Crystal structures of stapled and hydrogen bond surrogate peptides targeting a fully buried protein-helix interaction

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Supplementary Information

Contents:

Table S1 X-ray Data Collection and Refinement Statistics

Protein	*Pf*MTIPΔ60		
Ligand	pGly[801,805]	pGly[807,811]	HBS myoA
Reservoir conditions	200 mM NaCl 20% PEG 3,350	200 mM NH_4OAc 10 mM $Mg(OAc)_2.4H_2O$ 50 mM $(CH_3)_2AsO_2Na{\cdot}3H_2O$ 30% PEG 8,000	200 mM $MgCl_2.6H_2O$ 20% PEG 3,350
X-ray source	Diamond Light Source Beamline i02		DLS i04
Data collection			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P12_11$
Unit cell dimensions *a*, *b*, *c* (Å) α = β = γ = 90 °	37.38, 53.98, 75.63	37.98, 55.03, 75.83	49.04, 58.65, 59.21
Resolution (Å)	37.81 – 1.47 (1.51 – 1.47)*	37.98 – 1.82 (1.87 – 1.82)*	41.67 – 2.01 (2.12 – 2.01)*
Observations	316126	191156	106929
Unique reflections	26460	14865	21734
R_{merge}	0.045 (0.860)	0.069 (0.784)	0.092 (0.220)
$<I> / \sigma(I)$	33.4 (2.9)	26.2 (3.8)	8.6 (5.0)
Completeness (%)	99.6 (95.6)	100.0 (99.9)	96.6 (95.0)
Redundancy	11.9 (6.9)	12.9 (13.4)	4.9 (5.1)
Refinement			
Protein molecules in a.u.	1	1	2
R_{work} / R_{free} (%)	18.0 / 21.6	18.0 / 22.5	19.2 / 23.6
No. atoms			
Protein	1137	1111	2118
Ligand	151	149	284
Water	154	101	206
Mean *B*-factors (Å^2)			
Protein	19.9	22.8	18.4
Ligand	20.7	20.6	14.0
Water	32.1	31.1	25.3
R.m.s. deviations			
Bond lengths (Å)	0.007	0.008	0.011
Bond angles (°)	1.0	1.1	1.2
Ramachandran statistics			
% favored	99.4	100.0	99.7
% allowed	0.6	0.0	0.3
% outliers	0.0	0.0	0.0
PDB code	4MZJ	4MZK	4MZL

*Values in parentheses are for highest-resolution shell.

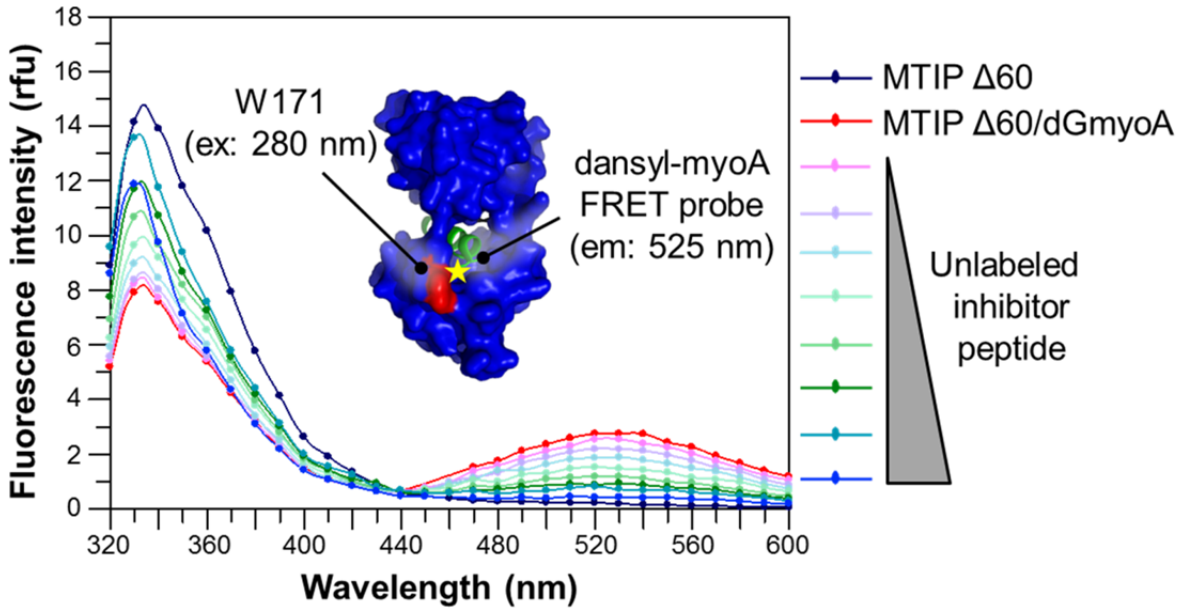


Figure S1 FRET inhibition assay: fluorescence spectra (excitation 280 nm) of *Pf*MTIPΔ60 and complexes. The protein contains one tryptophan (W171), which fluoresces at 330 nm; in complex with a dansylated myoA probe (dGmyoA), FRET between W171 and dGmyoA results in emission at 525 nm. As the probe is replaced by an unlabeled competitive inhibitor peptide, the FRET effect is diminished.

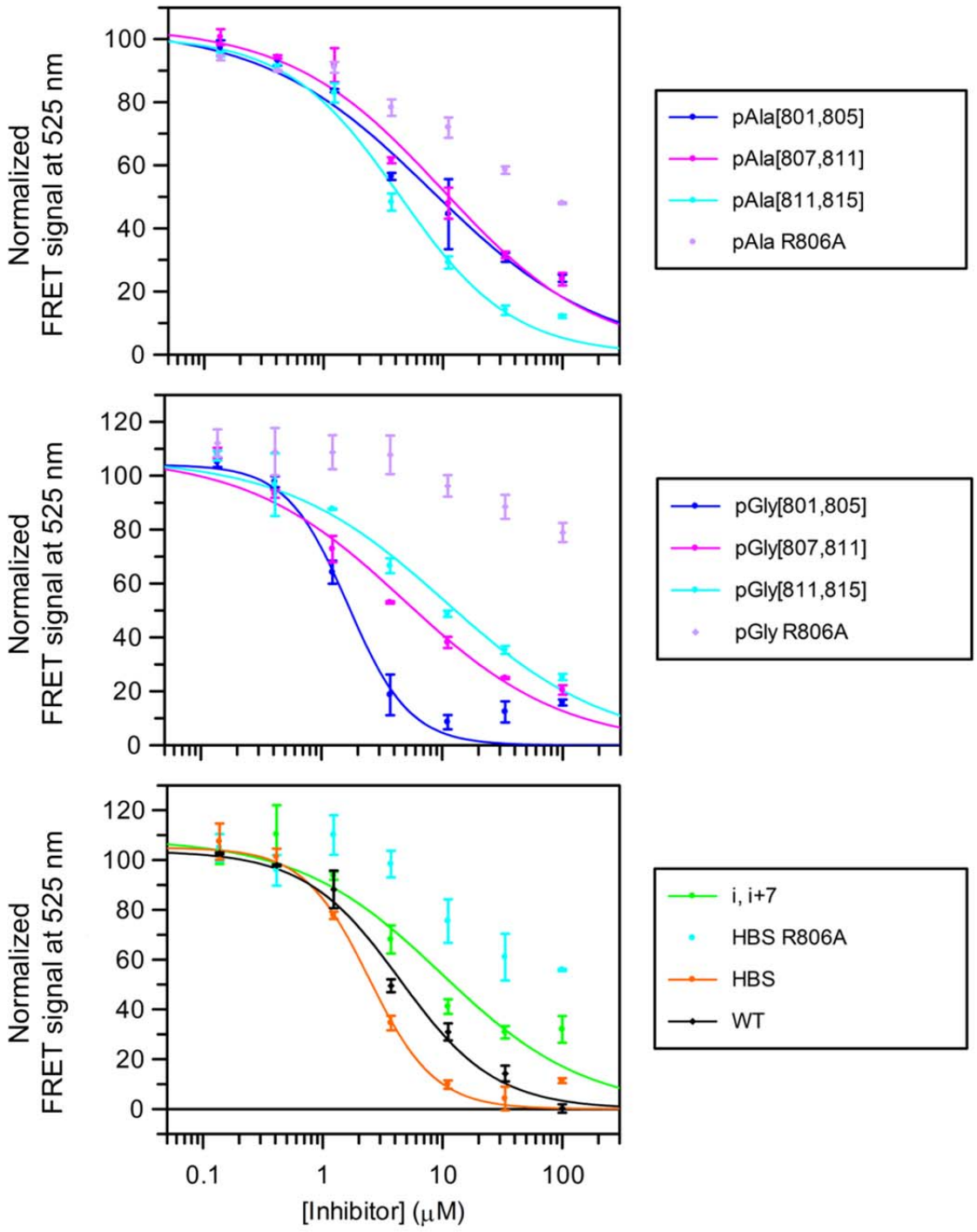
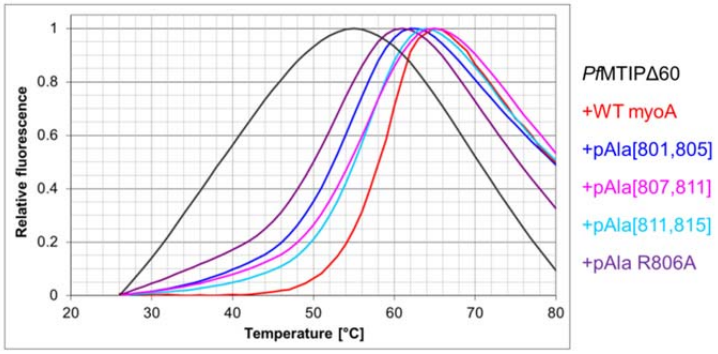
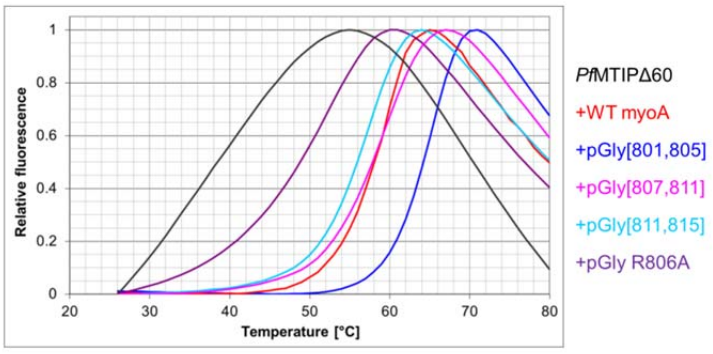


