Mechanism of Action of the Uridyl Peptide Antibiotics: An Unexpected Link to a Protein-protein Interaction Site in Translocase MraY

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S1. Chemical Synthesis

5'-succinyl-2', 3'-O-isopropylidene uridine

Method adapted from reference S1. Succinic anhydride (1.03 g, 1.03 x 10^{-2} mol, 0.9 eq) was dissolved in dry DCM (100 mL) and a solution of 2',3'-isopropylidene-uridine (3.101 g, 1.1 x 10^{-2} mol, 1 eq) in dry DCM (110 mL) was added. The mixture was treated with TEA (1.01 g, 1.4 mL, 1.0 x 10^{-2} mol, 0.90 eq) and was cooled to 0°C overnight. The reaction was allowed to warm to room temperature. The solvent was removed *in vacuo,* and the resulting residue was cooled to 0°C and dissolved in Na_2CO_3(*sat*). The aqueous layer was washed with Et_2O (3 x 100 mL), cooled to 0°C and acidified with 2M HCl to pH 1.6. The product was then extracted with DCM (4 x 50 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo*. The product was purified by flash chromatography using (8:2/EtOAc:$CHCl_3$) as the eluent (2.260 g, 5.88 x 10^{-3} mol, 57.0 %). **m.p.** 71-74°C $\mathbf{R_f}$ = 0.54 (8:2/EtOAc:$CHCl_3$); **^{1}H-NMR** (400MHz, DMSO-d_6) δ_H: 1.34 (s, 3H, H-6) 1.54 (s, 3H, H-6) 2.57 (m, 4H, H-1 H-2) 4.05 (m, 1H, H-4) 4.24 (d, 2H, *J*=5.0Hz, H-3) 4.81 (dd, 1H, *J*=4.0, 6.5Hz, H-5) 4.93 (dd, 1H, *J*=3.0, 6.5Hz, H-7) 5.54 (d, 1H, *J*=6.5Hz, H-8) 5.83 (d, 1H, *J*=4.0Hz, H-10) 7.62 (d, 1H, *J*=8.0Hz, H-9); **^{13}C-NMR** (100MHz, $CDCl_3$) δ_C: 25.5, 28.8, 28.9, 60.4, 64.0, 80.9, 84.6, 85.3, 94.9, 102.2, 142.5, 160.0, 164.4, 172.0, 176.4; **HRMS:** m/z (ESI) calculated for $C_{16}H_{20}N2NaO_9^+$: 407.1066 $[M+Na]^+$. Found: $[M+Na]^+$: 407.1061

General Scheme for Manual SPPS (according to reference S2)

Attachment of the first amino acid to the resin

In a dried RBF, 2eq* of Fmoc-protected amino acid was dissolved in minimum dried DCM. Following the addition of DIPEA (2.5eq)*, the reaction mixture was immediately added to the resin and agitated with $N_{2(g)}$ overnight. (Fmoc-Trp(PMC)-OH and Fmoc-Arg(PMC)-OH was agitated over two nights). To cap the unloaded resin beads, anhydrous MeOH (3x volume) was added to the resin and agitated under $N_{2(g)}$ for 1 hour. Vacuum filtration was applied to remove the solution from the resin. The resin was then washed with DCM (5 x 10 mL) and DMF (5 x 10 mL).

Fmoc-deprotection

Loaded resin was treated with 20% piperidine in DMF (10 mL) for 2 hours. Vacuum was applied to remove this solution from the resin. The resin was further washed with DMF (5 x 10 mL) and DCM (5 x 10 mL).

Peptide Coupling

In a dried RBF, 2eq* of Fmoc-protected amino acids and HATU (2eq)* were dissolved in minimum dried DMF. Following the addition of DIPEA (2.5eq)*, the reaction mixture was immediately

added to the resin and agitated with $N_{2(g)}$ overnight. The resin was then washed with DCM (5x10mL) and DMF (5x10mL). Fmoc-deprotection using the above procedure was used.

Cleavage/Isolation

The loaded resin was dried under vacuum for 30 minutes and transferred to a dried flask in preparation for resin cleavage. The dried resin was treated with 19:1/TFA:H_2O (1mL per 100mg of loaded resin), flushed with $N_{(g)}$, and stirred gently overnight. The resin was removed by reduced pressure filtration and washed with neat TFA. TFA was evaporated using a CO_2/acetone rotary evaporator. Cold Et_2O was used to precipitate the oligopeptide. The ether layer was decanted. The wet product was dried further with a high vacuum pump to remove any residual ether. The dried product was then dissolved in water and lyophilized overnight.

