Computational-Based Mechanistic Study and Engineering of Cytochrome P450 MycG for Selective Oxidation of 16-Membered Macrolide Antibiotics

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Generation of MycG-RhFRED mutants

Mutants were generated by whole-plasmid amplification using the pET28b_MycG-RhFRED plasmid[1] as template and the mutagenic primers listed in the table below.

Table S1.

Mutation(s)	Primer sequence (5'–3')
R380E	CGAGACGCAGCTGGAGTGGAGCGAAGG
K80A	CGTCCCGAGATGGTCGCGGGCGGGCTGCTGTCC
R140A	GGTGGCCATGTTCGCCGCGCAGTTGCCGGTCAGGG
R73A	CCCGCGACGAGCCTGCGACCCGTCCCGAGATGG
S170D	CCGGGGCCTTCCTCGATACCGCCGAGGTGACG
R163A	CCACGACCGCTTCACCGCGTGGTCCGGGGCCTTCC
L280F	CGCGGTGGGTGCCGTTTGGGGTGGGAACGG
D226A	GCAGGAACTGCTCGCGTTGGCCATTGGTC
L83N/L94N	GTCAAGGGCGGGAACCTGTCCATGGATCCGCCCGAGCACAGCCGAAACCGCCGACTCGTC
L227N	CAGGAACTGCTCGACAACGCCATTGGTCTGCTC
L83E/L94R	GTCAAGGGCGGGGAACTGTCCATGGATCCGCCCGAGCACAGCCGACGTCGCCGACTCGTC
L227E	GCAGGAACTGCTCGACGAAGCCATTGGTCTGCTCG
E237A	GCTCGTCGCGGGATACGCCAGCACGACGACCCAG
G281K	GCGGTGGGTGCCGCTGAAAGTGGGAACGGCGTTCC

Expression and purification of proteins for analytical-scale reactions

The pET28b plasmid containing the gene encoding MycG-RhFRED or mutants thereof was transformed into chemically competent *E. coli* C41(DE3) cells, and individual colonies were selected for overnight growth (37 °C) in 15 mL of LB containing kanamycin (50 μg/mL). 2 x 1.5 L of TB (2.8 L baffled Fernbach flasks) supplemented with kanamycin (50 μg/mL), thiamine (1 mM), and glycerol (4% v/v) were inoculated with the 15 mL overnight seed cultures and incubated at 37 °C (160 rpm). When the OD_{600} reached 0.6-1.0, the cultures were cooled in an ice-water bath (15-20 min) before addition of IPTG (0.1 mM) and δ-aminolevulinic acid (1 mM). The cultures were grown at 18 °C for 20 h before the cells were harvested and stored at -80 °C until used for protein purification.

All subsequent protein purification steps were performed at 4 °C. The cells were thawed and resuspended in 90 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol, 1 mM PMSF, pH 8) prior to lysis in two separate equal-volume batches using a sonic dismembrator (70% power, 5 s on, 5 s off, 5 min total on time). The crude lysate was centrifuged at 50,000 x g for 30 min to remove cellular debris, and the clarified lysate was incubated with 5 mL of Ni-NTA resin on a nutating shaker for 1 h. The resin slurry was loaded onto an empty column, and the lysate was pushed through with gentle syringe pressure. The resin was washed with 3 x 25 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol, pH 8) to remove bulk protein contaminants prior to elution of the protein of interest with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, 10% (v/v) glycerol, pH 8). The protein was subsequently concentrated using 50 kD MWCO centrifugal filters (Millipore) and desalted by loading onto PD-10 columns (GE Healthcare) and eluting with storage buffer (50 mM NaH_2PO_4, 1 mM EDTA, 0.2 mM DTT, 10% (v/v) glycerol, pH 7.3) according to the manufacturer's instructions. The purified enzyme was flash frozen in liquid N_2 and stored at -80 °C until used for analytical-scale reactions. All P450 concentrations were determined using the established CO-binding assay.[2]

Analytical-scale enzymatic reactions

Analytical-scale enzymatic reactions were carried out under the following conditions: 5 μM P450-RhFRED, 500 μM substrate (2.5% DMSO, final concentration), 1 mM $NADP^+$, 5 mM

glucose-6-phosphate, and 1 U/mL glucose-6-phosphate dehydrogenase in storage buffer (50 mM NaH_2PO_4, 1 mM EDTA, 0.2 mM DTT, 10% (v/v) glycerol, pH 7.3). The total volume of each reaction was 100 µL (1.7 mL Eppendorf tube), and reactions were incubated at 30 °C (200 rpm) for 3 h prior to quenching by addition of 200 µL of methanol. Quenched reactions were centrifuged at 17,000 x g for 10 min (4 °C), and the supernatant was removed for LC-MS analysis. All reactions were performed and analyzed in duplicate. Negative control reactions performed in parallel lacked P450-RhFRED.

LC-MS analysis was performed on an Agilent 6230 Time-of-Flight (TOF) mass spectrometer (Life Sciences Institute, University of Michigan) equipped with a Dual AJS ESI source and an Agilent 1290 Infinity series diode array detector, autosampler, and binary pump. For efficient separation of reaction components, a Waters XBridge C18 column with the following specifications was used: dimensions, 150 x 2.1 mm; particle size, 3.5 µm; pore size, 130 Å. HPLC conditions were as follows: mobile phase (A = deionized water + 0.1% formic acid, B = 95% acetonitrile/deionized water + 0.1% formic acid); 10% B (pre-run) for 2.5 min, 10% to 100% B over 12.5 min, 100% B for 2.5 min, 10% B (post-run) for 2.5 min; flow rate, 0.2 mL/min; injection volume, 0.5 µL. Percent conversion values were determined by integrating extracted-ion chromatogram (EIC) peaks corresponding to $[M + H]^+$ ions for unreacted substrate and potential products as previously described.[3] Retention times of substrates and observed products are shown in the table below.