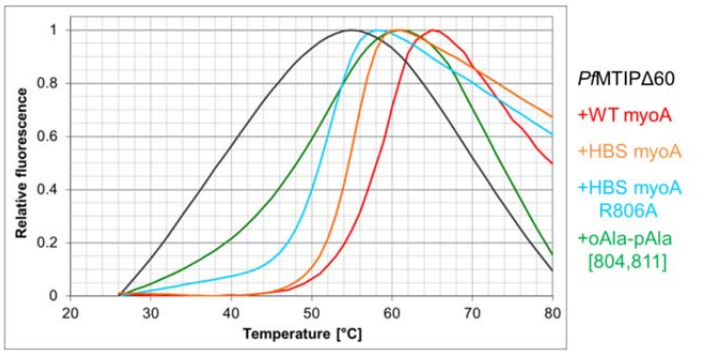
Figure S2 FRET IC_{50} plots for constrained peptide inhibitors against a complex of *Pf*MTIPΔ60 (600 nM) and dGmyoA (500 nM). Background-corrected IC_{50} values calculated by GraFit (Erithacus Software).



	WT	pAla [801,805]	pAla [807,811]	pAla [811,815]	pAla R806A
ΔT_m (°C)	19.3 ± 0.9	14.2 ± 1.4	15.9 ± 1.4	16.2 ± 0.9	12.5 ± 1.0
$\Delta\Delta T_m$ (°C)		**-5.1**	**-3.4**	**-3.1**	**-7.2**



	WT	pGly [801,805]	pGly [807,811]	pGly [811,815]	pGly R806A
ΔT_m (°C)	19.3 ± 0.9	25.9 ± 0.2	19.3 ± 0.4	16.3 ± 0.8	10.3 ± 0.8
$\Delta\Delta T_m$ (°C)		**+6.6**	**0.0**	**-3.0**	**-9.0**



	WT	HBS myoA	HBS myoA R806A	oAla-pAla [804,811]
ΔT_m (°C)	19.3 ± 0.9	16.1 ± 0.8	13.0 ± 0.7	9.8 ± 0.4
$\Delta\Delta T_m$ (°C)		**-3.2**	**-6.3**	**-9.5**

Figure S3 Differential Scanning Fluorimetry (DSF) melting curves of *Pf*MTIPΔ60 (5μM) in the absence (black) and presence (various, as labeled) of 200 μM myoA peptides. Under each set of melting curves are the T_m data compared to WT. Curves were analyzed in a customized 'DSF Analysis' MS Excel spreadsheet.[1]

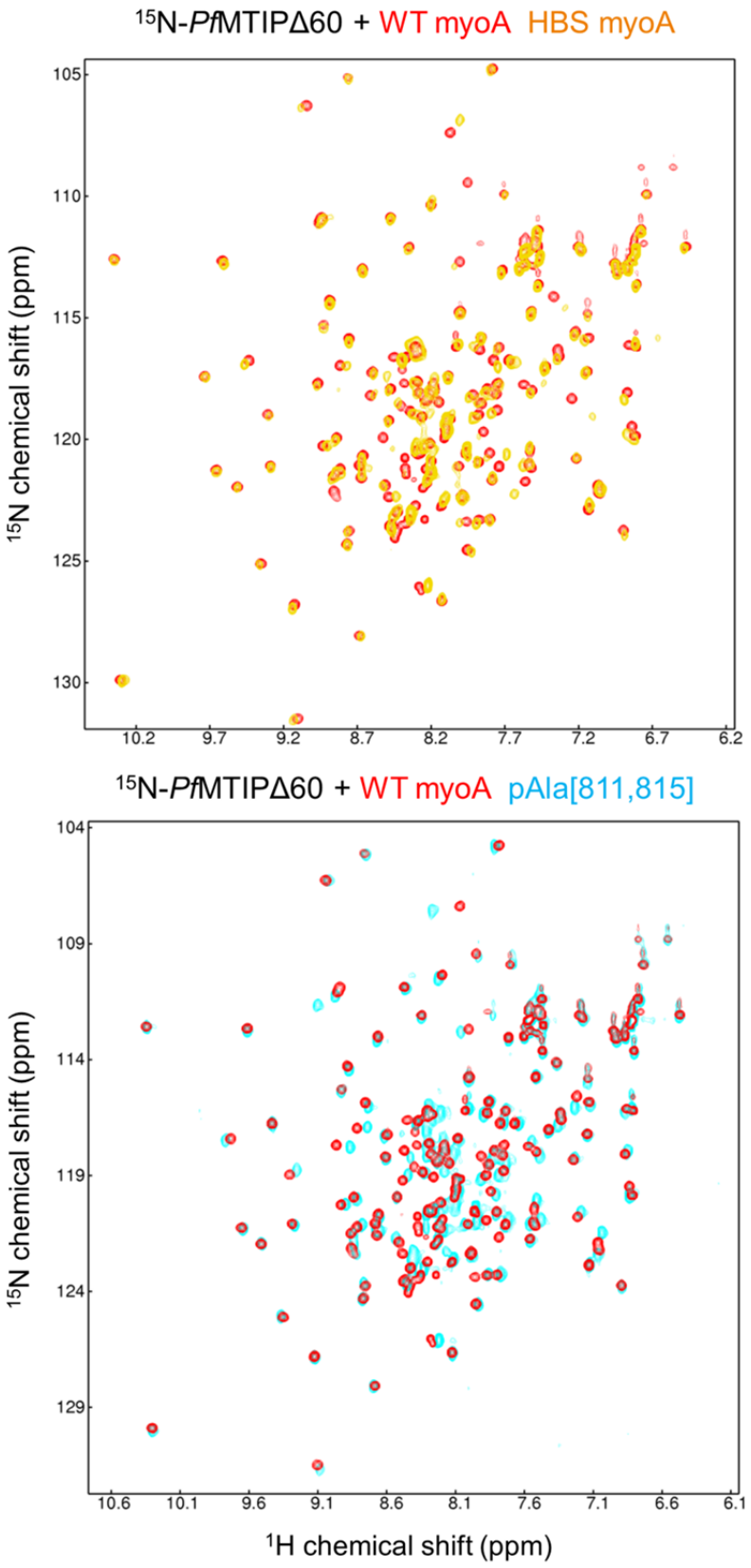


Figure S4 Overlaid solution ^{1}H, ^{15}N HSQC spectra of uniformly ^{15}N-labeled MTIP in complex with the WT myoA tail (red), HBS myoA (orange, top overlay) and pAla[811,815] (light blue, bottom overlay), recorded at 303 K and pH 7.0.