5'-Succinyl-2',3'-O-isopropylideneuridyl-glycyl-glycyl-glycyl-L-arginine [2]

Using the SPPS protocol above, with 2-chlorotrityl chloride resin: Fmoc-L-Arg, Fmoc-Gly, Fmoc-Gly, Fmoc-Gly were successively coupled to the resin, followed by 5'-succinyl-2', 3'-O-isopropylidene uridine. The product was deprotected as described above, and was purified by reverse phase HPLC at a flow rate of 20 mL/min. The method employed a binary mixture of eluents A (H_2O with 0.1 % TFA) and B (MeOH with 0.1 % TFA). HPLC gradient B: 5%-50%; 0-15 mins, 50%-100%; 15-20mins, 100%; 20-25mins, 100%-5%; 25-30mins, 5% 30- 40mins. Retention time was found to be at 9.8mins. (0.392 g, 5.8 x 10^{-4} mol, 96%) **^{1}H-NMR** (400MHz, MeOD) δ_H: 1.58 (m, 2H, H-15) 1.88 (m, 2H, H-14) 2.4-2.7 (m, 4H, H-8 H-9) 3.13 (m, 2H, H-16) 3.67 (t, 1H, *J*=3.1Hz, H-4) 3.80 (m, 6H, H-7 H-11 H-12) 3.90 (s, 2H, H-10) 4.07 (m, 1H, H-13) 4.29 (dd, 1H, *J*=2.8, 3.8Hz, H-5) 4.39 (q, 1H, *J*=4.6Hz, H-6) 5.63 (d, 1H, *J*=7.9Hz, H-3) 5.83 (d, 1H, *J*=4.5Hz, H-1) 7.91 (d, 1H, *J*=8.0Hz, H-2); **^{13}C-NMR** (100MHz, MeOD) δ_C: 24.9, 28.4, 29.1, 32.0, 41.5, 41.8, 42.0, 44.0, 55.3, 62.9, 70.1, 71.8, 81.8, 98.5, 104.2, 150.1, 157.0, 164.0, 170.8, 171.0, 171.6, 174.3, 172.8; **HRMS:** m/z (ESI) calculated for $C_{25}H_{38}N_9O_{13}^+$: 672.2589 $[M+H]^+$. Found: $[M+H]^+$: 672.2584.

5'-Succinyl-2',3'-O-isopropylideneuridyl-glycl-glycyl-L-tryptophyl-glycine [3]

Using the SPPS protocol above, with 2-chlorotrityl chloride resin: Fmoc-Gly, Fmoc-L-Trp, Fmoc-Gly, Fmoc-Gly were successively coupled to the resin, followed by 5'-succinyl-2', 3'-O-isopropylidene uridine. The product was deprotected as described above, and was purified by reverse phase HPLC at a flow rate of 20 mL/min. The method employed a binary mixture of eluents A (H_2O with 0.1 % TFA) and B (MeOH with 0.1 % TFA). HPLC gradient B: 5%-100%; 0-30 mins, 100%; 30-35mins, 100%-5%; 35-40mins, 5% 40-50mins. Retention time was found to be at 11.3 mins. (0.270 g, 3.8 x 10^{-4} mol, 91 %) **^{1}H-NMR** (400MHz, MeOD) δ_H: 2.5 (m, 6H, H-8 H-9 H-14)

3.69 (s, 1H, H-10) 3.72 (s, 1H, H-13) 3.74 (s, 1H, H-11) 3.81 (m, 1H, H-6) 4.01 (m, 1H, H-4) 4.23 (m, 3H, H-5 H-7) 4.61 (dd, 1H, *J*=5.1, 5.0Hz, H-12) 5.63 (d, 1H, *J*=8.0Hz, H-3) 5.71 (d, 1H, *J*=8.0Hz, H-1) 6.91 (t, 1H, *J*=7.5Hz, H-18) 7.00 (t, 1H, *J*=7.5Hz, H-17) 7.05 (d, 1H, *J*=5.5Hz, H-16) 7.21 (d, 1H, *J*=8.0Hz, H-2) 7.50 (d, 1H, *J*=8.0Hz, H-15) 7.60 (d, 1H, *J*=8.5Hz, H-19); **^{13}C-NMR** (400MHz, MeOD) δ_C: 16.6, 289, 30.3, 31.5, 43.8, 44.4, 55.9, 65.1, 71.6, 75.5, 83.4, 91.8, 103.3, 111.5, 112.6, 118.5, 119.6, 120.5, 123.0, 125.2, 129.2, 142.7, 158.9, 161.7, 164.0, 167.9, 171.3, 173.3, 182.0; **HRMS:** m/z (ESI) calculated for $C_{30}H_{35}N_7NaO_{13}^+$: 724.2185 $[M+Na]^+$. Found: $[M+Na]^+$: 724.2190.