Table S2.

Compound	Retention time (min)
M-IV	9.36
14-hydroxy-M-IV (M-V)	8.63
12,13-epoxy-M-IV (M-I)	9.27
12,13-epoxy-14-hydroxy-M-IV (M-II)	8.61
N-desmethyl-M-IV	9.21
N-desmethyl-14-hydroxy-M-IV	8.51
N-desmethyl-12,13-epoxy-M-IV	9.11
M-III	8.71
14-hydroxy-M-III (M-IX)	8.02
12,13-epoxy-M-III	8.64
12,13-epoxy-14-hydroxy-M-III	8.06
N-desmethyl-M-III	8.55
N-desmethyl-14-hydroxy-M-III	7.89
N-desmethyl-12,13-epoxy-M-III	8.46
M-VI	8.39
14-hydroxy-M-VI (M-XV)	7.72
12,13-epoxy-M-VI	8.32
12,13-epoxy-14-hydroxy-M-VI	7.74
N-desmethyl-M-VI	8.23
N-desmethyl-14-hydroxy-M-VI	7.61
N-desmethyl-12,13-epoxy-M-VI	8.14

Table S3. Mycinamicin IV (**M-IV**) Products with MycG Mutants*

MycG-RhFRED mutant	Hydroxylation (M-V)	Epoxidation (M-I)	Hydroxylation + epoxidation (M-II)	*N*-demethylation (starting material)	*N*-demethylation (oxidation products)	Total conversion
Wild-type	31.6 ± 0.8	39.2 ± 0.3	21.9 ± 1.5	0	0.6 ± 0.0	93.2 ± 0.4
R380E	19.8 ± 2.7	41.3 ± 0.4	32.7 ± 3.3	0	0.7 ± 0.0	94.4 ± 0.2
K80A	35.8 ± 0.9	19.9 ± 0.1	1.7 ± 0.1	0.8 ± 0.0	0.2 ± 0.0	58.3 ± 1.2
R140A	44.1 ± 1.3	28.8 ± 0.6	4.2 ± 0.6	0.4 ± 0.0	0.3 ± 0.0	77.8 ± 2.6
R73A	43.5 ± 0.8	23.3 ± 0.4	23.8 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	91.2 ± 0.2
S170D	24.7 ± 1.0	32.5 ± 2.0	4.6 ± 0.6	0	0.1 ± 0.0	61.9 ± 3.6
R163A	18.1 ± 3.8	39.2 ± 0.3	36.2 ± 4.2	0	0.9 ± 0.0	94.4 ± 0.1
L280F	25.2 ± 3.6	35.6 ± 0.0	30.7 ± 3.8	0	0.7 ± 0.0	92.3 ± 0.1
D226A	42.9 ± 0.3	29.9 ± 0.4	4.8 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	78.8 ± 0.7
L83N/L94N/L227N	0.4 ± 0.0	0.2 ± 0.1	0	3.6 ± 0.8	0	4.3 ± 0.9
L83E/L94R/L227E	1.0 ± 0.0	0.4 ± 0.0	0	0.8 ± 0.0	0	2.2 ± 0.0
E237A/G281K	0	0	0	0.8 ± 0.0	0	0.8 ± 0.0

Table S4. Mycinamicin III (**M-III**) Products with MycG Mutants*

MycG-RhFRED mutant	Hydroxylation (M-IX)	Epoxidation	Hydroxylation + epoxidation	*N*-demethylation (starting material)	*N*-demethylation (oxidation products)	Total conversion
Wild-type	15.1 ± 0.0	3.5 ± 0.1	0.1 ± 0.0	2.5 ± 0.0	0	21.1 ± 0.1
R380E	20.7 ± 0.0	5.6 ± 0.2	0.2 ± 0.0	2.0 ± 0.1	0	28.5 ± 0.2
K80A	1.8 ± 0.1	0.2 ± 0.0	0	1.6 ± 0.1	0	3.6 ± 0.2
R140A	9.6 ± 0.3	1.6 ± 0.0	0	2.6 ± 0.2	0	13.8 ± 0.5
R73A	25.5 ± 0.2	3.0 ± 0.0	0.2 ± 0.0	3.1 ± 0.1	0.1 ± 0.0	32.0 ± 0.3
S170D	28.5 ± 0.5	15.9 ± 0.2	0.6 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	45.4 ± 0.8
R163A	17.9 ± 0.5	4.1 ± 0.0	0.1 ± 0.0	2.4 ± 0.1	0	24.5 ± 0.6
L280F	15.1 ± 0.2	3.6 ± 0.1	0.1 ± 0.0	1.6 ± 0.0	0	20.3 ± 0.2
D226A	2.9 ± 0.0	0.4 ± 0.0	0	2.1 ± 0.0	0	5.5 ± 0.1
L83N/L94N/L227N	0	0	0	1.0 ± 0.0	0	1.0 ± 0.0
L83E/L94R/L227E	0	0	0	0.5 ± 0.0	0	0.5 ± 0.0
E237A/G281K	0	0	0	0.8 ± 0.2	0	0.8 ± 0.2