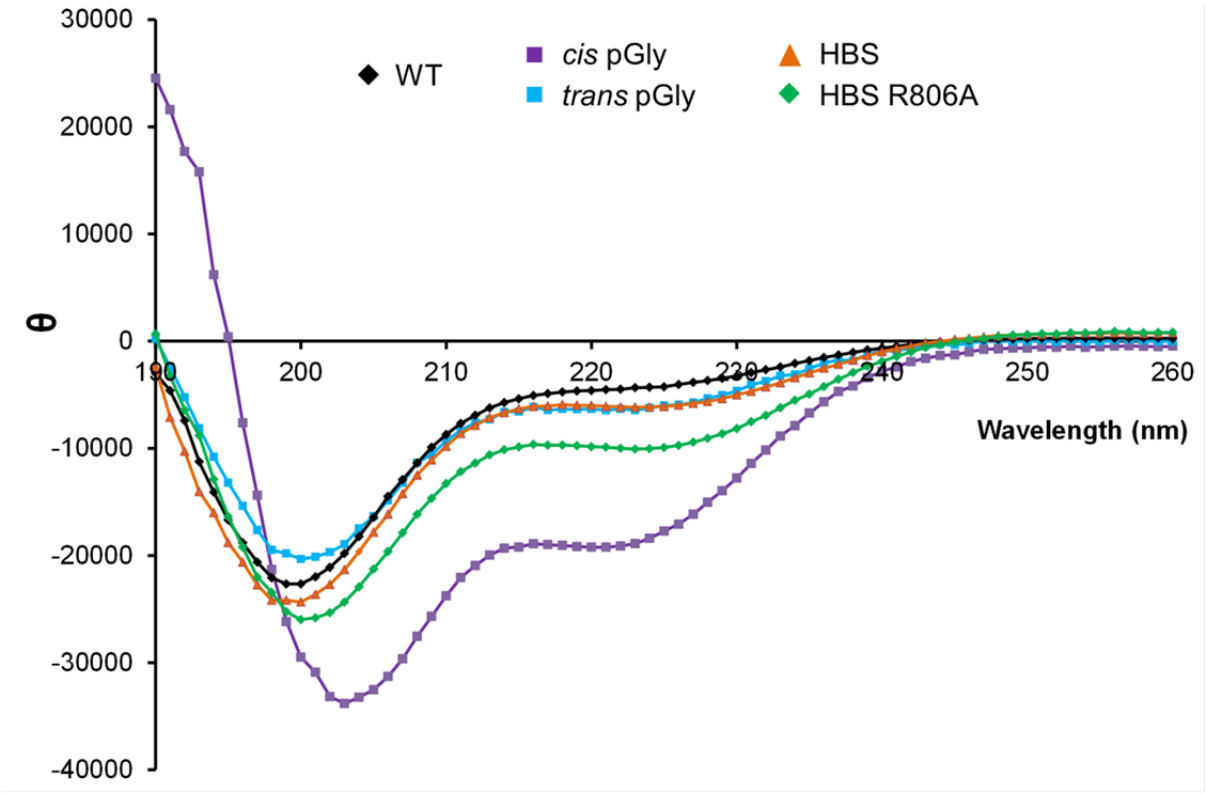


Figure S5 Circular Dichroism (CD) spectra of WT myoA (black), *cis* pGly R806A (purple), *trans* pGly R806A (blue), HBS myoA (orange) and HBS myoA R806A (green) from 190-260 nm. θ = molar ellipicity (deg.cm^2/mol.residue).

Experimental Details

1. Protein Expression and Purification

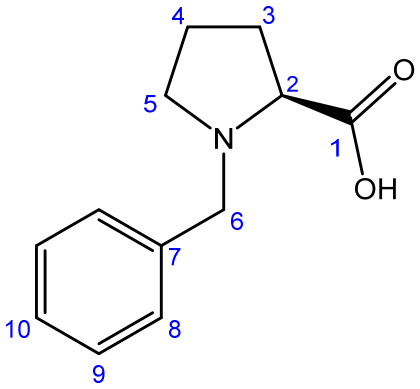
*Pf*MTIPΔ60 (i.e. lacking the N-terminal 60 amino acids, known to be dispensable for interaction with myoA[2]) for crystallography and biophysical analyses, and uniformly ^{15}N-labeled *Pf*MTIPΔ60 for NMR spectroscopy, were prepared as described previously.[3]

2. Peptide synthesis

2.1 Synthesis of (S)-*N*-Fmoc pentenyl glycine (pGly)

The following route uses the method reported by Gu *et al.*,[4] also applied recently by Yeo *et al.*[5]

Synthesis of (S)-N-benzylproline ((S)-BP)

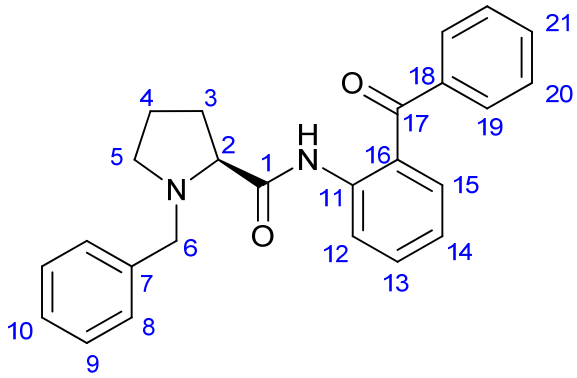


L-proline (6.55 g, 56.9 mmol) and KOH (11.2 g, 200 mmol) were added to i-PrOH (50 mL) with stirring at 40 °C. As soon as the solution became transparent, BnCl (8.9 mL, 63.9 mmol) was added drop wise over 30 min. The reaction was stirred for 6 h at 40 °C. The reaction mixture was neutralized with concentrated aqueous HCl to pH 6, followed by the addition of $CHCl_3$ (120 mL) with stirring. The resulting solid was removed by filtration and washed with $CHCl_3$. The $CHCl_3$ solutions were combined and evaporated, the residue was treated with acetone (200 mL) and a precipitate was formed. The precipitate was isolated by filtration, washed with acetone and dried *in vacuo* overnight to give (S)-BP as a colorless solid. (6.24 g, 30.4 mmol, 53% yield)

ν_{max} (solid)/cm^{-1} 1632 (C=O str.), 1450 (C=C str.), 1375 (C-H bend), 1305 (C-N str.) 1292 (C-O str.); δ_H/ppm (400 MHz, $CDCl_3$) 7.39 (5H, m, Ar-H), 4.32 (1H, d, J = 12.4

Hz, -C*H*$_2$-Ph), 4.13 (1H, d, *J* = 12.4 Hz, -CH$_2$), 3.79 (1H, dd, *J* = 8.8, 6.0 Hz, -NCH$_2$) 3.63-3.58 (1H, m, -NCH$_2$), 2.85 (1H, dd, *J* = 16.8, 8.8, -N-C*H*-CO$_2$H), 2.37-2.22 (2H, m, -CH-C*H*$_2$), 2.04-1.89 (2H, m, -CH-CH$_2$-C*H*$_2$); δ_C/ppm (100 MHz, CDCl$_3$) 171.5 (C$_1$), 130.5 (C$_7$), 130.3 (C$_8$), 129.5 (C$_{10}$), 129.3 (C$_9$), 67.4 (C$_2$), 58.6 (C$_6$), 54.4 (C$_5$), 29.2 (C$_3$), 23.4 (C$_4$); HRMS, found 206.1179 ($C_{12}H_{16}NO_2$, $[M+H]^+$, requires 206.1181).

Synthesis of (S)-2-[N-(N′-benzylprolyl)amino]benzophenone (BPB)

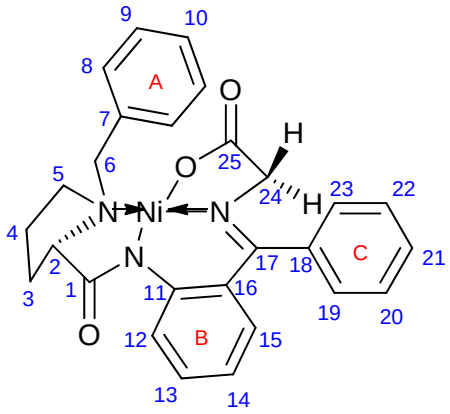


To a solution of (S)-BP (6.24 g, 30.4 mmol) in dry DCM (50 mL) was added SOCl$_2$ (4.46 g, 37.5 mmol), with stirring, at −20 °C within a period of 10 min. The stirring was continued at −10 °C until the reaction mixture became almost transparent. Then, a solution of 2-aminobenzophenone (3.70 g, 18.7 mmol) in dry DCM (25 mL) was added to the reaction mixture at −30 °C. The stirring was continued at ambient temperature overnight and then a solution of Na$_2$CO$_3$ (6.00 g, 56.6 mmol) in water (40 mL) was added to the reaction mixture at 0 °C. The dark orange organic layer was separated, the aqueous layer extracted with DCM (3 × 30 mL) and all organic fractions combined and concentrated. The resulting light brown residue was crystallized from EtOH to give a light brown solid. The crystalline product was filtered and dried *in vacuo* overnight to give BPB (6.67 g, 17.3 mmol, 93 % yield).

ν_{max} (solid)/cm^{-1} 1690 (C=O str.), 1646 (C=O str.), 1596 (C=C str.), 1513 (C=C str.), 1445 (C=C str.), 1371 (C-H bend), 1308 (C-N str.); δ_H/ppm (400 MHz, CDCl$_3$) 11.54 (1H, s, NH), 8.56 (1H, d, *J* = 8.8 Hz, Ar-H), 7.78 (2H, d, *J* = 6.8 Hz, Ar-H), 7.63-7.48 (5H, m, Ar-H), 7.37 (2H, dd, *J* = 5.6, 2.0 Hz, Ar-H), 7.16-7.07 (4H, m Ar-H), 3.92 (1H, d, *J* = 12.8 Hz, -C*H*$_2$-Ph), 3.59 (1H, d, *J* = 12.8 Hz, -C*H*$_2$-Ph), 3.31 (1H, dd, *J* = 10.4, 4.8 Hz, -N-C*H*-C(O)NH) 3.24-3.19 (1H, m, -N-C*H*$_2$-CH$_2$), 2.40 (1H, td, *J* = 9.2, 6.9 Hz, -N-C*H*$_2$-CH$_2$), 2.31-2.21 (1H, m, -C*H*$_2$-CH-N), 2.00-1.92 (1H, m, -C*H*$_2$-CH-N), 1.87-1.73 (2H, m, -N-CH$_2$-C*H*$_2$); δ_C/ppm (100 MHz, CDCl$_3$) 197.4 (C$_{17}$), 174.7 (C$_1$), 139.2 (C$_{11}$),

138.6 (C_{18}), 138.1 (C_7), 132.4 (C_{13}), 132.6 (C_{21}), 132.5 (C_{10}), 130.2 (C_{19}), 129.2 (C_8), 128.3 (C_{20}), 128.2 (C_9), 127.1 (C_{15}), 125.3 (C_{16}), 122.2 (C_{14}), 121.5 (C_{12}), 68.3 (C_2), 59.9 (C_6) 53.7 (C_5), 30.6 (C_3), 24.2 (C_4); HRMS, found 385.1917 ($C_{25}H_{25}N_2O_2$, $[M+H]^+$, requires 385.1916).