5'-Succinyl-2',3'-O-isopropylideneuridyl-glycyl-glycyl-L-tryptophanyl-L-arginine [4]

Using the SPPS protocol above, with 2-chlorotrityl chloride resin: Fmoc-L-Arg, Fmoc-L-Trp, Fmoc-Gly, Fmoc-Gly were successively coupled to the resin, followed by 5'-succinyl-2', 3'-O-isopropylidene uridine. The product was deprotected as described above, and was purified by reverse phase HPLC at a flow rate of 20 mL/min. The method employed a binary mixture of eluents A (H_2O with 0.1 % TFA) and B (MeOH with 0.1 % TFA). HPLC gradient B: 50%-100%; 0- 15 mins, 100%-50%; 15-20mins, 50%; 20-30mins. Retention time was found to be at 11.4mins (0.31 g, 3.9 x 10^{-4} mol, 64 %). **^{1}H-NMR** (400MHz, MeOD) δ_H: 1.85 (m, 2H, H-21) 1.90 (m, 2H, H-20) 2.5-2.8 (m, 8H, H-8 H-9 H-14 H-22) 3.5-4.4 (m, 11H, H-4 H-5 H-6 H-7 H-10 H-11 H-12 H-13) 5.89 (d, 1H, *J*=3.5Hz, H-3) 5.91 (d, 1H, *J*=3.5Hz, H-1) 6.91 (t, 1H, *J*=6.0Hz, H-18) 6.98 (t, 1H, *J*=8.0Hz, H-17) 7.1-7.4 (m, 2H, H-15 H-16) 7.56 (d, 1H, *J*=8.0Hz, H-2) 7.93 (d, 1H, *J*=8.0Hz, H-19); **^{13}C-NMR** (100MHz, MeOD) δ_C: 24.4, 30.3, 36.8, 41.6, 43.5, 44.4, 52.5, 53.4, 65.1, 65.7, 71.3, 72.2, 75.3, 76.1, 79.5, 80.3, 82.8, 83.4, 91.8, 103.6, 112.6, 119.6, 120.2, 122.7, 142.7, 169.6, 170.0, 171.9, 172.4, 190.1; **HRMS:** m/z (ESI) calculated for $C_{34}H_{46}N_{10}O_{13}^+$: 801.3162 $[M+H]^+$. Found: $[M+H]^+$: 801.3169.

S2. Biological Methods

Overexpression and isolation of membrane bound MraY enzymes

E. coli MraY was overexpressed as previously described (reference 15), using plasmid pJFY3c, transformed into *E. coli* C43. Assays were carried out using inner membranes from this overexpressing strain. Site-directed mutants F288L and E287A of *E. coli mraY* were generated as described in reference S3, and the mutant *mraY* genes were cloned into a pET52b vector (Novagen) and expressed in *E. coli* C43(DE3). A construct containing *P. aeruginosa* MraY overexpressed as a C-His_6 fusion protein was a gift of Prof. R. Levesque (Univ. Laval, Quebec, Canada); membranes containing the overexpressed *P. aeruginosa* MraY showed a specific activity of 4.91 FAU min^{-1} mg $protein^{-1}$. *B. subtilis mraY* and *S. aureus mraY* genes were cloned into vector pET52b (Novagen)