Table S5. Mycinamicin VI (**M-VI**) Products with MycG Mutants*

MycG-RhFRED mutant	Hydroxylation (M-XV)	Epoxidation	Hydroxylation + epoxidation	*N*-demethylation (starting material)	*N*-demethylation (oxidation products)	Total conversion
Wild-type	3.0 ± 0.2	1.1 ± 0.2	0	4.9 ± 0.3	0	9.1 ± 0.8
R380E	5.9 ± 0.3	2.9 ± 0.3	0	4.6 ± 0.1	0.1 ± 0.0	13.4 ± 0.7
K80A	0.1 ± 0.0	0	0	3.0 ± 0.1	0	3.2 ± 0.1
R140A	2.4 ± 0.1	0.7 ± 0.2	0	4.6 ± 0.3	0	7.7 ± 0.6
R73A	7.8 ± 0.1	1.1 ± 0.2	0	4.9 ± 0.1	0	13.8 ± 0.3
S170D	16.2 ± 0.6	21.0 ± 1.7	0.2 ± 0.0	1.0 ± 0.0	0.2 ± 0.0	38.5 ± 2.4
R163A	4.3 ± 0.1	1.6 ± 0.3	0	4.4 ± 0.2	0	10.4 ± 0.6
L280F	32.7 ± 1.1	4.6 ± 0.6	0.3 ± 0.0	3.6 ± 0.2	0.2 ± 0.0	41.4 ± 1.9
D226A	0.1 ± 0.0	0	0	4.3 ± 0.1	0	4.4 ± 0.1
L83N/L94N/L227N	0	0	0	1.8 ± 0.1	0	1.8 ± 0.1
L83E/L94R/L227E	0	0	0	1.8 ± 0.1	0	1.8 ± 0.1
E237A/G281K	0	0	0	1.5 ± 0.0	0	1.5 ± 0.0

* The values in each table are % of total reaction mixture (i.e., % conversion to a particular product). Errors are standard deviations of results from duplicate experiments.

References for Experimental Section

(1) Zhang, W.; Liu, Y.; Yan, J.; Cao, S.; Bai, F.; Yang, Y.; Huang, S.; Yao, L.; Anzai, Y.; Kato, F.; Podust, L. M.; Sherman, D. H.; Li, S. *J. Am. Chem. Soc.* **2014**, *136*, 3640.

(2) Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370.

(3) Li, S.; Chaulagain, M. R.; Knauff, A. R.; Podust, L. M.; Montgomery, J.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 18463.

Quantum mechanics methods and details

DFT calculations were performed using the Gaussian09 suite.[1] Conformational sampling of substrates and transition states were performed using Spartan.[2] The default settings for conformer distribution calculations were used. For transition states, the O_{Fe}-$H_{substrate}$ and $H_{substrate}$-$C_{substrate}$ bonds and were frozen and other substrate torsions were sampled, including rotation about the C-H bond being abstracted. Due to the large number of conformers of each substrate and transition state, the iron-oxo model and transition structures were computed at the quartet spin state. Geometry optimizations and frequency calculations were performed at the B3LYP level.[3] The 6-31G(d) basis set was used on all atoms except Fe, for which the LANL2DZ pseudopotential was used. Saddle point transition structures were confirmed by the presence of one imaginary frequency corresponding to the desired transformation. Thermal corrections were computed at 1 atm and 298.15 K. The quasi-harmonic correction, as described by Truhlar, was used to adjust the Gibbs free energy.[4] Single point calculations were performed at the B3LYP-D3(BJ)[5] level with CPCM[6] implicit solvation for water. LANL2DZ was used for Fe and 6-311+G(d,p) was used for all other atoms. All 3D images for DFT calculations were made with CYLview.[7]

MD simulations

The heme iron(IV)-oxo complex involved in the cytochrome P450-catalyzed oxidative hydroxylation cycle (compound I) was used to model the active form of the MycG cofactor. MD simulations were performed using the GPU code (pmemd) of the AMBER 14 package.[8,9,10] The Amber-compatible parameters developed by Cheatham et al. were used for compound I and its axial Cys ligand.[11] The parameters for the substrates (**M-IV**, **M-III**, and **M-VI**) were generated with the antechamber module using the general AMBER force field (gaff),[12,13] with partial charges set to fit the electrostatic potential generated at the HF/6-31G(d) level by the RESP model.[14] The charges were calculated according to the Merz-Singh-Kollman scheme using the Gaussian09.[1] MycG was immersed in a pre-equilibrated truncated octahexon with a 10 Å buffer of TIP3P water molecules using tleap module.[15] All subsequent calculations were done using Amber/ff99SBildn force field.[16] We performed 100 ns restrained simulations starting from the crystal structure (cocrystallized with **M-IV**) of MycG (PDB 2Y98). **M-III** and **M-VI** were manually docked into MycG with the same binding pose as **M-IV**. 500 kcal/(mol·Å)^2 harmonic restraints were added on the H_{C14}--O_{Fe} distance. The system was optimized and then gradually heated to 300 K, and was then allowed to equilibrate. Production trajectories were run for 100 ns under restraint and then for 400 ns without any restraint. Images within the manuscript were made using Chimera[17] and images in TOC graphic were made using PyMol.[18]

MetaDynamics

Metadynamics were conducted to quantify the difference in MycG activities toward each substrate. NAMD v2.9 was used for the Metadynamics simulations with ff99SBild force fields.[19,20] Well-tempered Metadynamics in conjunction with multiple-walker algorithm were used to accelerate the convergence of the free energy landscape computed from the Metadynamics simulations.[21] The Langevin thermostat controlled at 300 K and Langevin barostat kept the pressure at 1 atm.[22] We chose the H_{14}--O_{Fe} distance as the collective variable to describe the binding of substrate in MycG, with the range of the collective variable to be 7.9 – 1.1 Å.

