Supporting Information

Protein-Ligand Interactions: Thermodynamic Effects Associated with Increasing the Length of an Alkyl Chain

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MATERIALS AND EXPERIMENTAL METHODS

General for Synthetic Experiments. Solvents and reagents were reagent grade and were used without purification, unless otherwise noted. *N*, *N*-dimethylformamide (DMF) was dried and stored over activated molecular sieves. Dichloromethane (CH_2Cl_2), diisopropylethylamine ($i Pr_2NEt$), triethylamine (Et_3N), and pyridine were distilled from calcium hydride. Reaction temperatures are reported as the temperature of the bath surrounding the reaction vessel. Removal of solvent or concentration under reduced pressure was performed using a rotary evaporator with a bath temperature at 25–30 °C. Flash chromatography was preformed with the indicated solvents and Merck 250–400 mesh silica gel. HPLC was conducted using a binary solvent system, where solvent system A was 0.1% aqueous TFA and solvent system B was 0.1% TFA in acetonitrile, with a C18 column (10 mm particle size, 300 Å pore size), 22 mm diameter × 250 mm (flow rate of 10 mL/min), being used for preparative work and a C18 column (10 mm particle size, 300 Å pore size), 4.6 mm diameter × 250 (flow rate of 1 mL/min), being used for analytical work. Analytical TLC was preformed with Merck-60 TLC plates and the indicated solvents.

Melting points were determined on a melting point apparatus and are uncorrected. Proton (^1H) nuclear magnetic resonance (NMR) spectra were obtained at the indicated field strength as solutions in the indicated solvent. Chemical shifts are reported in parts per million (ppm, δ) referenced relative to the center of the residual ^1H resonance of the solvent (CD_3OD = 3.31 ppm; D_2O = 4.67 ppm; DMSO-d_6 = 2.50 ppm; or $CDCl_3$ = 7.26 ppm). Coupling constants are reported in hertz (Hz). Splitting patterns are designated as: s = singlet; d = doublet; dd = doublet of doublet; ddd = doublet of doublet of doublets; t = triplet; q = quartet; p = pentuplet; hep = heptet; m = multiplet; comp = overlapping multiplets of magnetically non-equivalent protons; br = broad; app = apparent. Carbon 13 (^{13}C) NMR spectra were obtained at the field indicated strength as solutions in the indicated solvent. Resonances are reported in ppm referenced from the center of the ^{13}C multiplet of the solvent (CD_3OD = 49.0 ppm; $CDCl_3$ = 77.2 ppm; or DMSO-d_6 = 39.5 ppm). Spectra taken in D_2O were referenced utilizing an external standard. Isothermal titration calorimetry was performed as previously described.[25,147]

General Procedure A: Solid Phase Peptide Synthesis of Tripeptides.

Peptide synthesis was performed on Rink Amide MBHA Resin using the standard Fmoc strategy; BOP was used as the coupling reagent in the presence of DIPEA in DMF. The following manual performed protocol was used: Rink Amide MBHA resin (Chem-Impex™, 0.72 mmol/g, 0.28 g) was swelled with CH_2Cl_2 (5 mL) for 20 min, whereupon the resin was filtered and washed with CH_2Cl_2 (3 x 5 mL). The resin was washed sequentially with DMF (2 x 3 mL), MeOH (2 x 3 mL), CH_2Cl_2 (2 x 3 mL), and DMF (2 x 3 mL). A solution of piperidine (20% v/v in DMF, 1% HOBt, 3 mL) was added to the washed resin, and the mixture was agitated for 20 min by bubbling N_2 through the mixture. The solvent was removed by filtration, and the resin was washed with DMF (2 x 3 mL). The resin was treated with piperidine and washed as described above one additional time.

To the resin was added N^{α}-Fmoc-N^{β}-trityl-L-asparagine (0.361 g, 0.604 mmol), BOP (0.268 g, 0.605 mmol), DMF (3 mL), followed by $^{i}Pr_2NEt$ (112 µL, 0.643 mmol). The resulting suspension was agitated (N_2) for 1 h, whereupon the resin was filtered and thoroughly washed with DMF (2 x 5 mL). N^{α}-Fmoc-N^{β}-trityl-L-asparagine was coupled a second time as above. The coupling was determined to be complete by a negative reaction to the Kaiser test;[164] in the event of a positive Kaiser test indicating the presence of a free amino group, the coupling reaction was repeated.

The resulting resin was deprotected with piperidine as reported above, twice. Then the desired Fmoc-Xaa-OH (0.604 mmol), BOP (0.268 g, 0.600 mmol), DMF (3 mL), followed by $^{i}Pr_2NEt$ (112 µL, 0.643 mmol) were added to the resin. The resulting suspension was agitated (N_2) for 1 h, whereupon the resin was filtered and thoroughly washed with DMF (2 x 5 mL). The desired Fmoc-Xaa-OH was coupled a second time as above.

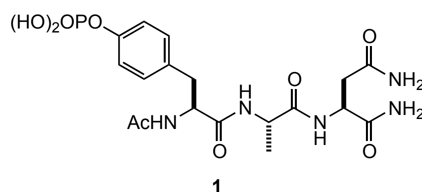
The resulting resin was deprotected with piperidine as reported above two times. The desired Fmoc-Tyr(OPO(OBn)$_2$)-OH (398 mg, 0.600 mmol), BOP (0.268 g, 0.605 mmol), DMF (3 mL), followed by $^{i}Pr_2NEt$ (112 µL, 0.643 mmol) were added to the resin. The resulting suspension was agitated (N_2) for 1 h, whereupon the resin was filtered and thoroughly washed with DMF (2 x 5 mL). Fmoc-Tyr(OPO(OBn)$_2$)-OH was coupled a second time as described.

After deprotection of the tripeptide resin, a solution of acetic anhydride (50% in CH_2Cl_2, v/v, 5 mL) was added, and the resulting suspension was agitated for 10 min. The resin was filtered, fresh acetic anhydride (50% in CH_2Cl_2, v/v, 5 mL) was added, and the suspension was agitated for 5 min, filtered and washed thoroughly with CH_2Cl_2 (5 x 5 mL).

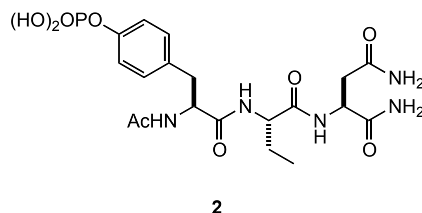
The resin was washed with DMF (2 x 3 mL) and MeOH (2 x 3 mL), whereupon a cleavage cocktail comprising TFA, TIPSH, and H_2O (95:2.5:2.5, 15 mL) was added, and the suspension was agitated for 1.5 h. The solution was collected, and the resin was rinsed with portions of cleavage

cocktail (2 x 10 mL). The combined cleavage cocktail was concentrated *in vacuo* to 1–2 mL, and a solution of Et_2O and hexanes (1:1, 20 mL) was then added to precipitate the peptide.