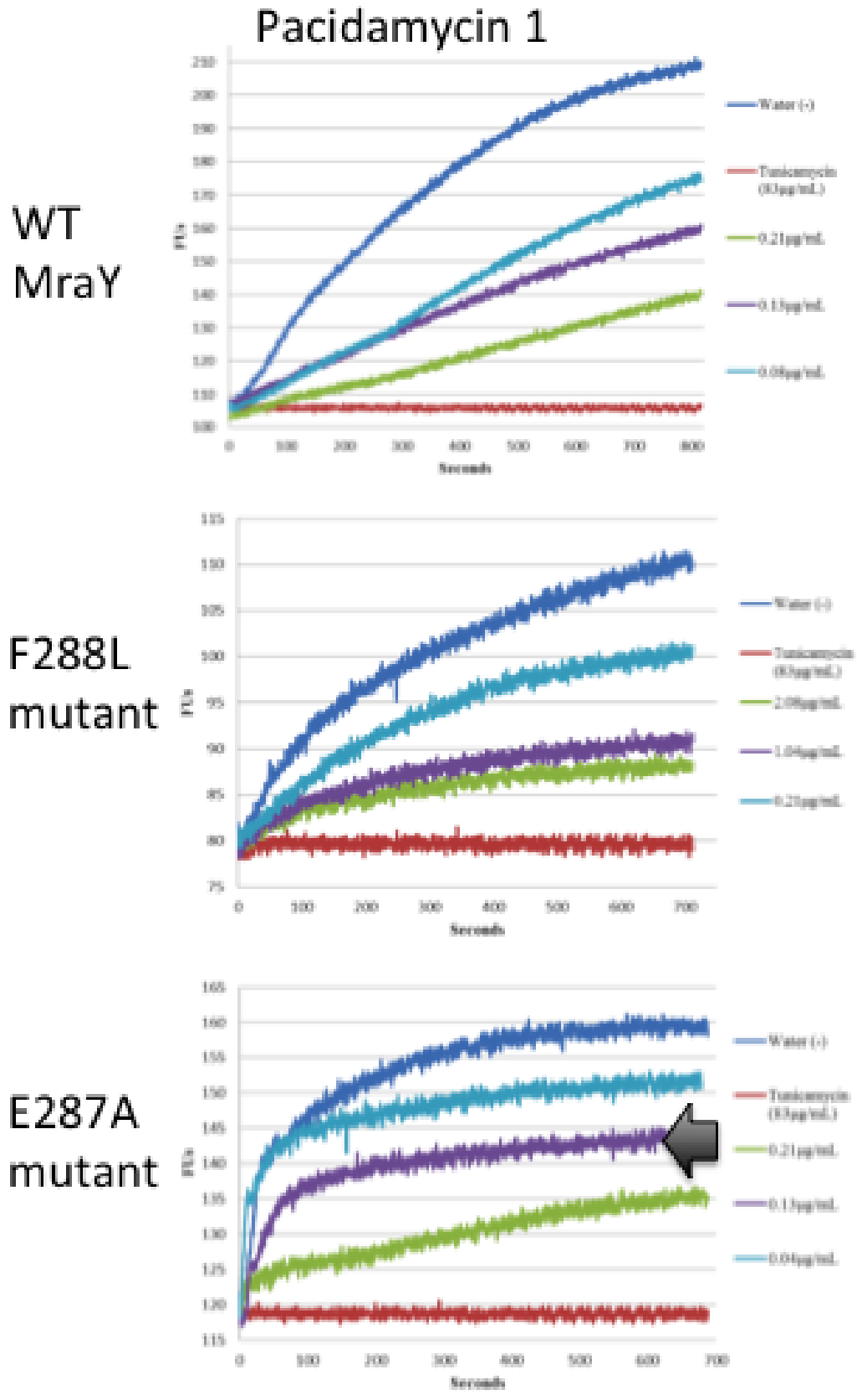
and expressed as N-terminal StrepTag fusion proteins in *E. coli* C43(DE3), as described in reference 15.

A 500 mL culture of each overexpression strain (containing *mraY* or *mraY* mutants) was grown in Luria-Bertani broth containing ampicillin (100 μg/mL) at 37 °C to an OD_{600} of 0.6, then induced with IPTG (1 mM) and allowed to grow for 4 hours at 37 °C with shaking. The cells were centrifuged at 4,400 g for 15 minutes at 4 °C. The pellet was transferred to a pre-weighed Falcon tube and resuspended in buffer (3 mL/gram of pellet) containing 50 mM Tris pH 7.5, 2 mM β-mercaptoethanol and 1 mM $MgCl_2$. 2.5 mg of egg white lysozyme and 25 μg of DNAse I (from bovine pancreas) was added to each 1mL of membrane buffer. The cells were then lysed using a Constant Systems Ltd. TS Series Cabinet cell disruptor. The lysed cells were centrifuged at 24,000 g for 20 minutes at 4 °C. The supernatant was then isolated and centrifuged at 40,000 rpm for 1 hour at 4 °C using an ultracentrifuge. The membrane pellet was homogenised in the above buffer (<2 mL) and flash frozen with liquid N_2 in 300 μL aliquots.