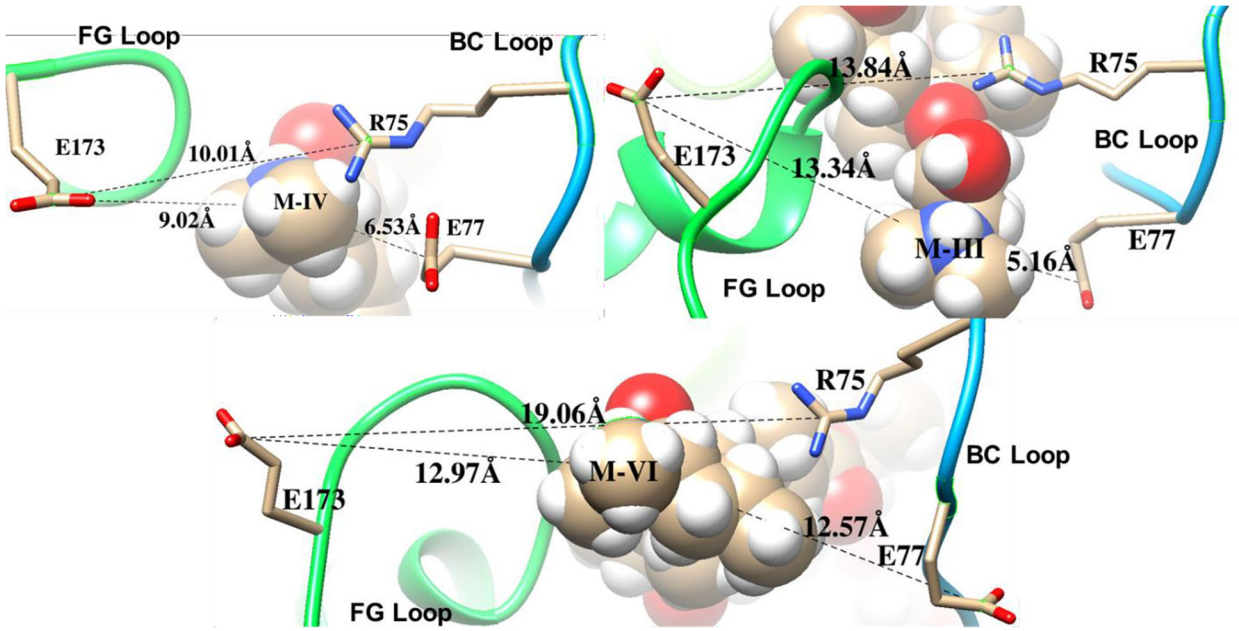

Figure S1. Average distance between each pair of salt bridge interactions in **M-IV**, **M-III**, and **M-VI**.

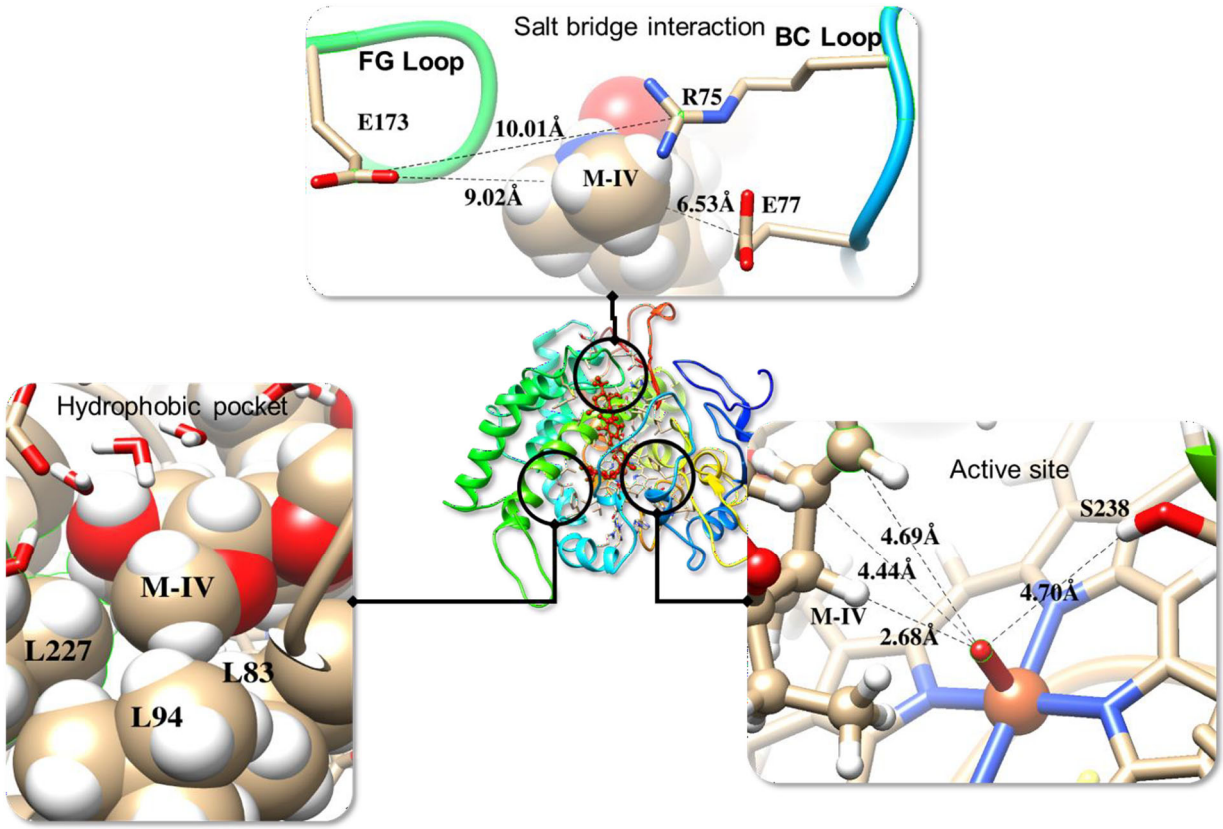

Figure S2. Three distant regions in MycG that have important interactions to collectively influence substrate recognition by the enzyme.