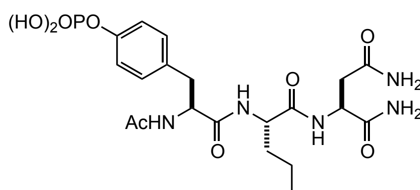
The tripeptide was purified by RP HPLC with a linear binary gradient of solvents A (H_2O, 0.1% TFA) and B (CH_3CN, 0.1% TFA). Products eluted as a single peak. Fractions containing product were collected in a 50 mL centrifuge tube. The solution was frozen and lyophilized until no solvent remained. Millipore water (20 mL) was added to the white solid in the centrifuge tube, which was again frozen and lyophilized until all water was removed, twice. Once all solvent was removed the white solid was pulverized with a spatula and lyophilized for an additional 6 h.



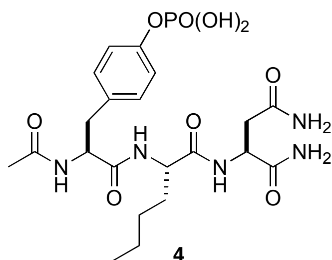
4-((*S*)-2-Acetamido-3-((*S*)-1-((*S*)-1,4-diamino-1,4-dioxobutan-2-ylamino)-1-oxopropan-2-ylamino)-3-oxopropyl)phenyl dihydrogen phosphate (1). Prepared according to **General Procedure A** to give 0.042 g (32%) of **1** as a white powder; ^{1}H NMR (D_2O, 400 MHz) δ 7.19 (d, J = 8.5 Hz, 2H), 7.09 (d, J = 8.5 Hz, 2H), 4.58 (dd, J = 8.0, 5.7 Hz, 1H), 4.50 (dd, J = 8.5, 6.3 Hz, 1H), 4.22 (q, J = 7.2 Hz, 1H), 3.06 (dd, J = 14.0, 6.3 Hz, 1H), 2.91 (dd, J = 14.0, 8.6 Hz, 1H), 2.77 (dd, J = 15.6, 5.7 Hz, 1H), 2.69 (dd, J = 15.6, 8.0 Hz, 1H), 1.89 (s, 3H), 1.28 (d, J = 7.2 Hz, 3H). ^{13}C NMR (D_2O, 125Hz) δ 175.0, 174.7, 174.6, 174.4, 173.5 150.7 (d, J_{PC} = 6.4 Hz), 132.5, 130.5, 120.6, 120.6, 55.2, 50.4, 49.9, 36.4, 36.3, 21.7, 16.4; mass spectrum (ESI –) *m/z* 486.1395 [$C_{18}H_{25}N_5O_9P$ (M–H) requires 486.1388].



4-((*S*)-2-Acetamido-3-(((*S*)-1-(((*S*)-1,4-diamino-1,4-dioxobutan-2-yl)amino)-1-oxobutan-2-yl)amino)-3-oxopropyl)phenyl dihydrogen phosphate (2). Prepared according to **General Procedure A** to give 98 mg (75%) of **2** as a white powder; mp = 160–166 ºC (dec); ^{1}H NMR (400 MHz; D_2O) δ 7.06 (d, J = 8.5 Hz, 2H), 6.97 (dd, J = 8.5, 1.1 Hz, 2H), 4.48 (dd, J = 8.1, 5.8 Hz, 1H), 4.40 (dd, J = 8.4, 6.5 Hz, 1H), 4.00 (dd, J = 8.1, 6.0 Hz, 1H), 2.93 (dd, J = 14.0, 6.5 Hz, 1H), 2.81 (dd, J = 14.0, 8.4 Hz, 1H), 2.66 (dd, J = 15.5, 5.8 Hz, 1H), 2.57 (dd, J = 15.5, 8.1 Hz, 1H), 1.79 (s, 3H), 1.67-1.46 (comp, 2H), 0.72 (t, J = 7.4 Hz, 3H); ^{13}C NMR (125 MHz; D_2O) δ 175.0, 174.7, 174.3, 173.8, 173.7, 151.0 (d, J_{pc} = 6.9 Hz), 132.3, 130.5, 120.7, 120.7, 55.6, 55.3, 50.4, 36.4, 36.4, 24.6, 21.8, 9.5; mass spectrum (ESI –) *m/z* 500.1552 [$C_{19}H_{27}N_5O_9P$ (M–H) requires 500.1551].

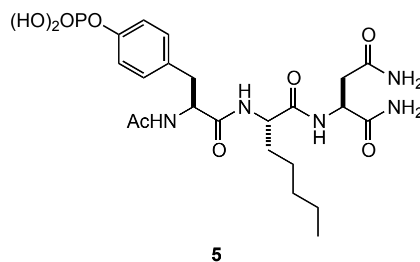


4-((*S*)-2-Acetamido-3-((*S*)-1-((*S*)-1,4-diamino-1,4-dioxobutan-2-ylamino)-1-oxopentan-2-ylamino)-3-oxopropyl)phenyl dihydrogen phosphate (3). Prepared according to **General Procedure A** to yield 0.027 g (26%) of **3** as a white powder; mp 161–166 ºC (dec); ^{1}H NMR (D_2O, 400 MHz) δ 7.20 (d, J = 8.5 Hz, 2H), 7.11 (dd, J = 8.5, 1.2 Hz, 2H), 4.61 (dd, J = 8.1, 5.8 Hz, 1H), 4.53 (dd, J = 8.4, 6.5 Hz, 1H), 4.22 (dd, J = 8.5, 5.9 Hz, 1H), 3.07 (dd, J = 14.1, 6.5 Hz, 1H), 2.94 (dd, J = 14.0, 8.5 Hz, 1H), 2.79 (dd, J = 15.5, 5.8 Hz, 1H), 2.70 (dd, J = 15.5, 8.1 Hz, 1H), 1.92 (s, 3H), 1.96–1.89 (m, 2H), 1.35–1.19 (m, 2H), 0.85 (t, J = 7.4 Hz, 3H); ^{13}C NMR (D_2O, 75 MHz) δ 175.0, 174.7, 174.3, 173.9, 173.7, 150.9 (d, J_{PC} = 6.6 Hz), 132.3, 130.5, 120.7, 120.6, 55.3, 53.9, 50.4, 36.4, 36.3, 33.0, 21.7, 18.4, 12.9; mass spectrum (ESI –) *m/z* 514.1708 [$C_{20}H_{29}N_5O_9P$ (M–H) requires 514.1706].