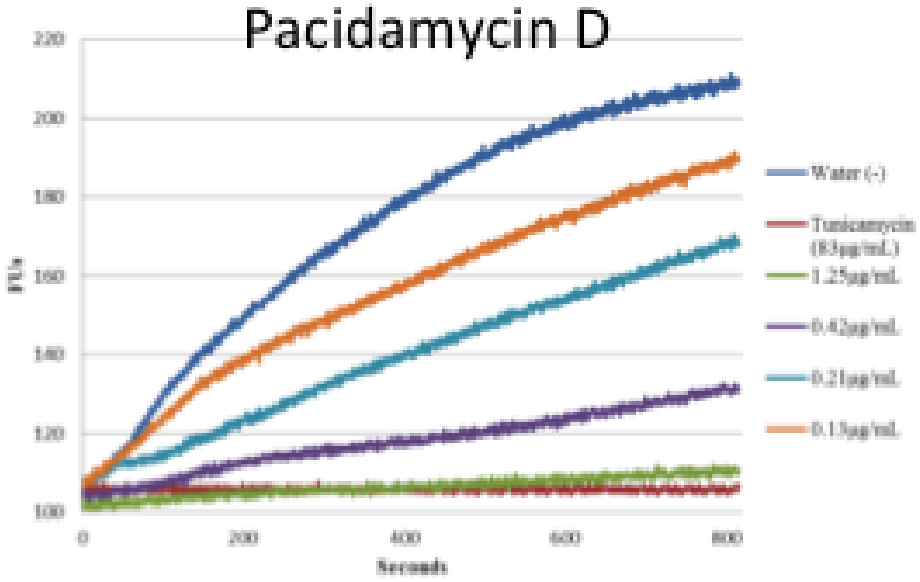
MraY Assays

UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(N^{ε}-dansyl)-D-Ala-D-Ala was prepared following the procedure of Brandish *et al* [reference 7]. The MraY catalysed reaction was monitored on a Perkin Elmer LS55 fluorimeter at an excitation wavelength of 340nm and an emission wavelength of 530nm. To monitor the formation of dansyl-lipid I, membrane-bound *E. coli* MraY (15 μL of 0.6 mg/mL stock) was incubated with 17.5 μM UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(N^{ε}-dansyl)-D-Ala-D-Ala, lipid carrier undecaprenyl phosphate (39 μM) or heptaprenyl phosphate (59 μM), 20 mM $MgCl_2$ in 83 mM Tris buffer pH 7.5, in a total volume of 0.5 mL. The final protein concentrations of membranes containing overexpressed *E. coli, P. aeruginosa, S. aureus, M. flavus* and *B. subtilis* MraY in this continuous assay were 0.6, 0.1, 0.28, 0.3, 0.3 and 0.9 mg/mL, respectively. Specific activities for the overexpressed *E. coli, P. aeruginosa, S. aureus,* and *B. subtilis* MraY enzymes were 2.3, 4.9, 3.0, and 2.7 FAU min^{-1} mg $protein^{-1}$ respectively. MraY inhibitors were added at 0.1-100 μg/mL final concentration, in duplicate assays, and IC_{50} values were determined from plots of activity vs. inhibitor concentration.

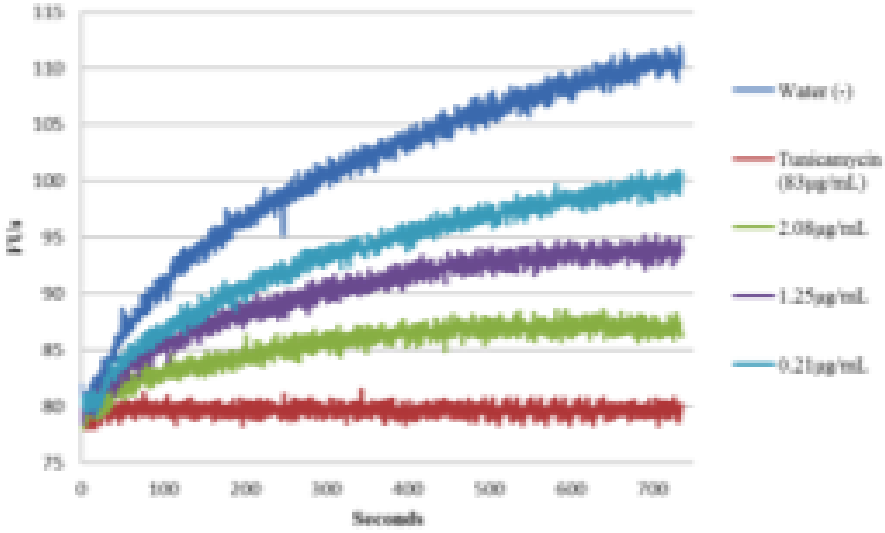
S3 Fluorescence Assay Progress curves for inhibition of *E. coli* MraY and F288L/E287A mutant enzymes by pacidamycin and caprazamycin uridyl peptide antibiotics. Instances of biphasic inhibition indicated by block arrow.