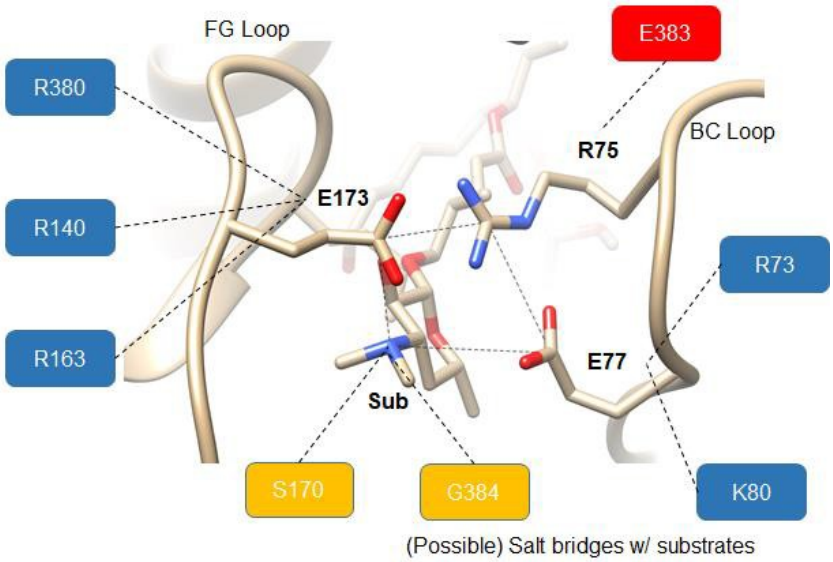

Figure S3. Proposed mutations to enhance salt bridge interactions around the desosamine group. R380/R140/R163 are adjacent to E173 and could potentially distract E173 away from R75 and desosamine; these residues are mutated to alanine/glutamic acid. R73 and K80 are near E77; these residues are mutated to alanine. S170 is mutated to aspartic acid to form a new salt-bridge interaction with desosamine.

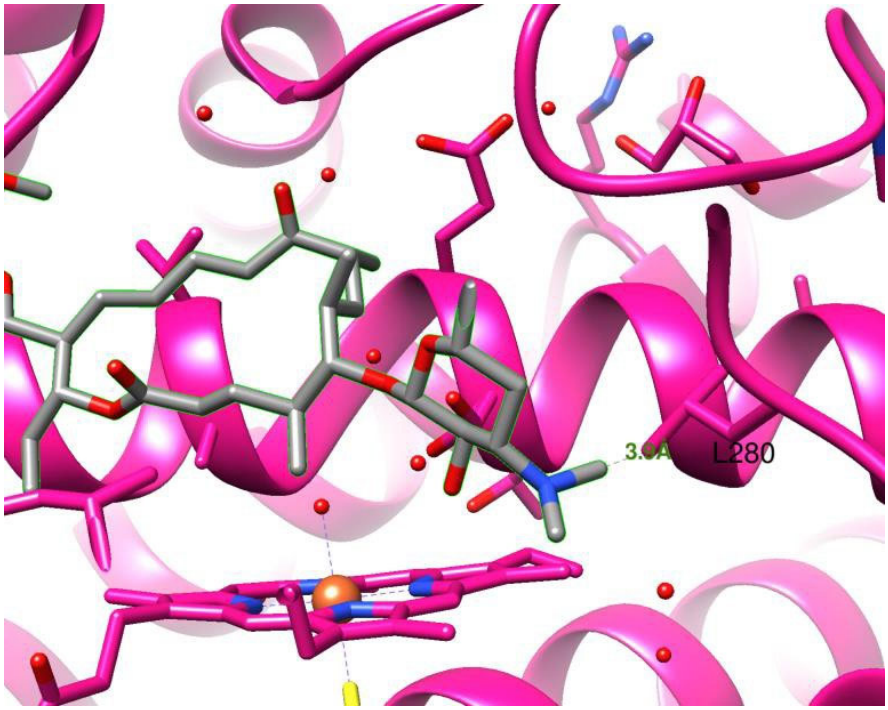

Figure S4. In the "desosamine-in" binding mode, the desosamine group is adjacent to L280 (with a distance of ~4 Å). The L280F mutation is designed to place a bulkier residue at this position, which disfavors the "desosamine-in" binding mode without affecting the "desosamine-out" binding mode.

Cartesian coordinates, energies, and corrections

A

C	1.95771	-0.66648	0.00000
H	1.91220	-1.75418	0.00000
C	0.82163	0.06465	0.00000
H	0.88375	1.15279	0.00000
C	-0.51461	-0.49784	0.00000
H	-0.59006	-1.58573	0.00000
C	-1.63837	0.24313	0.00000
H	-1.54796	1.33251	0.00000
C	-3.04355	-0.29469	0.00000
H	-2.99062	-1.39217	0.00000
C	-3.79740	0.15174	-1.26888
H	-3.28987	-0.19409	-2.17566
H	-4.81790	-0.24905	-1.27276
H	-3.86826	1.24571	-1.31834
C	-3.79740	0.15174	1.26888
H	-3.28987	-0.19409	2.17566
H	-3.86826	1.24571	1.31834
H	-4.81790	-0.24905	1.27276
C	3.33271	-0.12456	0.00000
O	4.28946	-0.88934	0.00000
C	3.54255	1.38451	0.00000
H	3.08726	1.84774	-0.88367
H	3.08726	1.84774	0.88367
H	4.61494	1.58771	0.00000

SCF energy: -426.723733 hartree
zero-point correction: +0.209037 hartree
enthalpy correction: +0.222103 hartree
free energy correction: +0.170212 hartree
quasiharmonic free energy correction: +0.171677 hartree