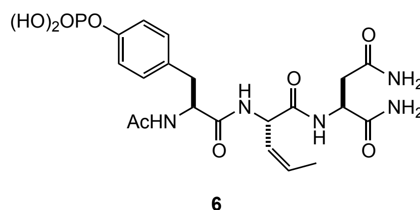


***N*-Acyl-tyrosyl(PO_3H_2)-norleucyl-asparagyl-NH_2 (4).** 1-*H*-Tetrazole (12 mg, 0.17 mmol) and dibenzyl diisopropylphosphoramidite (47 mg, 46 µL, 0.136 mmol) were added to a solution of **S5** (15 mg, 0.034 mmol) in DMF (10 mL) at 0 ºC, and the solution was stirred at 0 ºC for 1 h and then at room temperature for 15 h. The solution was cooled to 0 ºC, and 6 M *tert*-butyl hydroperoxide in decane (130 µL) was added. The resulting solution was stirred at 0 ºC for 30 min and then at room temperature for 5 h, whereupon it was cooled to 0 ºC and 5% aqueous $NaHSO_3$ (1.4 mL) was added. The solution was stirred at 0 ºC for 30 min and at room temperature for 2 h. The mixture was transferred to a separatory funnel containing H_2O (10 mL), and the layers were separated. The aqueous layer was extracted with CH_2Cl_2 (20 mL). DMF (5 mL) was added to the aqueous layer, which was again extracted with CH_2Cl_2 (15 mL), this was repeated one more time, then a final extraction of the aqueous layer with CH_2Cl_2 (15 mL) was done. The organic layers were combined and concentrated to dryness under reduced pressure. The residue was triturated with Et_2O (4 x 3 mL) to yield the crude benzyl-protected phosphotyrosine ligand as a white solid. The crude product was dissolved in MeOH/H_2O (18 mL, 15:3) containing 10% Pd/C (3 mg), and the mixture was purged four times with H_2. The suspension was stirred under H_2 (1

atm) for 14 h. The mixture was filtered through a pad of celite, and the pad was washed with MeOH (10 mL). The combined filtrate and washings were concentrated under reduced pressure to give a solid that was purified via preparative RP HPLC using a gradient of 0% B to 40% B over 30 min to yield 7 mg (39%) of the title compound **4** as a white solid over two-steps: mp 206-208 ºC (decomposed); ^{1}H NMR (500 MHz, D_2O) δ 7.13 (d, *J* = 8.5 Hz, 2 H), 7.04 (dd, *J* = 8.5, 1.3 Hz, 2 H), 4.54 (dd, *J* = 8.2, 5.8 Hz, 1 H), 4.47 (dd, *J* = 8.5, 6.5 Hz, 1 H), 4.14 (dd, *J* = 8.5, 5.9 Hz, 1 H), 3.00 (dd, *J* = 14.0, 6.5 Hz, 1 H), 2.88 (dd, *J* = 14.0, 8.5 Hz, 1 H), 2.73 (dd, *J* = 15.7, 5.8 Hz, 1 H), 2.63 (dd, *J* = 15.7, 8.2 Hz, 1 H), 1.86 (s, 3 H), 1.70-1.62 (m, 1 H), 1.62-1.53 (m, 1 H), 1.25-1.12 (comp, 4 H), 0.77 (app t, *J* = 6.9 Hz, 3 H); ^{13}C NMR (125 MHz, D_2O) δ 175.0, 174.7, 1754.3, 173.9, 173.7, 151.1, 132.2, 130.5, 120.7, 55.3, 54.1, 50.4, 36.4, 36.3, 30.7, 27.2, 21.8, 21.7, 13.3; MS (ESI –) 528.1865 *m/z* [$C_{21}H_{31}N_5O_9P$ (M–H) requires 528.1859].

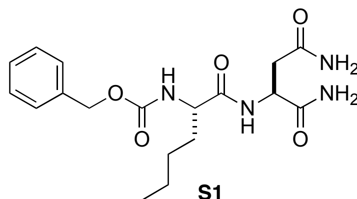


4-((*S*)-2-Acetamido-3-(((*S*)-1-(((*S*)-1,4-diamino-1,4-dioxobutan-2-yl)amino)-1-oxoheptan-2-yl)amino)-3-oxopropyl)phenyl dihydrogen phosphate (5). Prepared according to **General Procedure A** to give 28.8 mg (26%) of **5** as a white powder; mp = 165–167 ºC (dec); ^{1}H NMR (400 MHz; D_2O): δ 7.07 (dd, J = 8.5, 1.1, 2H), 6.98 (dd, J = 8.6, 1.1, 2H), 4.47 (ddd, J = 8.0, 5.8, 2.3, 1H), 4.41 (ddd, J = 8.3, 6.5, 1.8, 1H), 4.22-4.15 (m, 1H), 1.80 (s, 3H), 1.59-1.51 (m, 1H), 1.39-1.31 (m, 1H), 1.23-1.14 (m, 2H), 1.09-0.93 (m, 2H), 0.77-0.65 (m, 5H); ^{13}C NMR (150 MHz, D_2O) 174.7, 174.5, 174.1, 173.7, 173.5, 131.9, 131.2, 120.5, 120.4, 55.0, 53.8, 50.2, 36.1, 36.1, 30.7, 30.3, 24.3, 21.7, 21.5, 13.1; mass spectrum (ESI –) *m/z* 542.2022 [$C_{22}H_{33}N_5O_9P$ (M–H) requires 542.2021].

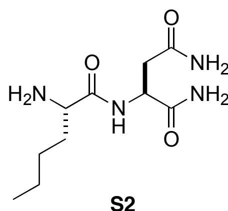


4-((*S*)-2-Acetamido-3-(((*S*,*Z*)-1-(((*S*)-1,4-diamino-1,4-dioxobutan-2-yl)amino)-1-oxopent-3-en-2-yl)amino)-3-oxopropyl)phenyl dihydrogen phosphate (6). Prepared according to **General Procedure A** utilizing 5 min coupling times and 5 min deprotection times to give 10.3 mg (10%) of **6** as a white powder; mp = 190–193 ºC; ^{1}H NMR (500 MHz; D_2O) δ 7.16 (d, *J* = 8.0 Hz, 2H), 7.06 (d, *J* = 8.0 Hz, 2H), 5.89-5.83 (m, 1H), 5.37 (t, *J* = 9.6 Hz, 1H), 4.97 (d, *J* = 9.0 Hz, 1H), 4.58 (app t, *J* = 6.5

Hz, 1H), 4.48 (app, t, J = 7.2 Hz, 1H), 3.04 (dd, J = 14.0, 6.6 Hz, 1H), 2.91 (dd, J = 14.0, 8.8 Hz, 1H), 2.76 (dd, J = 15.7, 5.5 Hz, 1H), 2.68 (dd, J = 15.7, 7.8 Hz, 1H), 1.88 (s, 3H), 1.62 (d, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 174.8, 174.7, 174.2, 173.3, 172.5, 151.0 (d, J_{pc} = 6.4 Hz), 133.0, 131.9, 130.3, 122.6, 120.5, 120.5, 55.1, 51.6, 50.3, 36.2, 35.9, 21.6, 12.7; mass spectrum (ESI –) m/z 512.1551 [$C_{20}H_{27}N_5O_9P$ (M–H) requires 512.1552].