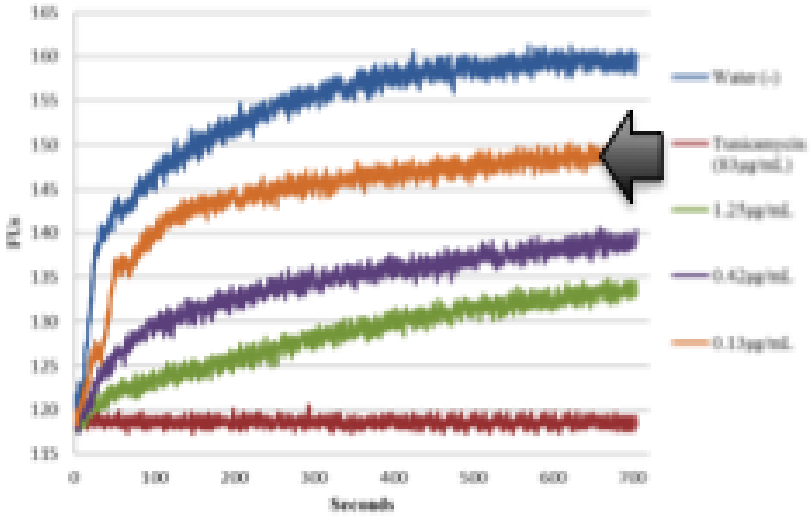
WT
MraY



F288L
mutant

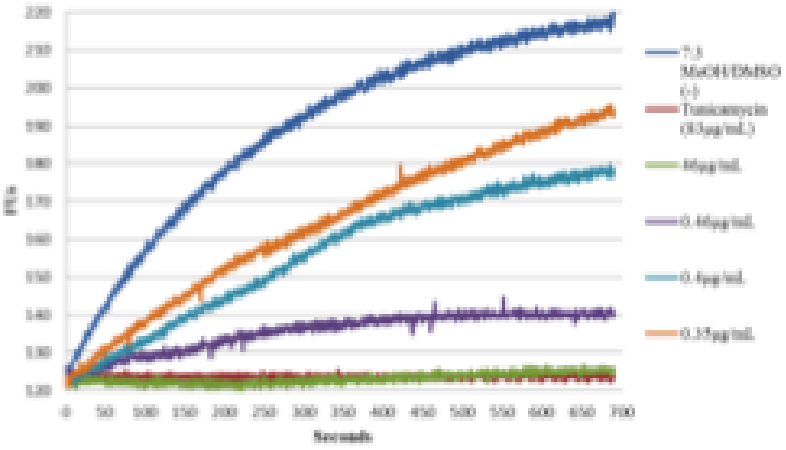


E287A
mutant

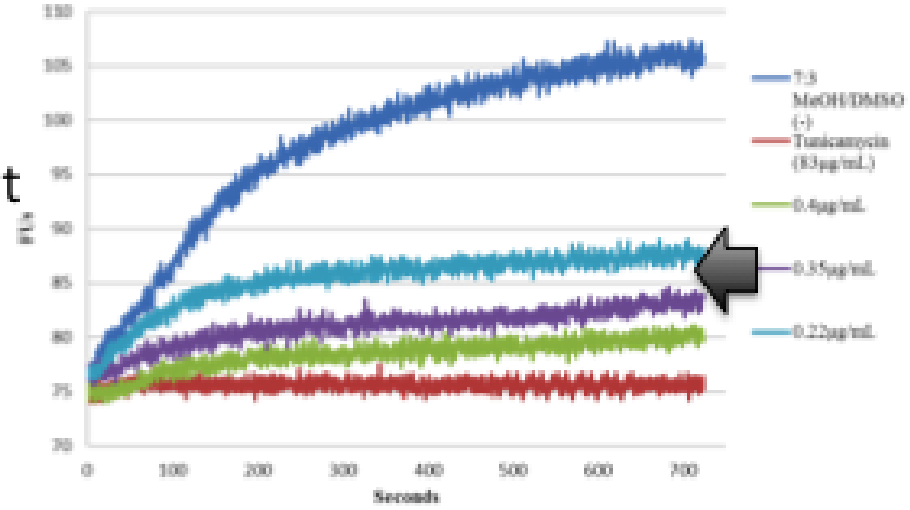


Hydroxyacylcaprazole HcaA

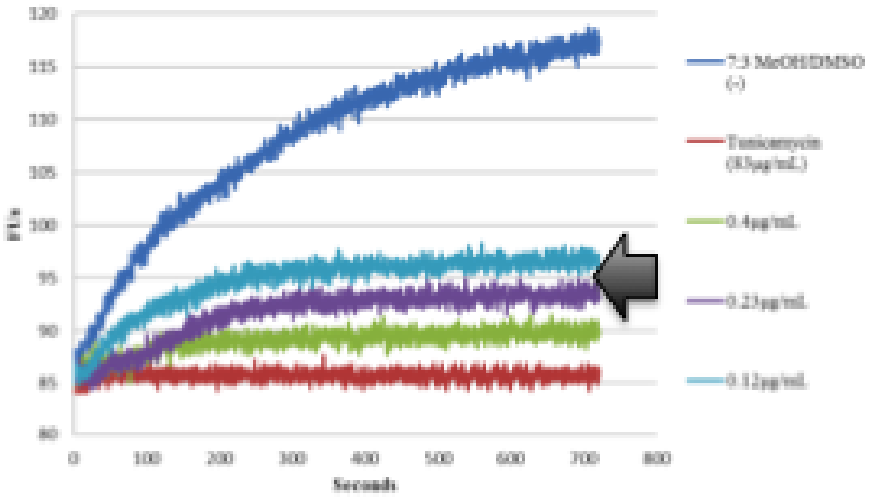
WT MraY



F288L mutant

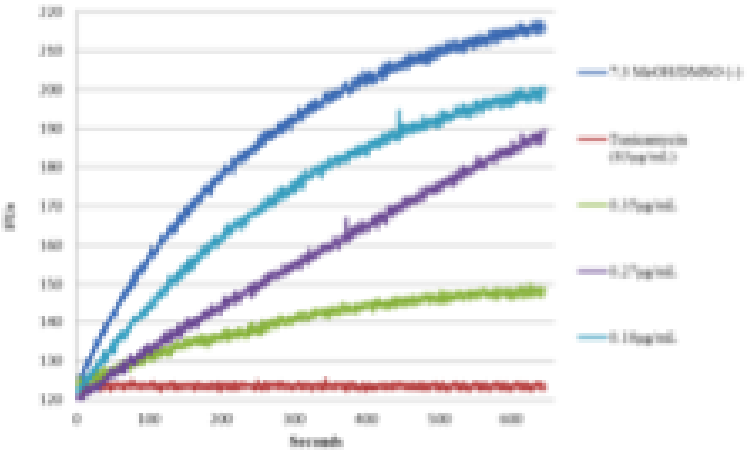