Synthesis of Gly-Ni-BPB

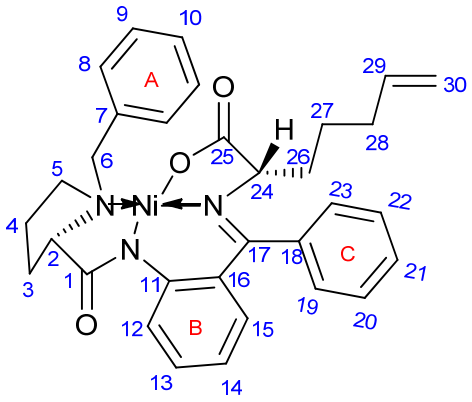


A solution of KOH (0.660 g, 11.8 mmol) in MeOH (6 mL) was poured into a stirred mixture of BPB (0.660 g, 1.71 mmol), $Ni(NO_3)_2{\cdot}6H_2O$ (0.980 g, 3.4 mmol) and glycine (0.626 g, 8.34 mmol) in MeOH (12 mL) under nitrogen gas at 45 °C. The resulting mixture was stirred at 60 °C for 1 h and then neutralized with AcOH to pH 7 and diluted with water (100 mL). The mixture was left overnight and the crystalline solid was then isolated by filtration and washed twice with water, giving Gly-Ni-BPB as a red solid. (0.650 g, 1.30 mmol, 76% yield.)

ν_{max} (solid)/cm^{-1} 1671 (C=O str.), 1638 (C=N str.), 1588 (C=C str.), 1475 (C=C str.), 1442 (C=C str.), 1361 (C-H bend), 1336 (C-N str.); δ_H/ppm (400 MHz, $CDCl_3$) 8.27 (1H, d, J = 8.9 Hz, Ar^B-H), 8.07 (1H, d, J = 7.4 Hz, Ar^A-H), 7.56-7.43 (3H, m, Ar^C-H), 7.45-7.41 (2H, m, Ar^A-H), 7.31 (1H, t, J = 7.5 Hz, Ar^A-H), 7.23-7.19 (1H, m, Ar^B-H), 7.10 (1H, d, J = 7.1 Hz, Ar^C-H), 6.99-6.97 (1H, m, Ar^C-H), 6.80 (1H, dd, J = 8.2, 1.7 Hz Ar^B-H), 6.71 (1H, t, J = 7.3 Hz, Ar^B-H), 4.49 (1H, d, J = 13.2 Hz, -CH_2-Ph), 3.76-3.65 (4H, m, -CH_2-Ph, -N-CH_2-CO_2Ni, -N-CH_2-CH_2), 3.49-3.45 (1H, m, -N-CH-C(O)NH), 3.41-3.30 (1H, m -N-CH_2-CH_2), 2.59-2.54 (1H, m, -N-CH_2-CH_2), 2.48-2.37 (1H, m, -N-CH_2-CH_2), 2.18-2.04 (2H, m, -CH_2-CH-N); δ_C/ppm (100 MHz, $CDCl_3$) 181.4 (C_1), 177.4 (C_{25}), 171.7 (C_{17}), 142.6 (C_{11}), 134.7 (C_{18}), 133.3 (C_7), 133.2 (C_{15}), 132.3 (C_{13}), 130.2 (C_8), 129.8 (C_{21}), 129.7 (C_{20}), 129.4 (C_{22}), 129.2 (C_{10}), 129.0 (C_9), 126.3 (C_{23}), 125.7 (C_{19}), 125.2 (C_{16}), 124.3 (C_{12}),

120.9 (C_{14}), 69.9 (C_2), 63.1 (C_{24}), 61.3 (C_6), 57.5 (C_5), 30.8 (C_3), 23.7 (C_4); HRMS, found 498.1347 ($C_{27}H_{26}N_3O_3$, $[M+H]^+$, requires 498.1328).

Alkylation of Ni(II) complex giving (S)-gly-Ni-(S)-BPB

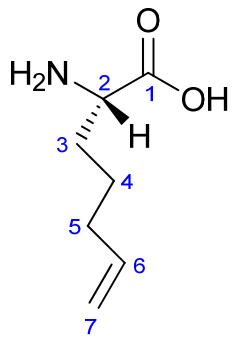


Gly-Ni-BPB (5.12 g, 10.3 mmol) and ground NaOH (4.10 g, 103 mmol) were added to a flask under argon. Anhydrous DMF (40 mL) was added and the mixture was allowed to stir for 5 min at room temperature before the addition of 5-bromo-1-pentene (1.19 mL, 10.0 mmol). A color change from green to red was observed. The reaction was then stirred at room temperature for 10 min. The solution was decanted into water (400 mL) containing 5% AcOH. The resulting suspension was dissolved in benzene (200 mL) and the emulsion was filtered through celite. The benzene solution was washed with brine (3 × 200 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel (DCM/acetone, 2:1, R_f = 0.52) affording (S)-gly-Ni-(S)-BPB (3.70 g, 6.54 mmol, 64% yield) as a red solid.

ν_{max} (solid)/cm^{-1} 1683 (C=O str.), 1632 (C=N str.), 1590 (C=C str.), 1471 (C=C str.), 1439 (C=C str.), 1353 (C-H bend), 1331 (C-N str.); δ_H/ppm (400 MHz, $CDCl_3$) 8.16 (1H, d, J = 8.4 Hz, Ar^B-H), 8.08 (2H, d, J = 7.8 Hz, Ar^A-H), 7.57-7.46 (3H, m, Ar_C-H), 7.38 (2H, t, J = 7.6 Hz, Ar^C-H), 7.27 (1H, br s, Ar^B-H), 7.23 (1H, t, J = 7.2 Hz, Ar^B-H), 7.20-7.15 (1H, m, Ar^A-H), 6.95 (1H, d, J = 7.2 Hz, Ar^B-H), 6.71-6.64 (2H, m, Ar^A-H), 5.81-5.71 (1H, m, CH_2=C*H*), 5.04-4.96 (2H, m, CH_2=CH), 4.48 (1H, d, J = 12.9 Hz, -CH_2-Ph), 3.94 (1H, dd, J = 8.4, 3.7 Hz, -N-CH_2-CO_2Ni), 3.64-3.54 (3H, m -N-C*H*-C(O)NH, -CH_2-Ph, -N-CH_2-CH_2), 3.51 (1H, dd, J = 10.7, 5.7 Hz, -N-CH_2-CH_2), 2.79 (1H, br s, -N-CH_2-CH_2), 2.59-2.53 (1H, m, -N-CH_2-CH_2), 2.26-2.17 (2H, m, -CH_2-CH-N), 2.11-1.94 (4H, m, CH_2=CH-CH_2-CH_2-CH_2), 1.76-1.67 (2H, m, CH_2=CH-CH_2-CH_2-CH_2); δ_C/ppm (100 MHz, $CDCl_3$) 180.4 (C_1), 179.4 (C_{25}), 167.4 (C_{17}), 137.8 (C_{30}), 135.4 (C_{11}), 133.8 (C_{18}), 133.3 (C_7),

133.2 (C_{15}), 132.2 (C_{13}), 131.6 (C_8), 129.8 (C_{21}), 129.0 (C_{20}), 128.9 (C_{22}), 127.6 (C_{10}), 127.2 (C_9), 126.6 (C_{23}), 126.3 (C_{19}), 125.2 (C_{16}), 123.7 (C_{12}), 120.8 (C_{14}), 115.3 (C_{29}), 70.4 (C_2), 70.24 (C_{24}), 63.08 (C_6), 57.0 (C_5), 34.8 (C_{28}), 33.3 (C_{27}), 30.8 (C_3), 24.7 (C_{26}), 23.7 (C_4); HRMS, found 566.1950 ($C_{32}H_{34}N_3O_3$, $[M+H]^+$, requires 566.1954).

