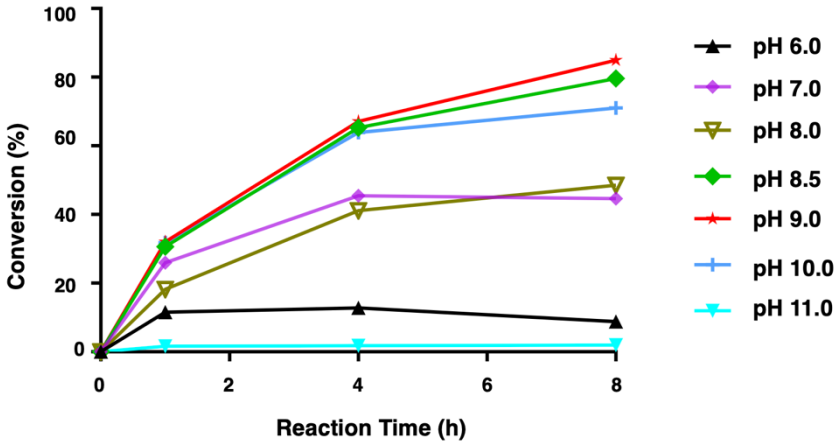


Figure S11. Effects of pH on lansoprazole sulfide oxidation reaction catalyzed by co-expressed cell lysates. The 10 mL reaction mixture (pH 6.0~11.0) contained 5 mM lansoprazole sulfide, 0.2 mM $NADP^+$, 100 mM HCOONa, 5% (*vol/vol*) methanol, cell-free extract of 0.1 g co-expressed wet cell. The reaction mixtures were incubated at 30°C, 180 rpm, and analyzed by HPLC.

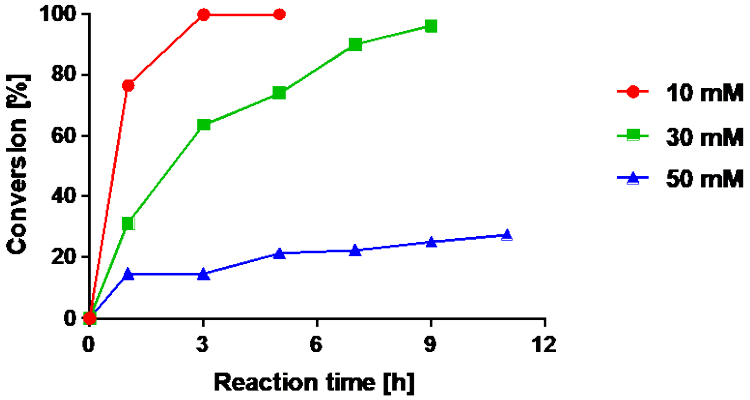


Figure S12. Effects of substrate loading on lansoprazole sulfide oxidation reaction. The 10 mL reaction mixture (100 mM KPB, pH 9.0) contained 10, 30 or 50 mM lansoprazole sulfide, 0.4 mM $NADP^+$, 100 mM HCOONa, 5% (*vol/vol*) methanol, and cell-free extract of 0.9 g co-expression wet cell. The reaction mixtures were incubated at 30°C, 180 rpm, and analyzed by HPLC.