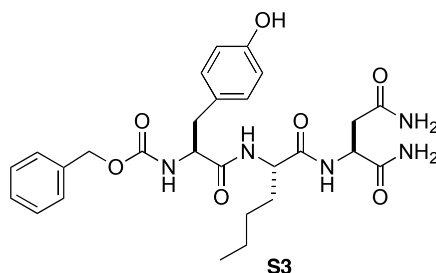


General procedure for amino acid coupling reaction (Method B): (*N*-Cbz-norleucyl)-asparagyl-NH$_2$ (S1). *N*-Methylmorpholine (NMM) (172 mg, 187 µL, 1.70 mmol) was added to a solution of Cbz-(*L*)-norleucine (150 mg, 0.566 mmol) and HCl•(*L*)-Asn-NH$_2$ (104 mg, 0.623 mmol) in DMF (9 mL) at 0 ºC. 1-(3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (119 mg, 0.623 mmol) and 1-hydroxybenzotriazole hydrate (HOBt) (153 mg, 1.13 mmol) were added, and the mixture was warmed to room temperature over 2 h and stirring continued for 15 h. The mixture was concentrated to dryness under reduced pressure. Saturated aq. $NaHCO_3$ (6 mL) was added, and the resultant solid was isolated by filtration and washed with 1 M HCl (3 x 5 mL) and H_2O (5 mL) to yield 212 mg (99%) of the title compound as a white solid. The crude product was found to be >95% pure by ^{1}H NMR and used without further purification: mp 239-240 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.07 (d, J = 7.9 Hz, 1 H), 7.50 (d, J = 7.5 Hz, 1 H), 7.40-7.26 (comp, 6 H), 7.06 (br s, 2 H), 6.88 (br s, 1 H), 5.04 (d, J = 12.5 Hz, 1 H), 4.98 (d, J = 12.5 Hz, 1 H), 4.50-4.41 (m, 1 H), 3.96-3.88 (m, 1 H), 2.56-2.40 (comp, 2 H, behind solvent peak), 1.67-1.55 (m, 1 H), 1.54-1.42 (m, 1 H), 1.31-1.16 (comp, 4 H), 0.88-0.78 (comp, 3 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.8, 171.71, 171.68, 156.2, 136.9, 128.4, 127.8, 127.7, 65.4, 54.9, 49.5, 36.9, 31.3, 27.5, 21.9, 13.9; mass spectrum (ESI +) m/z 379.1977 [$C_{18}H_{27}N_4O_5$ (M+H) requires 379.1981].

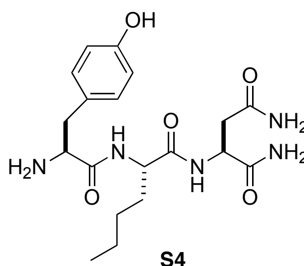


General procedure for Cbz-group removal (Method C): Norleucyl-asparagyl-NH$_2$ (S2). The benzyl carbamate **S1** (57 mg, 0.150 mmol) was dissolved in MeOH (10 mL) containing 10% Pd/C (16 mg, 10 mol %). The resulting mixture was purged four times with H_2, and the suspension was stirred under H_2 (1 atm) for 14 h. The mixture was filtered through a pad of celite, and the pad was washed

with MeOH (5 mL). The combined filtrate and washings were concentrated to dryness under reduced pressure to yield 28 mg (78%) of **S2** as a white solid. This material was found to be >95% pure by ^{1}H NMR and used without further purification: mp 176-178 ºC; ^{1}H NMR (400 MHz, CD_3OD) δ 4.70 (app t, *J* = 6.5 Hz, 1 H), 3.36-3.30 (m, 1 H), 2.70 (app d, *J* = 6.5 Hz, 2 H), 1.73-1.64 (m, 1 H), 1.58-1.47 (m 1 H), 1.40-1.28 (comp, 4 H) 0.96-0.88 (comp, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 177.8, 175.7, 175.0, 56.1, 51.1, 37.9, 35.8, 28.8, 23.7, 14.3; mass spectrum (ESI +) *m/z* 245.1620 [$C_{10}H_{21}N_4O_3$ (M+H) requires 245.1614].

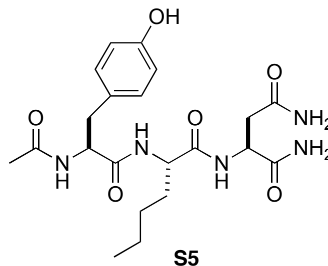


(*N*-Cbz-tyrosyl)-diethylglycyl-asparagyl-NH$_2$ (S3). Prepared from Cbz-(*L*)-Tyr and **S2** according to **Method B** to yield 146 mg (96%) of the title compound as a white solid. The crude product was found to be >95% pure by ^{1}H NMR and used without further purification; mp 222-223 ºC; ^{1}H NMR (400 MHz, CD_3OD) δ 7.34-7.24 (comp, 5 H), 7.06 (d, *J* = 8.5 Hz, 2 H), 6.68 (dd, *J* = 8.5, 1.2 Hz, 2 H), 5.10-5.02 (comp, 2 H), 4.64 (app t, *J* = 6.5 Hz, 1 H), 4.33 (dd, *J* = 9.6, 4.8 Hz, 1 H), 4.19 (dd, *J* = 8.5, 5.5 Hz, 1 H), 3.05 (dd, *J* = 14.0, 4.8 Hz, 1 H) 2.81-2.67 (comp, 3 H), 1.86-1.74 (m, 1 H), 1.71-1.59 (m, 1 H), 1.38-1.23 (comp, 4 H), 0.94-0.85 (comp, 3 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.8, 172.2, 171.8, 171.3, 155.9, 155.7, 137.0, 130.2, 128.4, 128.3, 127.7, 127.4, 114.8, 65.2, 56.5, 53.0, 49.5, 36.7, 36.5, 31.5, 27.3, 22.0, 13.9; mass spectrum (ESI +) *m/z* 542.2622 [$C_{27}H_{36}N_5O_7$ (M+H) requires 542.2615].



Tyrosyl-norleucyl-asparagyl-NH$_2$ (S4). Prepared by hydrogenolysis (**Method C**) of **S3** in MeOH (5 mL) to afford **S4** in 99% yield (37 mg, 0.092 mmol) as a white solid. This material was found to be >95% pure by ^{1}H NMR and used without further purification; mp 172-173 ºC; ^{1}H NMR (400 MHz, CD_3OD) δ 7.04 (d, *J* = 8.5 Hz, 2 H), 6.72 (d, *J* = 8.5 Hz, 2 H), 4.64 (app t, *J* = 6.2 Hz, 1 H), 4.19 (dd, *J* = 8.5, 5.1 Hz, 1 H), 3.60 (dd, *J* = 7.5, 5.1 Hz 1 H), 2.96 (dd, *J* = 13.7, 5.1 Hz, 1 H) 2.76-2.68 (comp, 3 H),

1.82-1.72 (m, 1 H), 1.68-1.58 (m, 1 H), 1.40-1.23 (comp, 4 H) 0.90 (app t, J = 6.8 Hz, 3 H); ^{13}C NMR (100 MHz, DMSO-d$_6$) 174.4, 172.8, 171.7, 171.2, 155.8, 130.2, 128.3, 114.9, 56.1, 52.4, 49.6, 36.7, 31.8, 27.1, 22.0, 13.9; mass spectrum (ESI +) *m/z* 408.2242 [$C_{19}H_{30}N_5O_5$ (M+H) requires 408.2254].