A-TS

C	3.00591	-0.98064	2.99560
H	3.26239	-0.87719	4.05421
H	2.49531	-1.94025	2.85642
S	1.88991	0.38247	2.54174
O	0.71063	0.07359	-1.43163
Fe	1.28220	0.19520	0.17248
N	2.86312	-0.98038	-0.21216
N	0.24169	-1.42330	0.84591
N	-0.24179	1.38133	0.77955
N	2.35602	1.81309	-0.34455
C	4.07106	-0.58915	-0.73339
C	4.90572	-1.74122	-0.97469
C	4.18155	-2.83446	-0.60848
C	2.90600	-2.35057	-0.13820
C	0.65323	-2.73090	0.80732
C	-0.37779	-3.59454	1.33379
C	-1.41828	-2.79137	1.69006
C	-1.02427	-1.43711	1.38259
C	-1.44591	0.99036	1.30927
C	-2.29109	2.13933	1.52486
C	-1.58459	3.22856	1.11408
C	-0.30815	2.74434	0.64646
C	1.93329	3.11496	-0.32514
C	2.97153	3.98573	-0.83020
C	4.02735	3.19046	-1.14810
C	3.63172	1.83459	-0.83912
C	4.44048	0.71982	-1.01530
H	5.43448	0.88131	-1.42114
C	1.88715	-3.16766	0.33759
H	2.07409	-4.23730	0.35021
C	-1.81742	-0.31872	1.59164
H	-2.80854	-0.47694	2.00439
C	0.69881	3.55431	0.13478
H	0.50446	4.62185	0.09513
H	5.90949	-1.70083	-1.37827
H	4.46841	-3.87773	-0.64720
H	-0.29816	-4.67120	1.41583
H	-2.36780	-3.07198	2.12799
H	-3.28978	2.09798	1.94057
H	-1.88304	4.26925	1.12220
H	2.88542	5.06146	-0.91652
H	4.99076	3.47488	-1.55175
H	3.91203	-0.97535	2.38484
H	-0.37207	-0.46889	-1.83795
C	-1.28602	-0.97457	-2.62141
C	-2.50966	-0.32574	-2.17210
C	-0.76289	-0.47370	-3.96790
C	-1.16831	-2.48221	-2.44448
C	-3.50455	-0.86091	-1.40621
H	-2.61455	0.72184	-2.46183
H	-0.80163	0.61909	-4.02943
H	0.27690	-0.78116	-4.11527
H	-1.36117	-0.88339	-4.79468
H	-1.41923	-2.80494	-1.42994
H	-1.83401	-3.00934	-3.14360
H	-0.14324	-2.80687	-2.65126
C	-4.65451	-0.10770	-0.98686
H	-3.45248	-1.90170	-1.09452
C	-5.66956	-0.60259	-0.23160
H	-4.68887	0.93346	-1.30748
H	-5.65159	-1.64011	0.09780
C	-6.85320	0.14967	0.21802
O	-7.69894	-0.39717	0.91881
C	-7.01437	1.60882	-0.19443
H	-6.17458	2.21837	0.16076
H	-7.05237	1.70989	-1.28571
H	-7.94245	1.99120	0.23445

SCF energy: -2052.329903 hartree
zero-point correction: +0.521043 hartree
enthalpy correction: +0.558186 hartree
free energy correction: +0.450292 hartree

quasiharmonic free energy correction: +0.459248 hartree

B

C	2.01444	0.34206	-0.54861
H	1.90840	1.05571	-1.36402
C	0.95802	-0.36604	-0.11722
H	1.06156	-1.08902	0.69026
C	-0.40289	-0.23014	-0.66833
C	-1.60547	-0.35783	0.18869
H	-1.44382	-0.56252	1.25203
C	-2.90762	0.34877	-0.13681
H	-2.91727	0.52855	-1.22063
C	-2.97992	1.69961	0.59122
H	-2.14345	2.35171	0.31452
H	-3.91111	2.22253	0.34664
H	-2.95384	1.56394	1.68004
C	-4.10019	-0.55602	0.21064
H	-4.03047	-1.51212	-0.31845
H	-4.13564	-0.76459	1.28778
H	-5.04624	-0.07762	-0.06724
H	-0.49591	0.37105	-1.57423
O	-1.21016	-1.42908	-0.66800
C	3.39223	0.25609	-0.00335
C	3.68802	-0.72216	1.12407
H	3.8857	-0.49229	2.01314
H	3.5140	-1.75056	0.82615
H	4.74740	-0.65390	1.37739
O	4.27025	0.96947	-0.46766

SCF energy: -501.943007 hartree
zero-point correction: +0.213884 hartree
enthalpy correction: +0.227562 hartree
free energy correction: +0.174044 hartree
quasiharmonic free energy correction: +0.175773 hartree