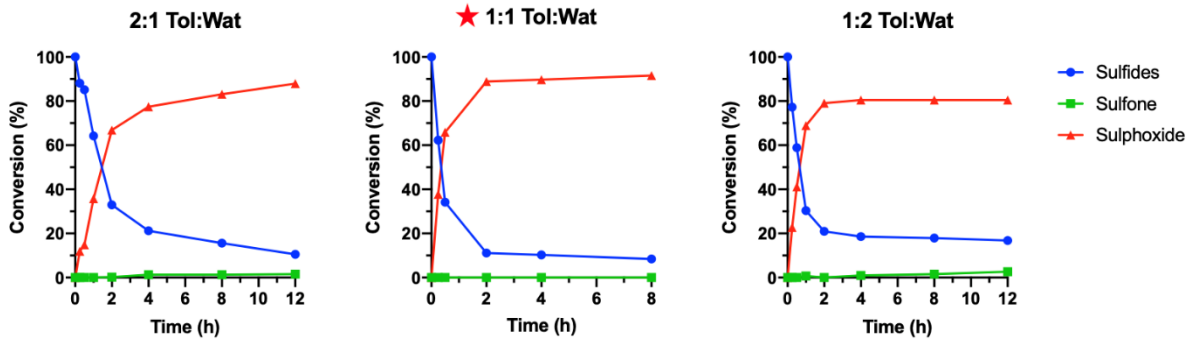


Figure S13. Effects of two-phase system on lansoprazole sulfide oxidation reaction catalyzed by co-expressed cell lysates. The total volume of the two-phase system was 10 mL, which contained 50 mM lansoprazole sulfide, 0.4 mM $NADP^+$, 100 mM HCOONa, and cell-free extract of 0.9 g co-expression wet cell. The buffer for the aqueous phase was 100 mM KPB Buffer (pH 9.0). For the different two-phase proportion of reaction, 3.3 mL, 5 mL or 6.7 mL toluene was added to the different reaction systems. The reaction mixtures were incubated at 30 ℃, 180 rpm, and analyzed by HPLC.

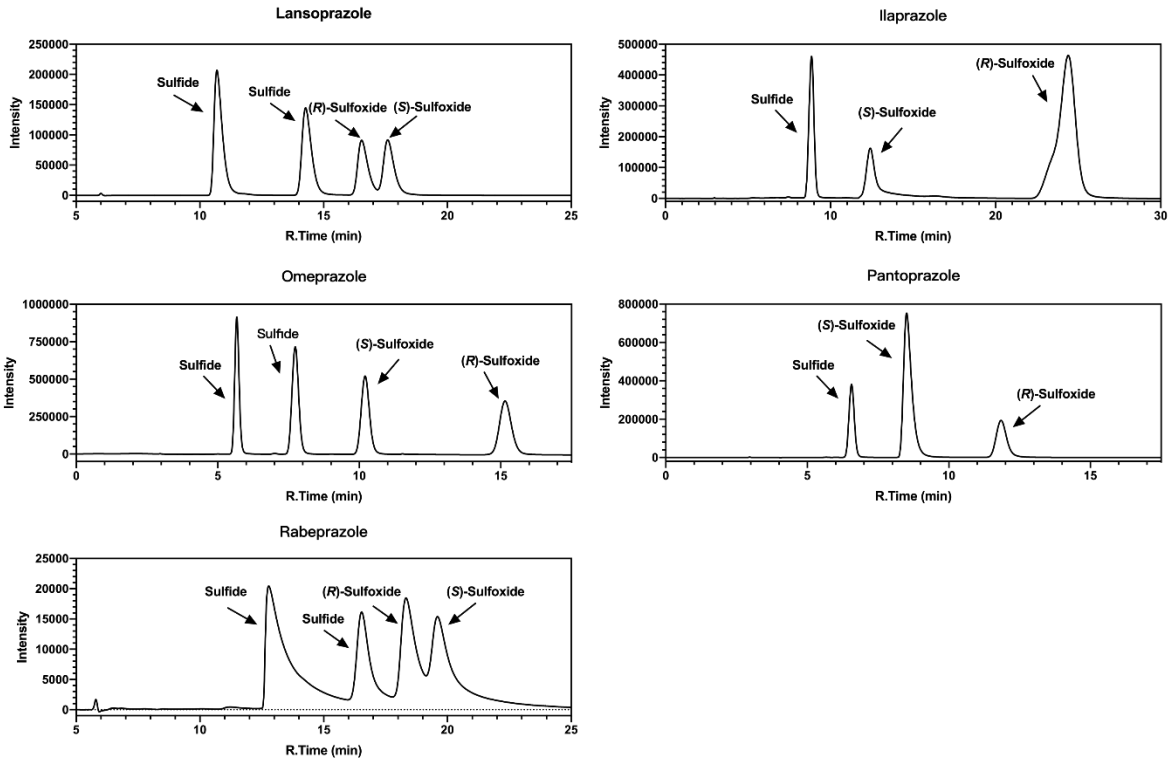


Figure S14. Liquid chromatograms of substrates **1a-5a** and their corresponding oxidation products. The samples were analyzed by HPLC with a mobile phase of *n*-heptane and ethanol in the ratio of 7:3 (*vol/vol*). The separation of the samples was carried out on a CHIRALPAK® IA column at 40°C, and the detection of the substrates and products was performed at 300 nm.