(N-Acyl-tyrosyl)-norleucyl-asparagyl-NH$_2$ (S5). Prepared from AcOH and **S4** according to the general procedure (Method B) to yield 52 mg (70%) of the title compound as a white solid. The crude product was found to be >95% pure by ^{1}H NMR and used without further purification; mp 227–229 ºC; ^{1}H NMR (400 MHz, CD$_3$OD) δ 7.08 (d, J = 8.6 Hz, 2 H), 6.70 (d, J = 8.6 Hz, 2 H), 4.64 (t, J = 6.2 Hz, 1 H), 4.55 (dd, J = 9.2, 5.1 Hz, 1 H), 4.19 (dd, J = 8.9, 5.1 Hz, 1 H), 3.07 (dd, J = 14.4, 5.1 Hz, 1 H), 2.80 (dd, J = 14.4, 9.2 Hz, 1 H), 2.73 (d, J = 6.2 Hz, 2 H), 1.90 (s, 3 H), 1.86-1.76 (m, 1 H), 1.70-1.60 (m, 1 H), 1.40-1.25 (comp, 4 H), 0.96-0.86 (comp, 3 H); ^{13}C NMR (150 MHz, CD$_3$OD) δ 175.5, 175.1, 174.6, 174.0, 173.5, 157.3, 131.3, 129.1, 116.2, 56.5, 55.5, 51.4, 37.8, 37.4, 32.2, 29.0, 23.4, 22.4, 14.2; mass spectrum (ESI +) *m/z* 450.2347 [$C_{21}H_{32}N_5O_6$ (M+H) requires 450.2341].

JMM03-140a-2 Ala ligand

Pulse Sequence: s2pul

Solvent: D2O
Ambient temperature
Mercury-400BB "nmr6"

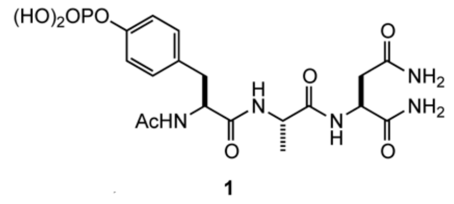
Relax. delay 2.000 sec
Pulse 16.4 degrees
Acq. time 2.856 sec
Width 5602.2 Hz
17 repetitions
OBSERVE H1, 400.2680071 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 0 min, 0 sec

$(HO)_2OPO$ AcHN H O N O H N O NH_2 NH_2

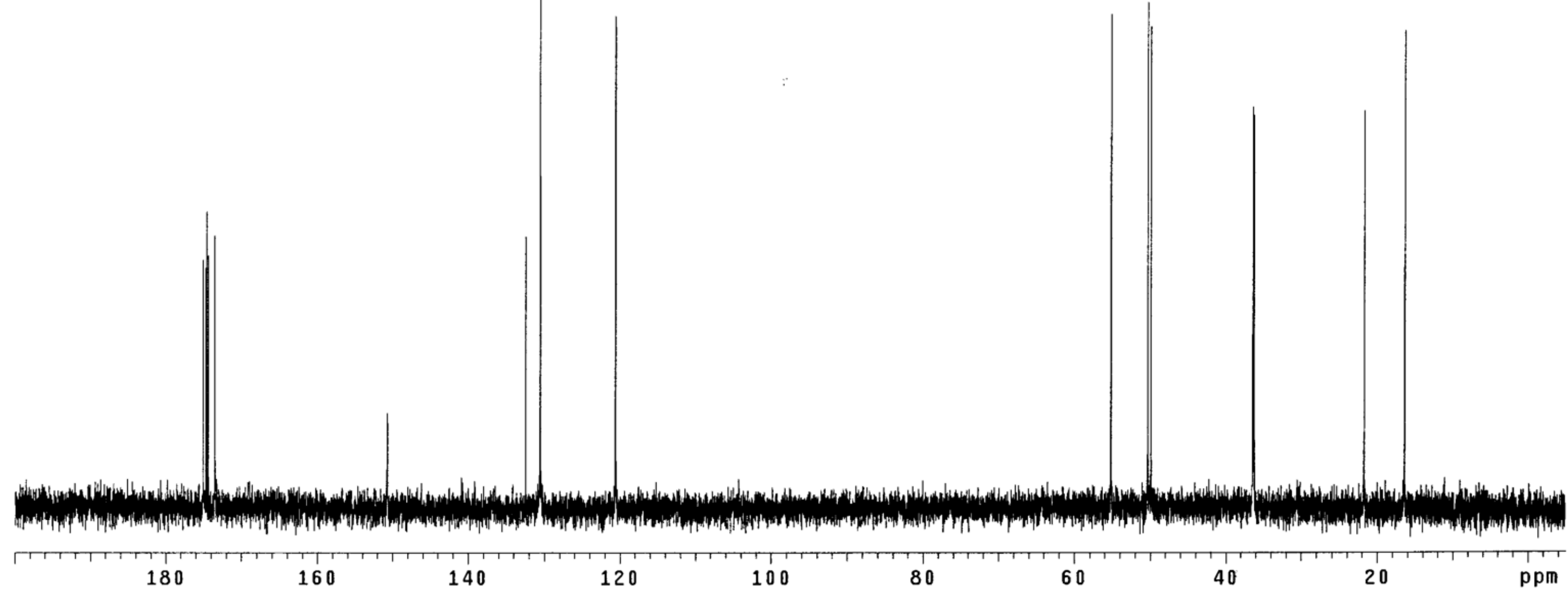
1

9 8 7 6 5 4 3 2 1 ppm

2.00 2.00 1.02 1.03 1.00 1.02 1.07 1.08 1.01 2.94 2.94



(HO)2OPO
AcHN
H
N
O
NH2
NH2
1



JMM03-140a-13C
exp4 Carbon
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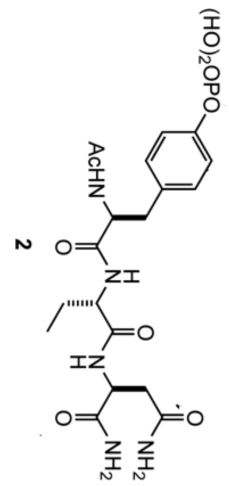
date Aug 28 2009
solvent d2o
file exp
ACQUISITION
sw 30143.2
at 1.062
np 64024
fb 17000
bs 64
ss 128
d1 2.000
nt 20000
ct 1312
TRANSMITTER
tn C13
sfrq 125.587
tof 1254.3
tpwr 51
pw 8.000
DECOUPLER
dn H1
dof 0
dm yyy
dmm w
dpwr 39
dmf 12600
SPECIAL
temp 27.0
gain 50
spin 20
hst 0.008
pw90 15.500
alfa 10.000
FLAGS
il n
in n
dp y
hs nn
PROCESSING
lb 1.00
fn not used
DISPLAY
sp -628.8
wp 25742.4
rfl 1886.3
rfp 0
rp 98.1
lp -221.3
PLOT
wc 250
sc 0
vs 12158
th 68
ai cdc ph
jw
180
160
140
120
100
80
60
40
20
ppm