Hydrolysis of alkylation product of Ni(II)-complex giving (S)-2-aminohept-6-enoic acid (pentenyl glycine, pGly)

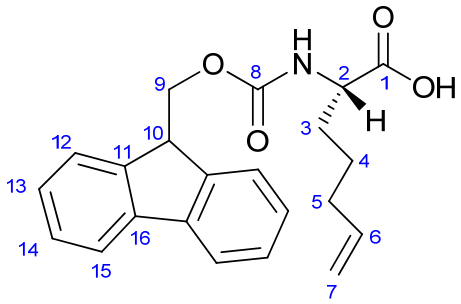


(S)-gly-Ni-(S)-BPB (3.70 g, 6.54 mmol) was dissolved in a methanol and DCM mixture (2:1, 20 mL) and added dropwise to a solution of HCl (3M, 13 mL) and methanol (13 mL) at 60 °C. The solution was refluxed for 1 h. The solution was concentrated and the resulting residue dissolved in water (15 mL) and evaporated to remove the HCl. This was repeated three times. Hydroxylamine (15 mL) was added, followed by water (15 mL) and the mixture was concentrated *in vacuo* to dryness. The residue was then dissolved in water (15 mL) and extracted into $CHCl_3$ (15 mL). The organic phase was separated and the water phase was washed with $CHCl_3$ (2 x 15 mL). The combined organic phases were washed with brine and dried over $MgSO_4$, and then concentrated *in vacuo* to give (*S*)-BPB (2.37 g, 6.15 mmol, 94% yield). The aqueous phase was loaded onto an ion-exchange column (55 g DOWEX 50Wx8 resin in a water slurry) which was activated with HCl (2M, 400 mL) and then washed with water (600 mL) to pH 5. The column was then washed with ammonium hydroxide/water/ethanol (4:1:2, 800 mL) to wash out the amino acid. The aqueous solution collected from the column was concentrated and colorless (S)-2-aminohept-6-enoic was collected after lyophilization (0.766 g, 5.35 mmol, 82% yield).

ν_{max} (solid)/cm^{-1} 2221 (O-H str.), 1576 (C=O str.), 1451 (C=C str.), 1405 (C=C str.), 1361 (C-H bend), 1342 (C-N str.); δ_H/ppm (400 MHz, D_2O) 5.81 (1H, ddt, J = 10.7, 9.8, 7.2 Hz, CH_2=C*H*), 5.04-4.99 (1H, m, C*H*$_2$=CH), 4.98-4.95 (1H, m, C*H*$_2$=CH), 3.67 (1H, t, J = 6.3 Hz, CO_2H-C*H*), 2.06 (2H, q, J = 7.5 Hz, CH_2=CH-C*H*$_2$), 1.87-1.70 (2H, m, CH_2=CH-CH_2-C*H*$_2$-CH_2), 1.50-1.33 (2H, m, CH_2=CH-CH_2-CH_2-C*H*$_2$); δ_C/ppm (100 MHz,

D_2O) 174.9 (C_1), 138.4 (C_7), 114.6 (C_6), 54.6 (C_2), 32.4 (C_5), 29.7 (C_4), 23.4 (C_3); HRMS, found 144.1018 ($C_7H_{14}NO_2$, $[M+H]^+$, requires 144.1025).

Fmoc protection of (S)-2-aminohept-6-enoic acid to give Fmoc-pGly, ready for incorporation in solid phase synthesis



(S)-2-aminohept-6-enoic acid (0.351 g, 2.45 mmol) and sodium carbonate (0.522 g, 4.93 mmol) were dissolved in water (4.5 mL) and cooled to 4 °C with stirring. A solution of Fmoc-OSu (1.24 g, 3.69 mmol) in para-dioxane (5 mL) was also cooled to 4 °C and added dropwise to the stirring amino acid solution. The reaction was stirred at 0 °C for 1 h and then allowed to warm to room temperature overnight with stirring. An excess volume of water (20 mL) was added and the mixture extracted with ethyl acetate (3 x 20 mL). The combined organic fractions were back-extracted with saturated sodium bicarbonate solution (3 x 20 mL) and the aqueous layers acidified to pH 1 with 3M HCl. All aqueous fractions were then extracted with ethyl acetate (3 x 15 mL), and all organic fractions combined, dried over sodium sulphate and concentrated *in vacuo*. The crude product was purified via flash chromatography on silica gel (DCM/MeOH/AcOH, 96:3:1, R_f = 0.35) to yield N-Fmoc-(S)-2-aminohept-6-enoic acid (0.408 g, 1.12 mmol, 46% yield) as a white solid.

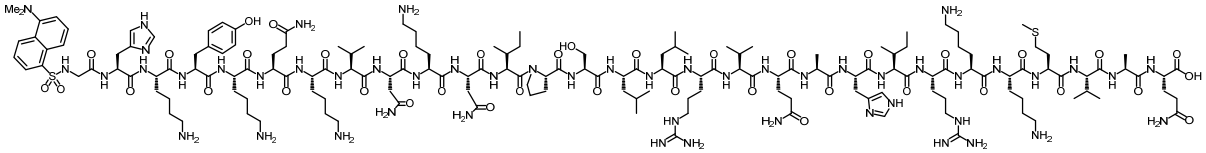
$[\alpha]_D^{22}$= +35.0º (c = 0.002, $CHCl_3$); ν_{max} (solid)/cm^{-1} 1679 (C=O str.), 1531 (C=C str.), 1450 (C=C str.), 1251 (C-O str.); δ_H/ppm (400 MHz, CD_3OD) 7.80 (2H, d, *J* = 7.5 Hz, Ar-H), 7.68 (2H, t, *J* = 6.8 Hz, Ar-H), 7.39 (2H, t, *J* = 6.4 Hz, Ar-H), 7.31 (2H, t, *J* = 7.5 Hz, Ar-H), 5.82 (1H, ddt, *J* = 10.6, 9.5, 7.0 Hz, CH_2=C*H*), 5.02 (1H, d, *J* = 17.0 Hz, CH_2=CH), 4.97 (1H, d, *J* = 10.0 Hz, CH_2=CH), 4.35 (2H, d, *J* = 7.4 Hz, -CH_2-Ph), 4.23 (1H, t, *J* = 7.0 Hz, CO_2H-C*H*), 4.14 (1H, dd, *J* = 8.5, 5.4 Hz, C*H*-CH_2-O), 2.11-2.07 (2H, m, CH_2=CH-CH_2-CH_2-CH_2), 1.90-1.81 (1H, m, CH_2=CH-CH_2-CH_2-CH_2), 1.73-1.64 (1H, m, CH_2=CH-CH_2-CH_2-CH_2), 1.54-1.45 (2H, m, CH_2=CH-CH_2-CH_2-CH_2); δ_C/ppm (100 MHz, CD_3OD) 176.0 (C_1), 158.7 (C_8), 152.4 (C_{14}), 145.4 (C_{13}), 145.2 (C_{15}), 142.6 (C_{12}), 139.4 (C_7),

128.2 (C_{16}), 126.3(C_{11}), 120.9 (C_{10}), 115.4 (C_6), 68.0 (C_9), 55.2 (C_2), 34.3 (C_5), 32.2 (C_4), 26.3 (C_3); HRMS, found 366.1704 ($C_{22}H_{24}NO_4$, $[M+H]^+$, requires 366.1705).

2.2 Solid phase synthesis

The amino acid sequences of the synthesized peptides are shown in Figure 1c (main text), with the exception of dGmyoA:

[dansyl]-GHKYKQKVNKNIPSLLRVQAHIRKKMVAQ-[OH]



This sequence corresponds to *P. falciparum* 3D7 myoA residues 791-818, with an N-terminal dansyl functionality connected to the sequence via a glycine spacer.

All the peptides were capped with an acetyl group on the N-terminus to remove unnatural charge (except HBS myoA, HBS myoA R806A and dGmyoA). All the peptides have C-terminal amide groups (except WT myoA and dGmyoA, which extend to the natural C-terminus of *P. falciparum* myoA and were therefore left as free acids).

WT myoA and dGmyoA(791-818)

WT myoA(799-818) and dGmyoA(791-818) were synthesized essentially as reported previously using optimized Fmoc SPPS protocols.[2] Briefly, preloaded Fmoc-Gln(Trt)-Wang resin (20 μmol) was swelled in DMF and transferred to fritted reaction columns in the automated ResPepSL synthesizer (Intavis). Deprotection of the N-terminal Fmoc group was achieved using 20% (v/v) piperidine in DMF (400 μL, 3 × 10 min), followed by washing with copious amounts of DMF. The incoming Fmoc-protected amino acid (87.5 μmol, 4.4 eq.; 175 μL of a 0.5M stock in NMP) was preactivated with HBTU (85 μmol, 4.25 eq.; 170 μL from a 0.5M stock in NMP) and NMM (200 μmol, 10 eq.; 50 μL from a 4M stock in NMP) before addition to the resin, and coupling was allowed to take place over 30-45 min. The resin was washed and the coupling repeated (i.e. the amino acid was 'double-coupled'). Any unreacted N-terminal amines were acetylated by addition of 'capping mixture' (5% v/v Ac_2O in DMF, 400 μL, 10 min) to prevent deletion sequences, followed by washing with copious amounts of DMF. The process of deprotection-coupling-capping was repeated for each amino acid in sequence; after the final (N-terminal) amino acid coupling, Fmoc

deprotection and washing gave the crude, resin-bound, fully side-chain-protected peptide. The peptide was then acetylated or dansylated using the method reported previously.[2]