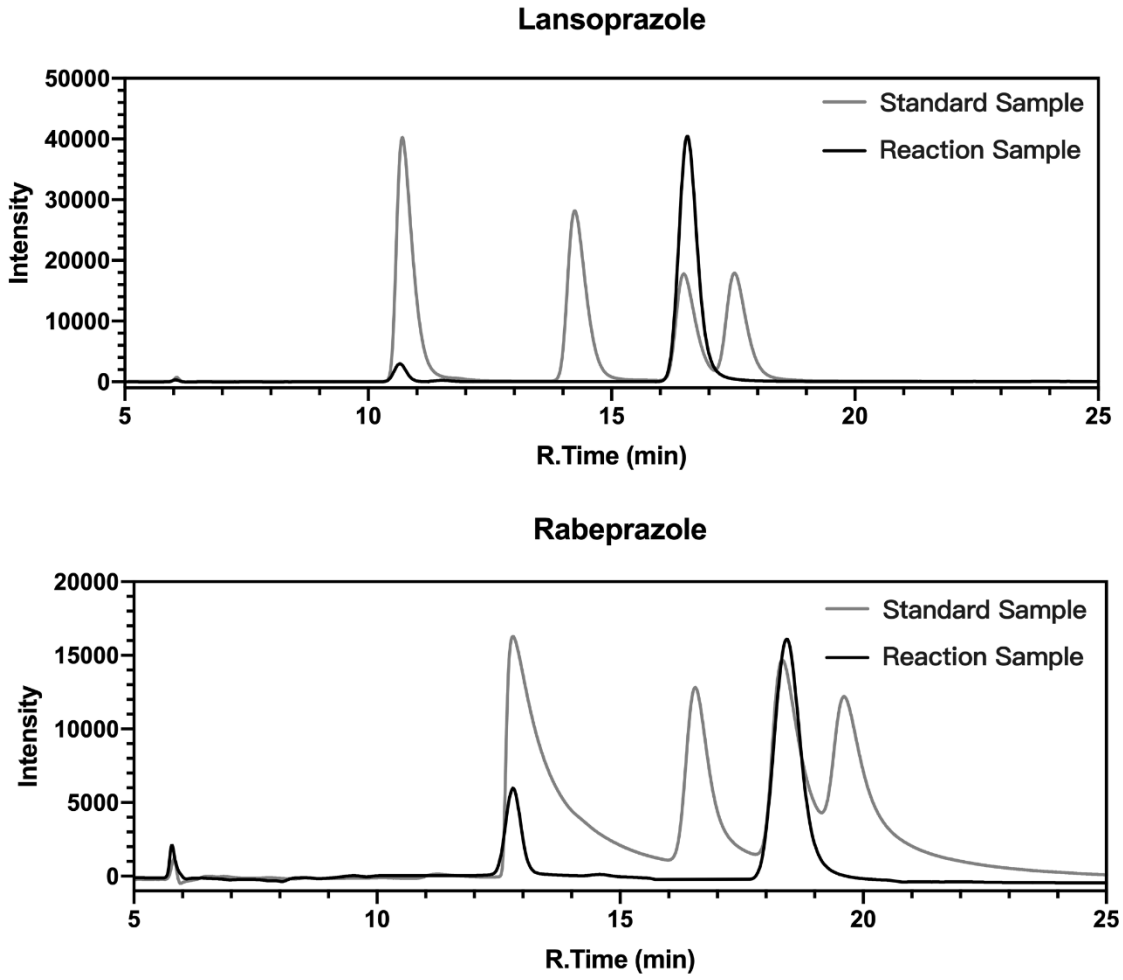


Figure S15. Liquid chromatographic results of the reactions catalyzed by *Cb*BVMO$_{V3}$ in two-phase system for substrates **1a** and **3a**.

4. References

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