(HO)$_2$OPO
AcHN
2
NH$_2$
NH$_2$

500 MHz nmr0

JMM03-109a-1

exp4 Carbon

```
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date   Jul 15 2009  temp          27.0
solvent        d2o  gain            50
file /home/service~ spin            20
/vnmrsys/data/jmm0~ hst          0.008
  3_109a_1_c13.fid  pw90         9.500
    ACQUISITION     alfa        10.000
sw         30165.9          FLAGS
at           1.958  il               n
np          118154  in               n
fb           17000  dp               y
bs              16  hs              nn
d1           2.000        PROCESSING
nt           12000  lb            1.00
ct           12000  fn        not used
   TRANSMITTER             DISPLAY
tn             C13  sp          -628.7
sfrq       125.705  wp         28280.5
tof         1255.4  rfl         1885.3
tpwr            53  rfp              0
pw           3.163  rp           -30.5
    DECOUPLER       lp          -189.2
dn              H1            PLOT
dof              0  wc             250
dm             yyy  sc               0
dmm              w  vs           29254
dpwr            37  th               5
dmf          10582  ai   cdc  ph
```
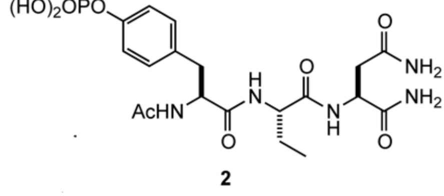
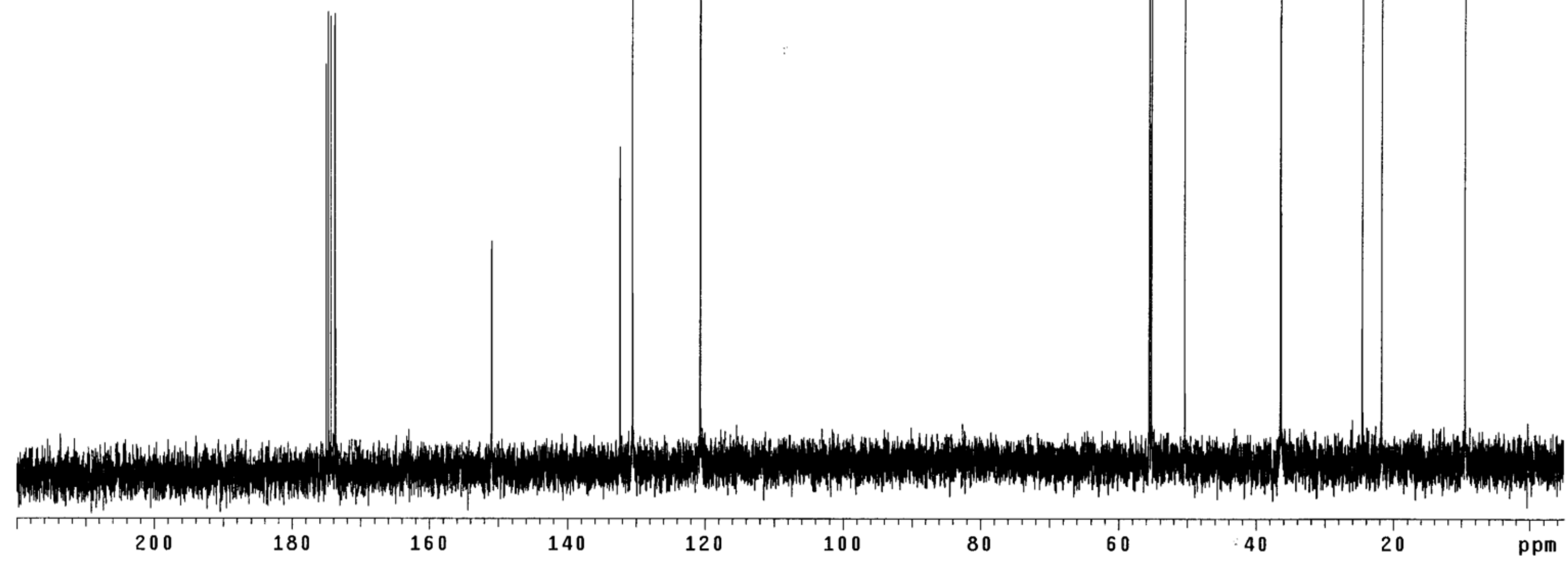
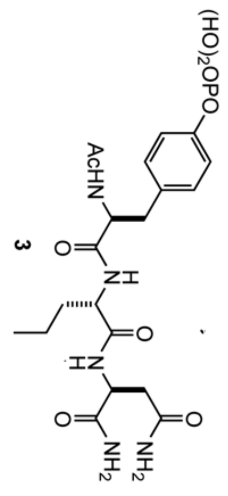