HBS myoA and HBS myoA R806A

Rink Amide AM resin (20 μmol) was swelled in DMF and transferred to fritted reaction columns in the automated ResPepSL synthesizer (Intavis). Couplings of natural amino acids were achieved using the protocol described above for the WT sequence. Incorporation of the HBS functionality, and subsequent microwave-assisted ring-closing metathesis using Hoveyda-Grubbs II, followed 'method B' in the protocol published by Patgiri *et al.* (i.e. myoA residues Pro802 and Lys799 were replaced by *N*-allyl-glycine [†] and 4-pentenoic acid [X] respectively).[6]

***i, i+4* pGly 'stapled' peptides**

Rink Amide AM resin (20 μmol) was swelled in DMF and transferred to fritted reaction columns in the automated ResPepSL synthesizer (Intavis). Couplings of natural amino acids and final acetylation were achieved using the protocol described above for the WT sequence. Elongation with (S)-*N*-Fmoc pentenyl glycine (pGly [γ], synthesized as described above) was identical except that 3 equivalents of amino acid were used, HATU (2.95 eq.) was used to preactivate, and the double coupling was allowed to proceed for 2 × 2 h. Ring-closing metathesis was achieved using Grubbs first generation catalyst as described in the protocol published by Kim *et al.* [7]

***i, i+4* and *i, i+7* pAla/oAla 'stapled' peptides**

Rink Amide AM resin (20 μmol) was swelled in DMF and transferred to fritted reaction columns in the automated ResPepSL synthesizer (Intavis). Couplings of natural amino acids and final acetylation were achieved using the protocol described above for the WT sequence. Elongation with (R)-*N*-Fmoc (4'-octenyl) alanine (oAla [ω], a kind gift from Pfizer plc.), or (S)-*N*-Fmoc (4'-pentenyl) alanine (pAla [*]) was identical except that 3 equivalents of amino acid were used, HATU (2.95 eq.) was used to preactivate, and the double coupling was allowed to proceed for 2 × 2 h with shaking. Addition of the amino acid after either pAla or oAla was identical except that the double coupling was allowed to proceed for 2 × 2 h. Ring-closing metathesis was achieved using Grubbs first generation catalyst as described in the protocol published by Kim *et al.* [7]

Washing of resin treated with ruthenium-based catalysts

To wash the resin of residual ruthenium-containing impurities, the following method was applied, which was adapted from those used by Galan *et al.* and Hossain *et al*.[8,9]

4.4 equivalents of potassium isocyanoacetate in a 1:1 mixture of DCM/MeOH was added to the protected resin-bound peptides and gently agitated for 1 h. The reagent mixture was removed by filtration and further extensive washing of the resin was carried out using DMF/DMSO (1:1, 5 × 3 mL).

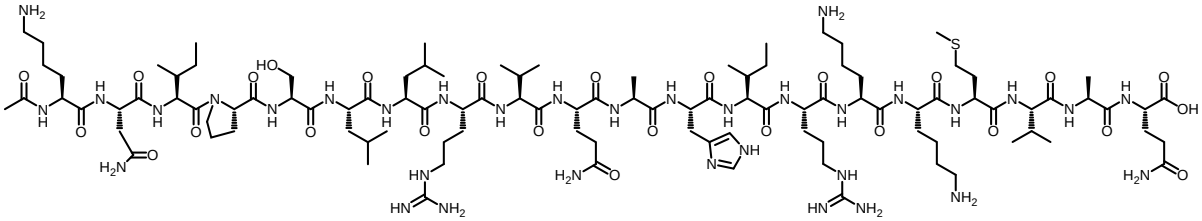
Deprotection, cleavage and purification of all peptides

The resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), MeOH (5 × 3 mL) and Et_2O (5 × 3 mL), before desiccation *in vacuo*. Cleavage from the resin and deprotection of side chains was achieved by adding 1.5 mL of deprotection mix (94% v/v TFA, 2.5% v/v H_2O, 2.5% w/v DTT, 1% v/v TIS) to the resin and shaking vigorously for 3 h at room temperature. The liquid containing the desired product was then filtered into a 15-mL centrifuge tube and 5 mL cold TBME added to precipitate the crude peptide. A pellet was formed by centrifugation (15 min, 4,500 rpm, 4 °C) and washed several times with cold TBME by resuspension/centrifugation, before being desiccated *in vacuo* overnight. Once dry, the crude peptide was dissolved in H_2O and purified by semi-preparative LC-MS, using a focused gradient of H_2O (0.1% HCOOH): MeOH (0.1% HCOOH) over 18 mins which was dependent on the retention time of the crude product as measured by analytical LC-MS runs. The LC-MS platform consisted of a Waters HPLC system (Waters 2767 autosampler for sample injection and collection; Waters 515 HPLC pump to deliver the mobile phase; XBridge C_{18} column with dimensions 19 mm × 100 mm for preparative runs and 4.6 mm × 100 mm for analytical runs) coupled to a Waters 3100 mass spectrometer (with ESI) and a Waters 2998 Photodiode array (detection between 200-600 nm).

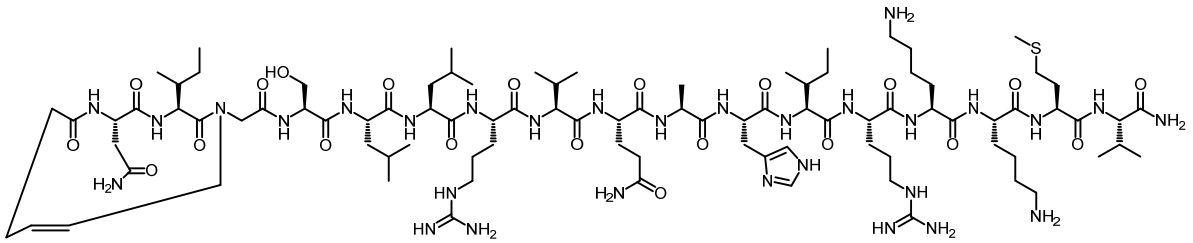
After purification, the solvent was removed using a centrifugal evaporator before lyophilization.

2.3 Peptide chemical structures and characterization

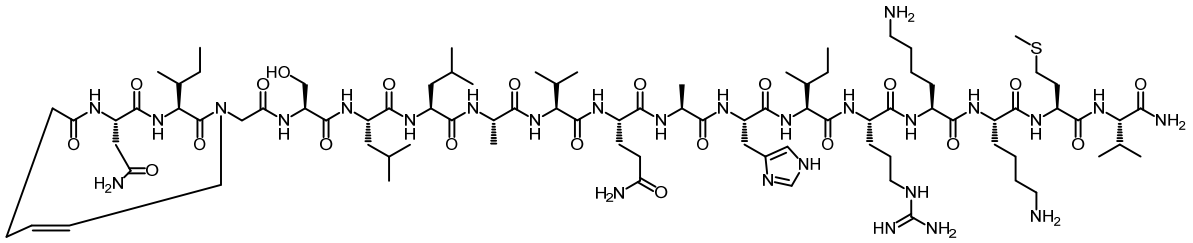
WT myoA Ac-KNIPSLLRVQAHIRKKMVAQ-OH



HBS myoA XNI†SLLRVQAHIRKKMV-NH_2

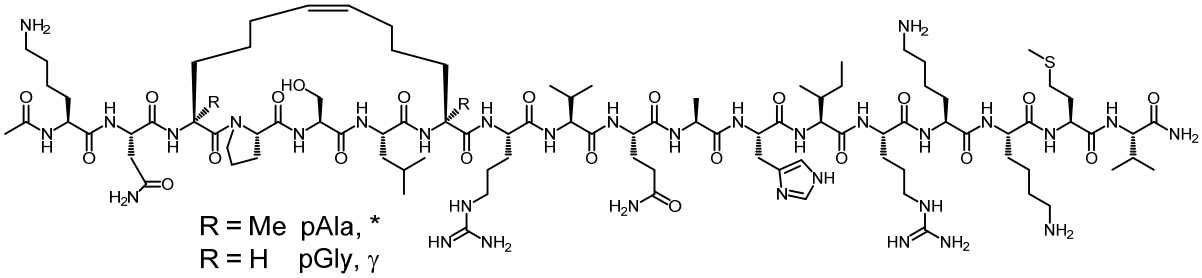


HBS myoA R806A XNI†SLLAVQAHIRKKMV-NH_2



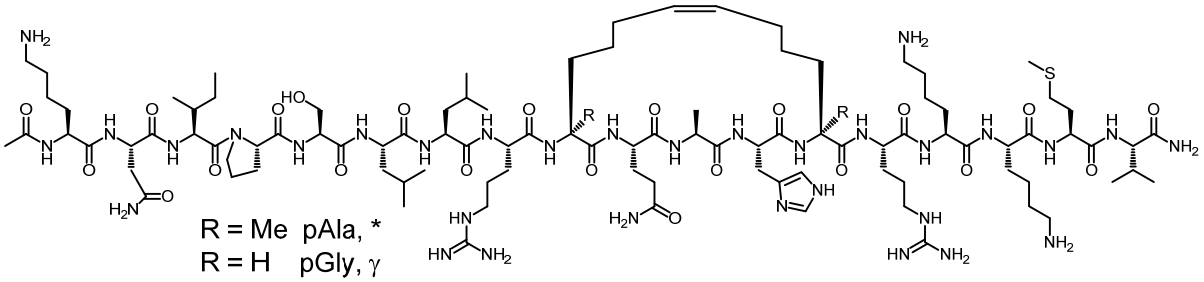
pAla[801,805] Ac-KN*PSL*RVQAHIRKKMV-NH_2