B-TS

C	1.02393	1.95427	-3.25275
H	1.46206	2.21291	-4.22132
H	0.07908	1.42802	-3.42988
S	2.17929	0.85801	-2.37532
O	0.63077	-0.33299	1.31289
Fe	1.25498	0.25146	-0.19373
N	-0.03612	1.79018	-0.24626
N	-0.02345	-0.85171	-1.34720
N	2.62568	-1.22221	-0.36357
N	2.56268	1.37749	0.83084
C	0.10945	3.00494	0.37551
C	-1.09095	3.79111	0.21970
C	-1.97207	3.03160	-0.48958
C	-1.30439	1.78322	-0.77284
C	-1.28034	-0.48346	-1.75721
C	-1.88369	-1.54433	-2.52975
C	-0.97195	-2.55480	-2.58838
C	0.18783	-2.11354	-1.85069
C	2.47384	-2.44016	-0.98172
C	3.66018	-3.23979	-0.79790
C	4.52596	-2.49452	-0.05678
C	3.86902	-1.23879	0.21338
C	3.81376	1.00633	1.24374
C	4.42877	2.07467	2.00116
C	3.53298	3.09643	2.03187
C	2.37109	2.65082	1.29422
C	1.23297	3.41726	1.08188
H	1.21217	4.41306	1.51421
C	-1.88633	0.73798	-1.48164
H	-2.90359	0.88944	-1.82916
C	1.34543	-2.85990	-1.67509
H	1.37355	-3.85053	-2.11863
C	4.42677	-0.20641	0.95946
H	5.42783	-0.36230	1.35017
H	-1.22907	4.78850	0.61774
H	-2.98933	3.25678	-0.78371
H	-2.87187	-1.50031	-2.96975
H	-1.05811	-3.51230	-3.08642
H	3.79593	-4.23955	-1.19050
H	5.51947	-2.75595	0.28483
H	5.41828	2.02992	2.43816
H	3.63107	4.06783	2.49968
H	0.81671	2.85849	-2.67559
H	-0.10368	-1.26291	1.45786
C	-0.91665	-2.19326	2.03754
C	-2.27440	-1.76989	1.57588
C	-0.48028	-3.54294	1.49276
C	-0.60892	-1.99425	3.51025
C	-3.01666	-0.60389	2.12806
H	-2.51068	-2.02854	0.54346
O	-3.41040	-1.93616	2.46439
H	-0.68941	-3.64094	0.42309
H	0.59297	-3.69920	1.64620
H	-1.01220	-4.35446	2.01453
H	-0.72298	-0.95078	3.81888
H	-1.27559	-2.60867	4.13133
H	0.42422	-2.28675	3.72221
C	-3.89954	0.19032	1.25350
H	-2.54065	-0.05158	2.93917
C	-5.06774	-0.22747	0.73995
H	-3.54269	1.18556	0.99742
H	-5.47047	-1.19963	1.01394
C	-5.83235	0.62543	-0.20330
C	-7.23593	0.16060	-0.55037
O	-5.36487	1.64944	-0.69227
H	-7.20842	-0.84496	-0.98979
H	-7.85051	0.09244	0.35646
H	-7.69644	0.85618	-1.25452

SCF energy: -2127.541796 hartree
zero-point correction: +0.525713 hartree
enthalpy correction: +0.563533 hartree

free energy correction: +0.455236 hartree
quasiharmonic free energy correction: +0.463002 hartree

C

C	2.28734	-0.85018	0.00000
H	2.26383	-1.93860	0.00000
C	1.13960	-0.13687	0.00000
H	1.17742	0.94912	0.00000
C	-0.17212	-0.74658	0.00000
H	-0.20221	-1.83530	0.00000
C	-1.36904	-0.12612	0.00000
C	-2.70405	-0.83531	0.00000
H	-2.45825	-1.90585	0.00000
C	-3.52068	-0.53854	-1.27278
H	-2.95274	-0.78646	-2.17569
H	-4.44097	-1.13323	-1.27230
H	-3.80107	0.51848	-1.32506
C	-3.52068	-0.53854	1.27278
H	-2.95274	-0.78645	2.17569
H	-3.80107	0.51848	1.32506
H	-4.44097	-1.13323	1.27230
C	3.65302	-0.28064	0.00000
O	4.62094	-1.03065	0.00000
C	3.83339	1.23110	-0.00000
H	3.36861	1.68495	-0.88347
H	3.36861	1.68496	0.88347
H	4.90158	1.45497	-0.00000
Cl	-1.45636	1.64020	-0.00000

SCF energy: -886.388450 hartree
zero-point correction: +0.200005 hartree
enthalpy correction: +0.214104 hartree
free energy correction: +0.159274 hartree
quasiharmonic free energy correction: +0.161207 hartree

C-TS

C	-1.24792	-0.53796	3.73829
H	-1.65268	-0.38269	4.74290
H	-1.15244	-1.60623	3.52991
S	-2.34660	0.28228	2.54454
O	-0.91400	-0.08963	-1.35685
Fe	-1.48950	-0.02303	0.25207
N	-0.10751	1.33344	0.89272
N	-0.27620	-1.51265	0.86377
N	-2.91012	-1.35917	-0.23284
N	-2.77178	1.48309	-0.11973
C	-0.21371	2.70307	0.84208
C	0.99128	3.31403	1.35004
C	1.82356	2.29927	1.71478
C	1.12983	1.06716	1.42383
C	0.99759	-1.39935	1.36000
C	1.57517	-2.70611	1.55949
C	0.63827	-3.61124	1.16391
C	-0.51019	-2.85661	0.72371
C	-2.79085	-2.72268	-0.21377
C	-4.00835	-3.33874	-0.69299
C	-4.86536	-2.32669	-0.99199
C	-4.16810	-1.09414	-0.69903
C	-4.05314	1.36878	-0.59879
C	-4.61921	2.67621	-0.82349
C	-3.66018	3.58232	-0.48856
C	-2.51003	2.82999	-0.05051
C	-1.32103	3.40225	0.38338
H	-1.26424	4.48653	0.38824
C	1.65125	-0.20358	1.63827
H	2.66003	-0.26860	2.03351
C	-1.67792	-3.42493	0.22831
H	-1.72236	-4.50892	0.18441
C	-4.71124	0.17414	-0.86055
H	-5.72774	0.23794	-1.23670
H	1.15757	4.38150	1.42166
H	2.81601	2.36020	2.14288
H	2.57192	-2.88780	1.94052
H	0.70552	-4.69164	1.15633
H	-4.16763	-4.40632	-0.77583
H	-5.87683	-2.38731	-1.37306
H	-5.61964	2.85836	-1.19487
H	-3.71098	4.66312	-0.52503
H	-0.25217	-0.08171	3.69244
H	0.19570	0.28488	-1.78647
C	1.17298	0.64416	-2.60863
C	2.36836	-0.00542	-2.07142
C	0.60694	0.10211	-3.92096
C	1.16451	2.17009	-2.55895
C	3.37590	0.61316	-1.38044
Cl	2.46267	-1.75818	-2.29107
H	0.45471	-0.97781	-3.89076
H	-0.35915	0.57339	-4.12323
H	1.28312	0.33205	-4.75715
H	1.38307	2.56345	-1.56319
H	1.89309	2.59261	-3.26596
H	0.17280	2.53431	-2.84361
C	4.52259	-0.00135	-0.78248
H	3.29374	1.69084	-1.27680
C	5.49650	0.68441	-0.12776
H	4.63331	-1.08091	-0.84440
H	5.45028	1.76958	-0.04489
C	6.64943	-0.01019	0.48414
O	6.76357	-1.23084	0.46723
C	7.69882	0.87079	1.14593
H	8.13474	1.56497	0.41577
H	7.24771	1.48264	1.93792
H	8.48802	0.24799	1.57142