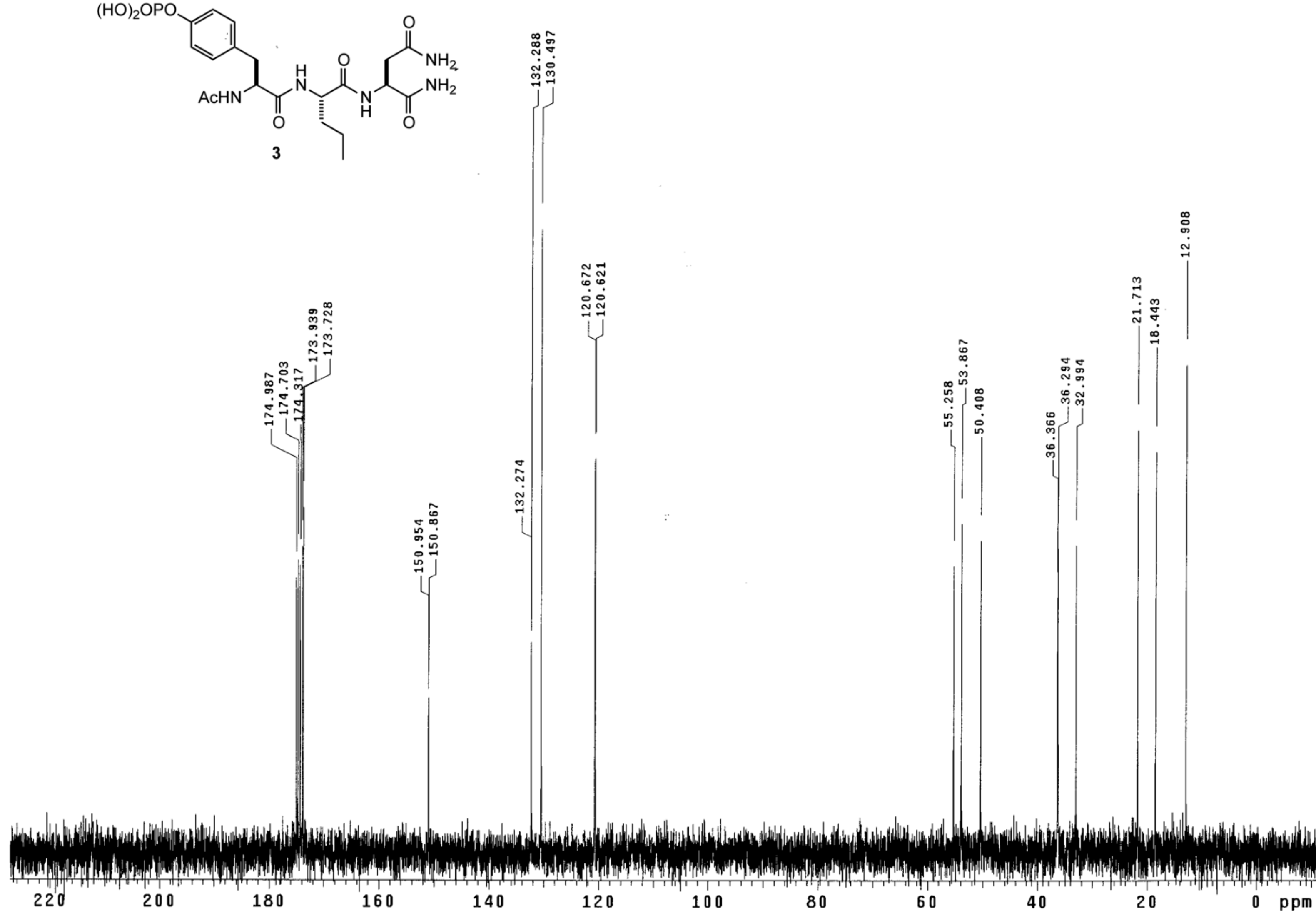
8

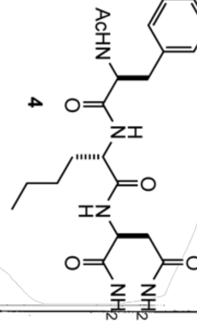
6

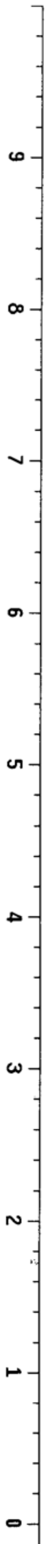
4

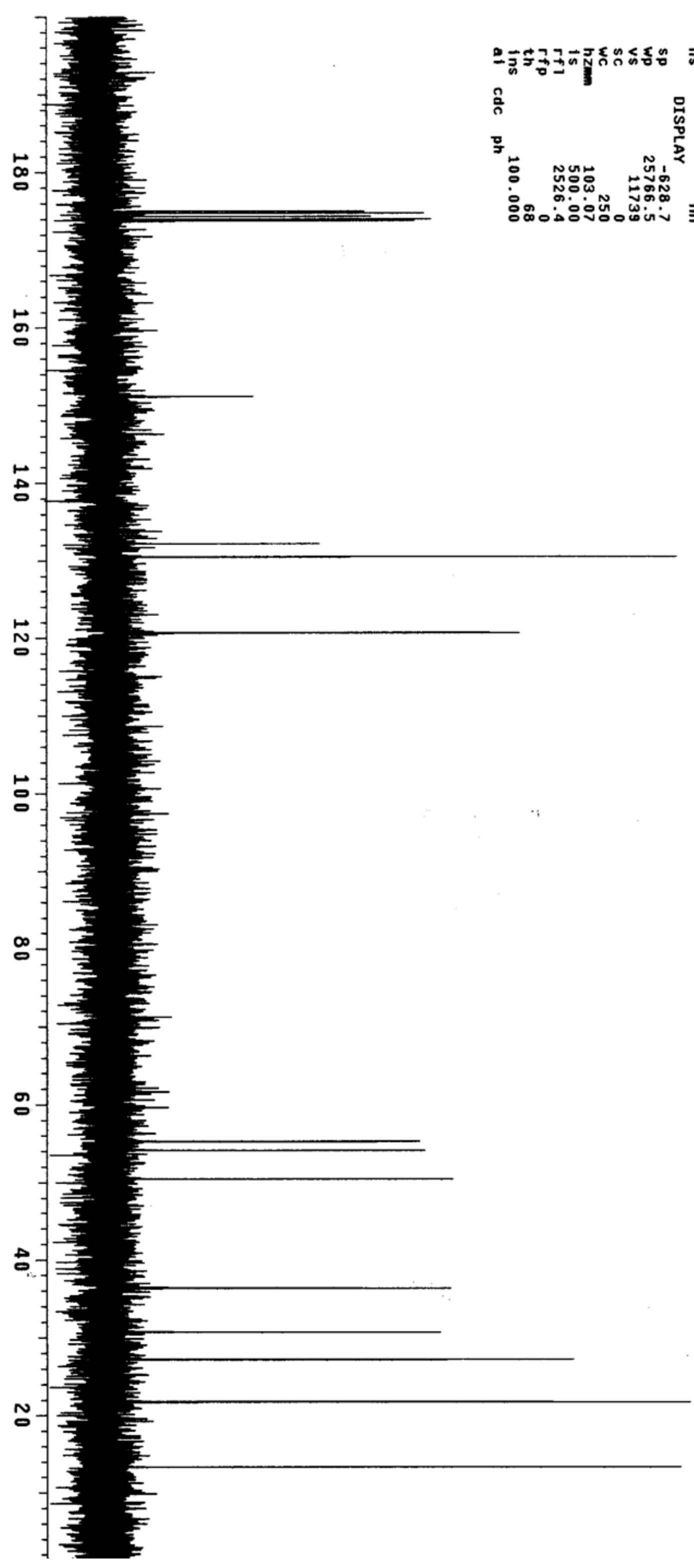
2

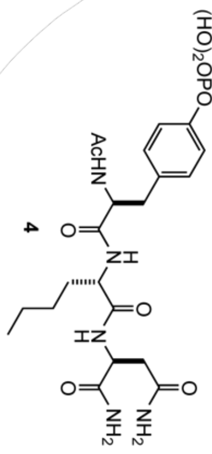
PPM

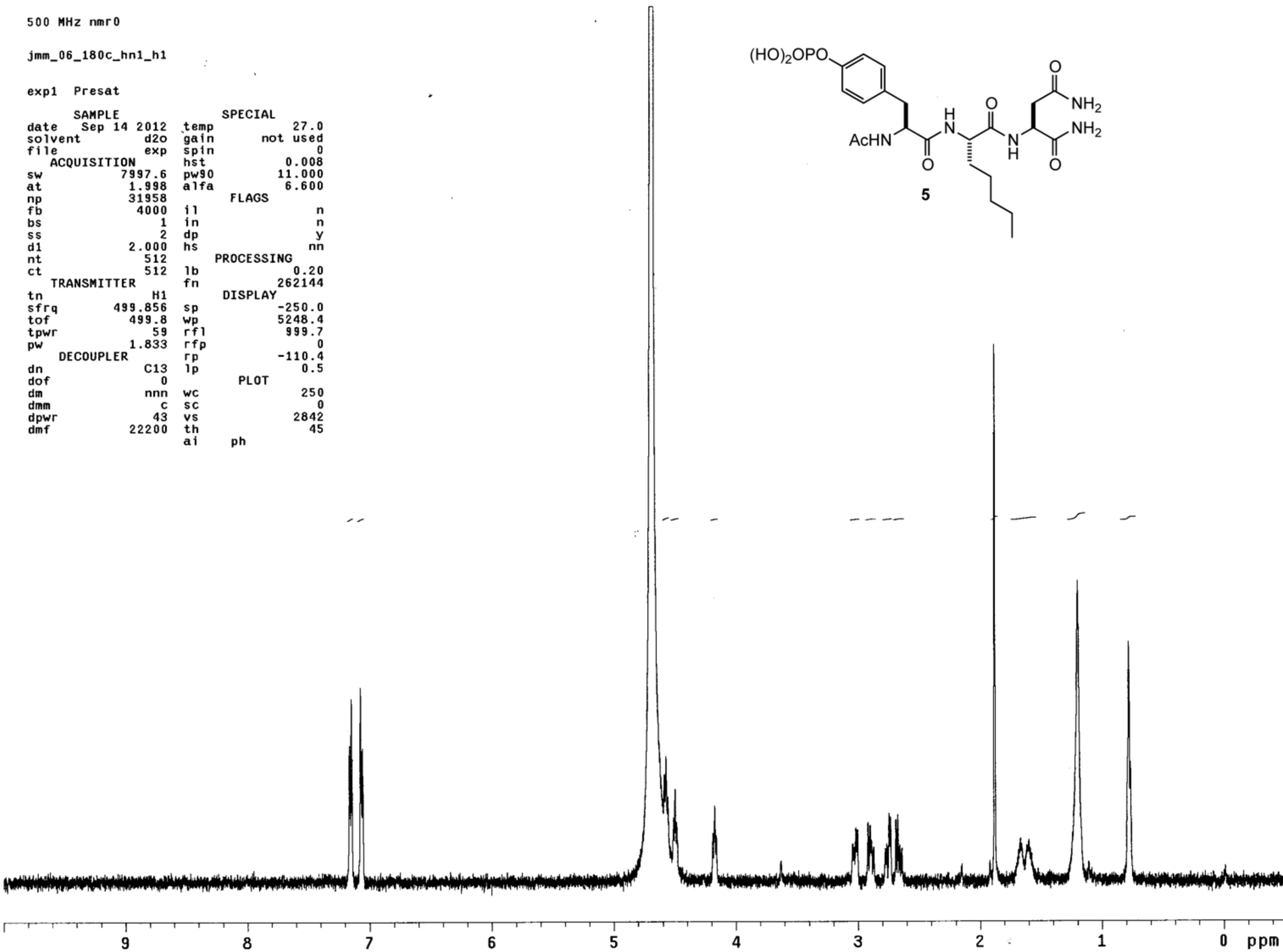

(HO)2OPO
AcHN
NH2
NH2
3
174.987
174.703
174.317
173.939