pGly[801,805] Ac-KNγPSLγRVQAHIRKKMV-NH_2



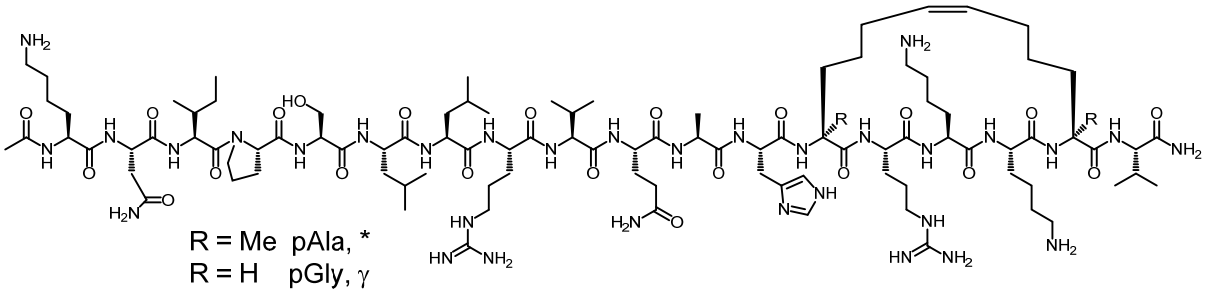
pAla[807,811] Ac-KNIPSLLR*QAH*RKKMV-NH_2

pAla[807,811] Ac-KNIPSLLRγQAHγRKKMV-NH_2



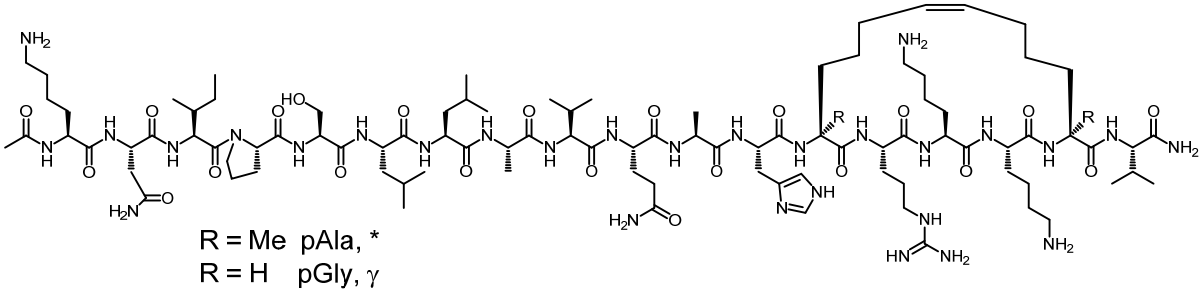
pAla[811,815] Ac-KNIPSLLRVQAH*RKK*V-NH_2

pGly[811,815] Ac-KNIPSLLRVQAHγRKKγV-NH_2

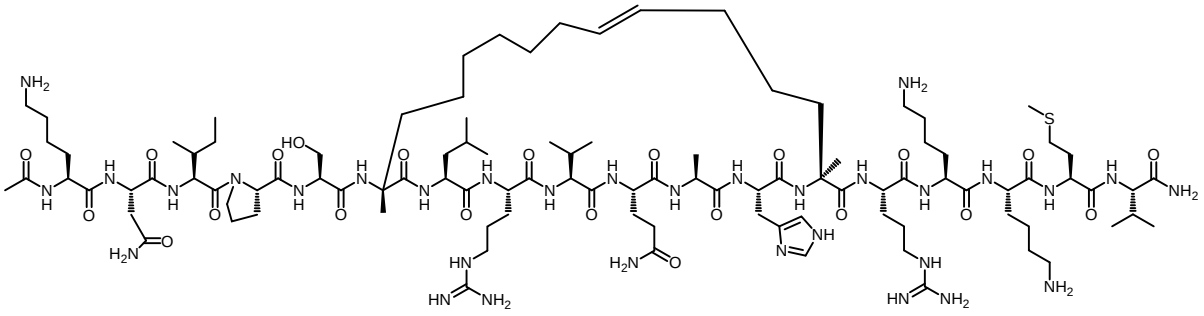


pAla R806A Ac-KNIPSLLAVQAH*RKK*V-NH_2

pGly R806A Ac-KNIPSLLAVQAHγRKKγV-NH_2



oAla-pAla[804,811] Ac-KNIPSωLRVQAH*RKKMV-NH_2



Peptide	N-	C-	Calculated mass	R_t (min)	ES+ peaks (m/z)
WT myoA	Ac-	-OH	2373	8.85	792 (m/3+1), 594 (m/4+1)
dGmyoA	dansyl-	-OH	3647	8.60	913 (m/4+1), 731 (m/5+1), 609 (m/6+1)
HBS myoA	n/a	$-NH_2$	2057	9.11	1030 (m/2+1), 687 (m/3+1), 515 (m/4+1)
HBS myoA R806A	n/a	$-NH_2$	1971	9.85	987 (m/2+1), 658 (m/3+1), 494 (m/4+1)
pAla[801,805]	Ac-	$-NH_2$	2197	8.95	733 (m/3+1), 550 (m/4+1)
pAla[807,811]	Ac-	$-NH_2$	2211	9.27	738 (m/3+1), 554 (m/4+1)
pAla[811,815]	Ac-	$-NH_2$	2178	9.49	727 (m/3+1) 546 (m/4+1)
pAla R806A	Ac-	$-NH_2$	2093	9.94	1048 (m/2+1) 699 (m/3+1) 524 (m/4+1)
oAla-pAla[804,811]	Ac-	$-NH_2$	2239	9.64	747 (m/3+1) 561 (m/4+1)
pGly[801,805]	Ac-	$-NH_2$	2168	8.39, 8.55	724 (m/3+1) 543 (m/4+1)
pGly[807,811]	Ac-	$-NH_2$	2183	8.77, 8.89	729 (m/3+1) 547 (m/4+1)
pGly[811,815]*	Ac-	$-NH_2$	2150	8.70 (*trans*), 8.96 (*cis*)	718 (m/3+1) 539 (m/4+1)
pGly R806A*	Ac-	$-NH_2$	2065	9.24 (*trans*), 9.53 (*cis*)	689 (m/3+1) 517 (m/4+1)

Table S2 Characterization data for purified peptides used in this study. Peptides bearing C-terminal acids (-OH) were synthesized using pre-loaded Wang resin; peptides bearing C-terminal amides ($-NH_2$) were synthesized using Rink Amide AM resin. Analysis of the peptides was performed with a Waters LC-MS system using an isocratic gradient of 5-98% H_2O/MeOH (0.1% HCOOH) over 18 min. * *cis/trans*- isomers could be isolated.

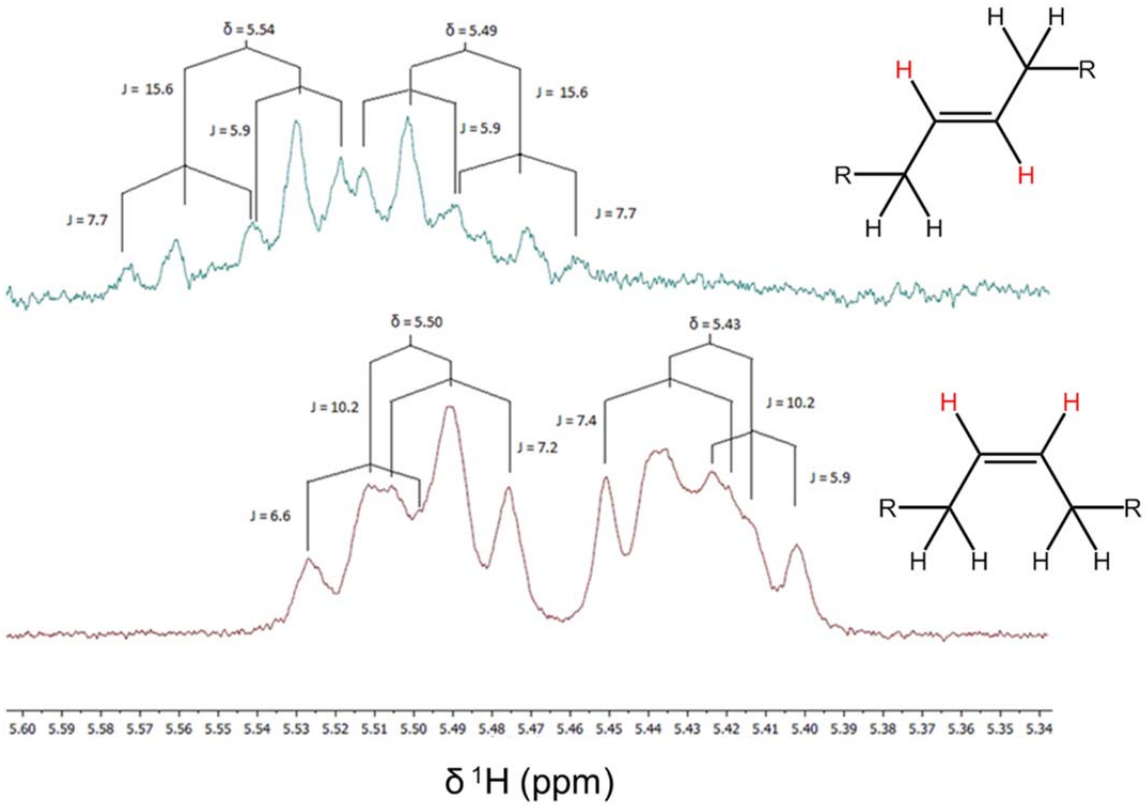


Figure S6 Portions of ^{1}H NMR spectra (400 MHz, D_2O) of LC-MS purified pGly R806A isomers between 5.34-5.60 ppm. Top: minor product at R_t = 9.24 min corresponds to the *trans* olefin. Bottom: major product at R_t = 9.53 min corresponds to the *cis* olefin.