SCF energy: -2511.957673 hartree
zero-point correction: +0.511765 hartree
enthalpy correction: +0.549957 hartree
free energy correction: +0.438713 hartree

quasiharmonic free energy correction: +0.449106 hartree

D

C	-1.92955	-0.26659	-0.58962
H	-1.82691	-0.43329	-1.66087
C	-0.86967	-0.38869	0.22457
H	-0.99514	-0.21378	1.29412
C	0.52286	-0.73735	-0.21419
H	0.53403	-0.93874	-1.29303
C	1.53892	0.37533	0.12112
H	1.53376	0.55882	1.20634
C	2.98429	0.08697	-0.33368
H	2.95542	-0.13554	-1.41160
C	3.59925	-1.12460	0.38382
H	3.03992	-2.04765	0.19620
H	4.62955	-1.29317	0.04920
H	3.62607	-0.96319	1.46981
C	3.86146	1.33277	-0.13507
H	3.45804	2.19710	-0.67581
H	3.92386	1.60314	0.92725
H	4.88300	1.15895	-0.49308
C	-3.30803	0.08804	-0.17068
O	-4.19511	0.16831	-1.00930
C	-3.59998	0.34791	1.30113
H	-3.36728	-0.53294	1.91137
H	-2.99569	1.17965	1.68250
H	-4.65827	0.59199	1.40960
H	1.19744	1.30735	-0.34890
H	0.82570	-1.66794	0.28685

SCF energy: -427.982238 hartree
zero-point correction: +0.232646 hartree
enthalpy correction: +0.246155 hartree
free energy correction: +0.192849 hartree
quasiharmonic free energy correction: +0.194992 hartree

D-TS

C	3.94010	0.01867	-2.32580
H	4.40628	0.19254	-3.30010
H	4.53061	0.48512	-1.53355
S	2.24856	0.68417	-2.37181
O	0.24115	-0.02184	1.21630
Fe	1.14546	0.31004	-0.22794
N	1.10527	-1.62627	-0.89691
N	2.89608	-0.05115	0.68873
N	1.23706	2.23361	0.32939
N	-0.49660	0.69162	-1.34490
C	0.16988	-2.20209	-1.72274
C	0.49926	-3.58905	-1.95630
C	1.64616	-3.84174	-1.26770
C	2.01619	-2.60950	-0.60990
C	3.53524	-1.26073	0.80224
C	4.70144	-1.12932	1.64219
C	4.75067	0.17031	2.04548
C	3.61394	0.83329	1.45318
C	2.18024	2.81497	1.13247
C	1.86542	4.21080	1.34729
C	0.72293	4.46291	0.65601
C	0.34014	3.22069	0.01986
C	-1.15128	1.89323	-1.43589
C	-2.31590	1.76246	-2.27703
C	-2.36114	0.46332	-2.68669
C	-1.22387	-0.19965	-2.09601
C	-0.92456	-1.54633	-2.26992
H	-1.58983	-2.12629	-2.90245
C	3.14086	-2.44688	0.19275
H	3.76602	-3.31975	0.35590
C	3.29255	2.16998	1.65617
H	3.95993	2.75290	2.28366
C	-0.76711	3.07135	-0.80404
H	-1.38754	3.94714	-0.96819
H	-0.07782	-4.26408	-2.57559
H	2.20259	-4.76817	-1.20168
H	5.38029	-1.93702	1.88519
H	5.47719	0.64997	2.68905
H	2.45630	4.89084	1.94765
H	0.17739	5.39391	0.56837
H	-2.99740	2.56918	-2.51562
H	-3.08483	-0.01444	-3.33508
H	3.90432	-1.06163	-2.14593
H	-0.30502	-1.04946	1.49852
C	-1.07613	-1.96180	2.14651
C	-2.45048	-1.77681	1.51300
C	-0.46662	-3.32599	1.88623
C	-0.93702	-1.50090	3.58698
C	-3.06045	-0.36551	1.66350
H	-2.37893	-2.02684	0.44591
H	-3.14599	-2.51468	1.95011
H	-0.53346	-3.61364	0.83255
H	0.58728	-3.35556	2.18437
H	-0.99444	-4.09537	2.47427
H	-1.18953	-0.44383	3.70921
H	-1.60028	-2.08618	4.24510
H	0.08949	-1.64090	3.94248
C	-4.28588	-0.18922	0.81764
H	-3.30242	-0.16213	2.71268
H	-2.30004	0.36505	1.35397
C	-5.51877	0.07616	1.27868
H	-4.12784	-0.29290	-0.25638
H	-5.70014	0.18668	2.34682
C	-6.73058	0.25176	0.44463
O	-7.80667	0.50058	0.97230
C	-6.62211	0.11470	-1.06956
H	-5.88543	0.81439	-1.48127
H	-6.30206	-0.89685	-1.34765
H	-7.60085	0.31533	-1.50934

SCF energy: -2053.546152 hartree

zero-point correction: +0.544435 hartree
enthalpy correction: +0.581986 hartree
free energy correction: +0.473761 hartree
quasiharmonic free energy correction: +0.482373 hartree

Full reference for Gaussian09

Gaussian 09, Revision D.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, T. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, and D. J. Fox, Gaussian, Inc., Wallingford CT, 2013.

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