E287A mutant

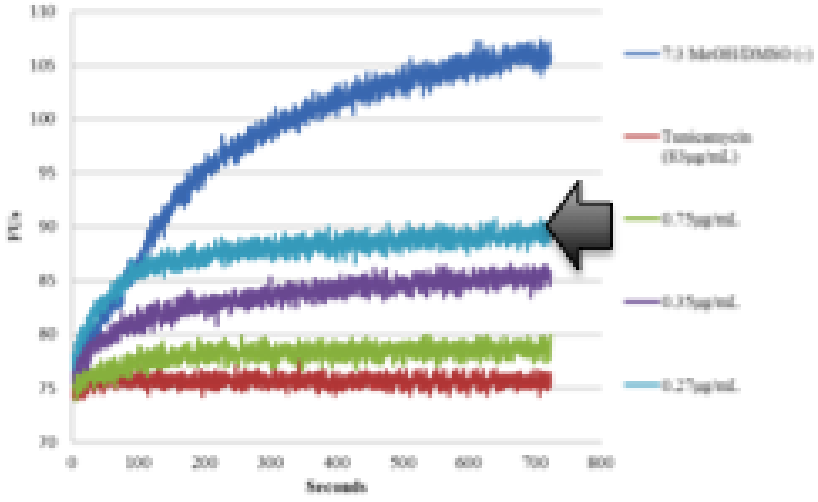


Hydroxyacylcaprazole HcaE

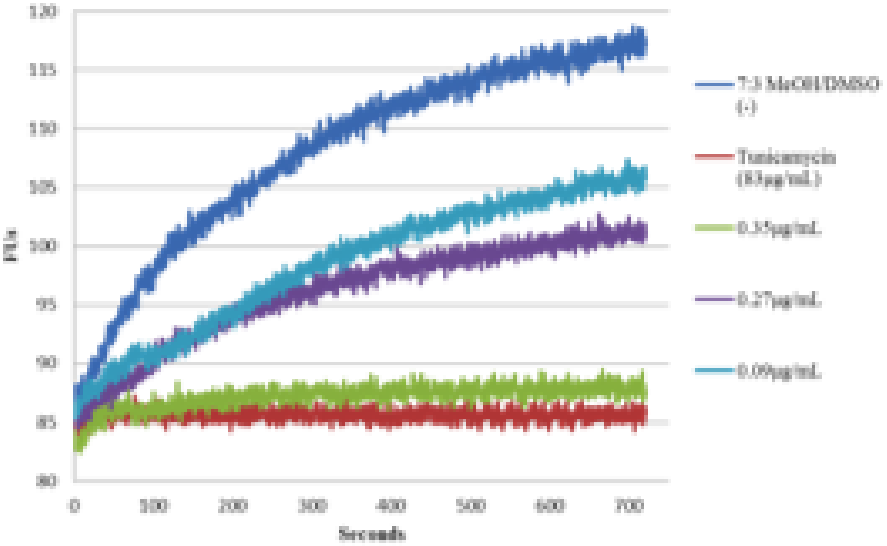
WT MraY



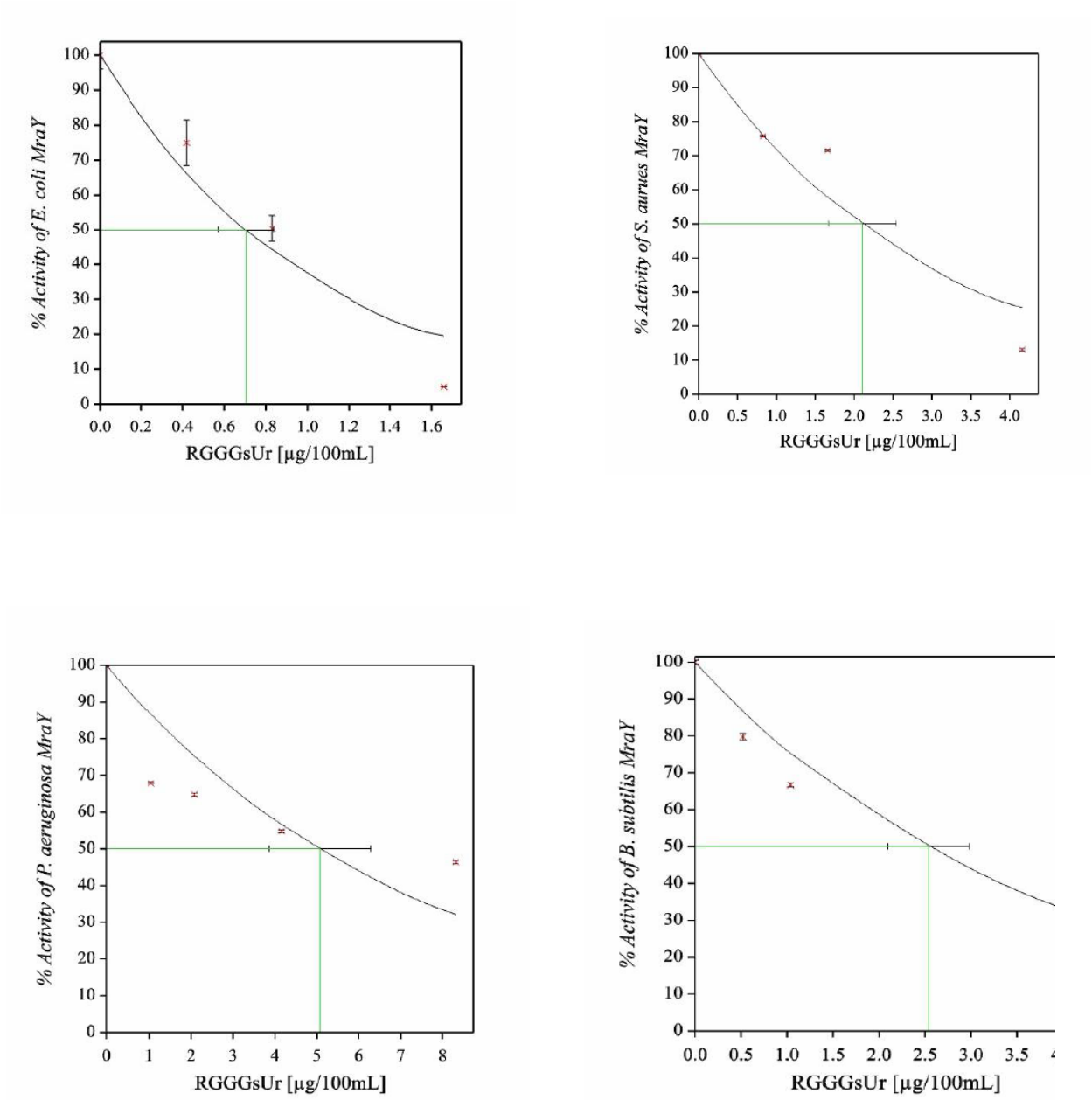
F288L mutant



E287A mutant



S4. IC_{50} plots for inhibition or recombinant MraY enzymes by uridine-peptide analogue 2



S1. A.E. Trunkfield, S.S. Gurcha, G.S. Besra, and T.D.H. Bugg, *Bio-Org. Med. Chem.*, 2010, **18**, 2651-2663.

S2. Chan, W. and White, P. (2000) *Fmoc Solid Phase Peptide Synthesis; A Practical Approach*; Oxford University Press, Oxford, UK.