3. X-ray crystallography

3.1 Sample preparation

The protocol for sample preparation was essentially as described previously.[3] Briefly, *Pf*MTIPΔ60 was concentrated to 5-10 mg mL^{-1} in the presence of five molar equivalents of the ligand of interest, buffered by a solution containing 20 mM HEPES pH 7.5, 50 mM NaCl, 1 mM TCEP.

3.2 Data collection and structure determination

Conditions were screened using the sitting-drop vapor-diffusion method with commercially available screens; hits were cryoprotected with paraffin oil, frozen in liquid N_2 and taken directly to Diamond Light Source for data collection. The reservoir conditions that yielded the crystals that were used for data collection are listed in Table S1.

Data were collected at 100 K at 1 ° oscillations (pGly[801,805] and pGly[811,815]) or 0.5 ° oscillations (HBS myoA). Data were processed with the xia2 bundle [10] (running

XDS,[11] XSCALE, Pointless and Aimless[12,13]) or indexed in Mosflm[14] and scaled in Scala.[12] Phasing of the data by molecular replacement was achieved in Phaser[15] using our previously reported structure of WT *Pf*MTIP/myoA (pdb code: 4AOM) as the search model.[3] Inspection of electron density maps was done in COOT.[16] Initial refinement was done in REFMAC,[17] and final refinement steps, including TLS parameters, were carried out in Phenix.[18] Restraints for the non-natural linkages in the ligands were generated in JLigand.[19] Table S1 shows statistics for data collection and refinement.

4. NMR spectroscopy

4.1 Sample preparation

The protocol for sample preparation was as described previously.[3] Briefly, samples consisted of 500 µL protein solution containing an excess of ligand and 10% D_2O, buffered by a solution containing 20 mM MOPS pH 7.0, 50 mM NaCl, 1 mM TCEP and 0.005% NaN_3 in a standard 5 mm NMR tube.

4.2 ^{1}H, ^{15}N-HSQC spectra

Spectra were recorded at 303 K on a Bruker 600 MHz Avance III spectrometer equipped with a TCI Cryoprobe (Cross Faculty NMR Centre, Imperial College), processed with NMRPipe[20] and viewed in NMRView (One Moon Scientific).

5. Biophysical Assays

5.1 Differential Scanning Fluorimetry

DSF experiments were carried out in 96-well real-time PCR plates (Eppendorf) with a Stratagene MX3005P real-time PCR machine (Agilent Technologies) running MxPro QPCR software. 20 µL samples were arrayed in triplicate and contained 5 µM protein ± 200 µM peptide and a buffer of 20 mM HEPES pH 7.5, 50 mM NaCl and 1 mM TCEP, supplemented with SYPRO Orange dye (Sigma) at a nominal 10× concentration. Fluorescence of the SYPRO Orange was monitored over 25-95 °C and melting curves analyzed in a customized MS Excel spreadsheet.[1]

5.2 Circular Dichroism Spectroscopy

100 µM of peptide solution (200 µL in pH 7.0 sodium phosphate buffer) was placed in a 1-mm quartz cuvette and the CD spectrum recorded between 190-260 nm at 25 °C using a Chirascan spectropolarimeter (Applied Photophysics) at a band width of 1 nm, step size of 1 nm and 1 s per step. The spectra presented represent an average of five scans.

5.2 Fluorescence Resonance Energy Transfer (FRET) assay

Measurements were recorded on a SpectraMax M2e Microplate Reader (Molecular Devices). See Figure S7 for the plate layout, described in the assay protocol below.

Assays were carried out in triplicate in a 96-well microplate, allowing four separate inhibitors able to be tested simultaneously. 150 µL buffer (25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, 0.1% Tween) was added to wells B–G in rows 12, 9, 6 and 3. 225 µL of Inhibitor 1 was added to well A12, Inhibitor 2 to A9, Inhibitor 3 to A6 and Inhibitor 4 to A3. Tripling dilutions using 75 µL of each of these inhibitors were carried out between wells A-G to give a concentration range between 100-0.14 µM. 50 µL was transferred from row 12 into rows 11 and 10, 50 µL from row 9 into rows 8 and 7, 50 µL from row 6 into rows 5 and 4, and 50 µL from row 3 into rows 2 and 1 to set up triplicate experiments. 50 µL of buffer was added to wells 1–12 of column H which were to be used for controls.

7.5 mL dGMyoA (1.3 µM) was mixed with 7.5 mL *Pf*MTIPΔ60 (1.6 µM); 350 µL dGMyoA (1.3 µM) was mixed with 350 µL buffer, and 350 µL *Pf*MTIP Δ60 (1.6 µM) was mixed with 350 µL buffer. Each of these solutions was incubated at 37 °C for 10 min along with the plate containing the various concentrations of inhibitor. 150 µL of the *Pf*MTIPΔ60/dGMyoA complex was then added to wells A–G of rows 1–12 to give wells containing the preformed complex (final concentrations: 0.6 µM *Pf*MTIPΔ60; 0.5 µM dGmyoA) with various concentrations of inhibitor. 150 µL was also added to wells H1–H3 to be used as controls. 150 µL of *Pf*MTIPΔ60 (0.8 µM) mixed with plate assay buffer was added to wells H4–H6, and 150 µL dGMyoA (0.65 µM) mixed with assay buffer was added to wells H7–H9. 150 µL plate assay buffer was added to H10–H12 to complete the control lane. See Figure S7 for the final plate layout. The plate was incubated at 37 °C for 30 min before fluorescence was scanned between 320–600 nm (excitation 280 nm, cutoff 325 nm) and single point readings were taken at emission 320 nm (excitation 280nm, no cutoff) and 525 nm (excitation 280 nm, cutoff 325 nm). Measurements were taken at 37 °C. The readings at 525 nm (corresponding to the FRET signal) were normalized and plotted against inhibitor concentration to generate IC_{50} curves. The IC_{50}s were fit according to the 'background corrected IC_{50}' four-parameter equation in GraFit (Erithacus Software):

$$y = \frac{Range}{1 + (\frac{x}{IC_{50}})^s}$$

in which *y* is the FRET signal, *x* is inhibitor concentration, *s* is the Hill slope and '*Range*' is the difference between fitted uninhibited signal and the background fluorescence.

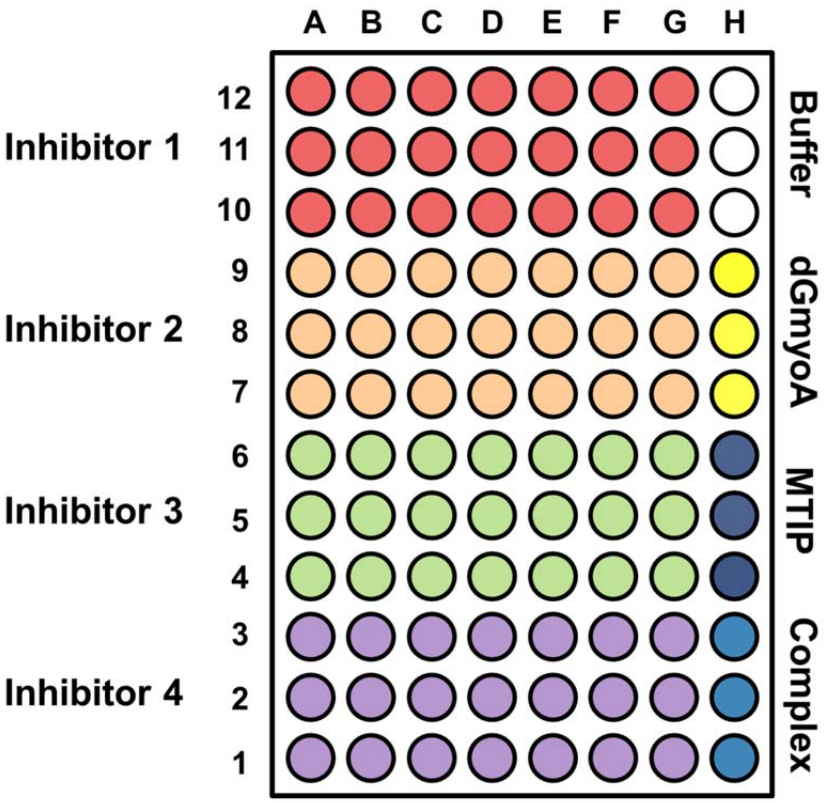


Figure S7 Plate layout for the FRET inhibition assay.

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