173.728
150.954
150.867
132.274
132.288
130.497
120.672
120.621
55.258
53.867
50.408
36.366
36.294
32.994
21.713
18.443
12.908
220
200
180
160
140
120
100
80
60
40
20
0
ppm



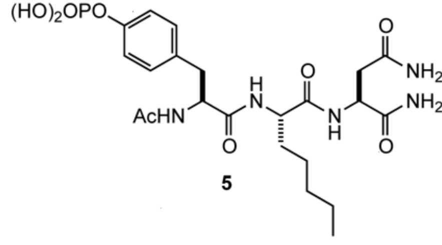




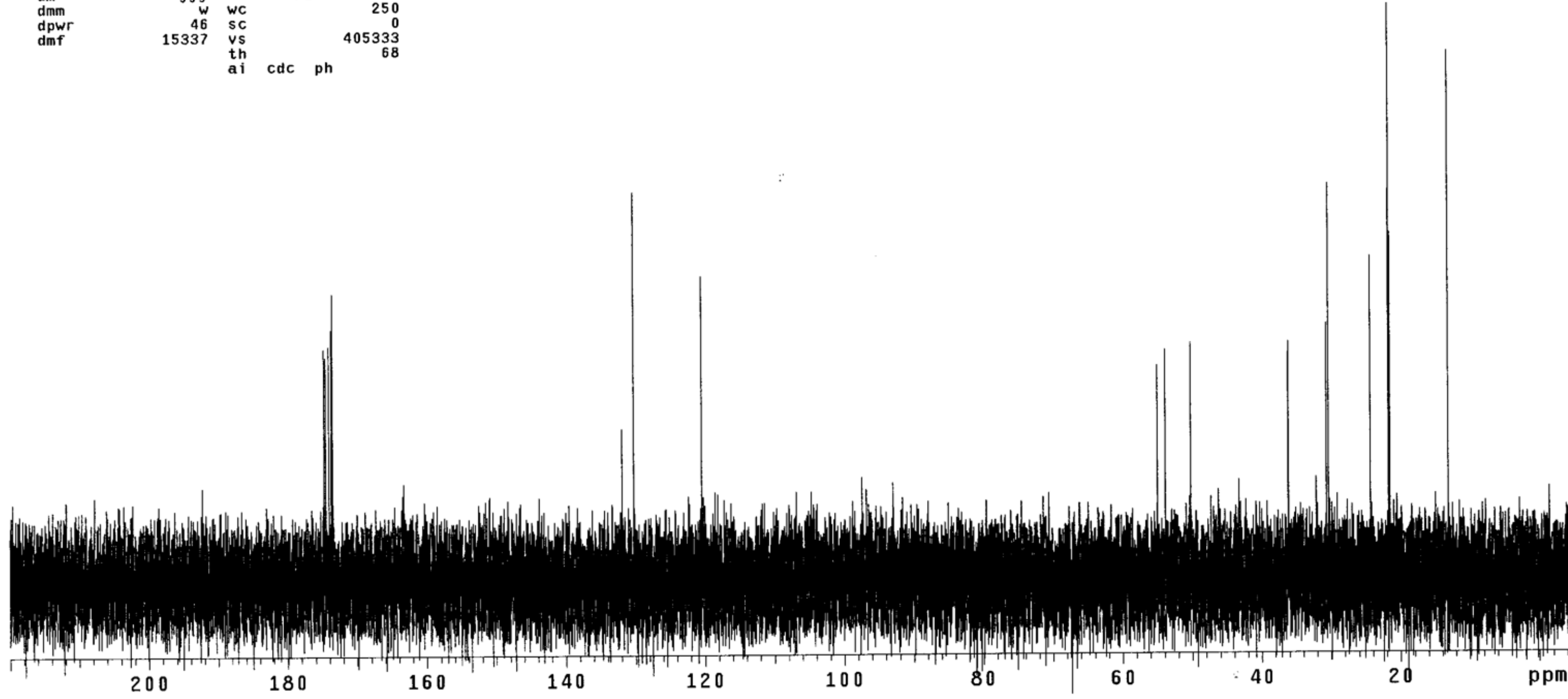




500 MHz nmr0
jmm_06_180c_hn1_h1
exp1 Presat
SAMPLE
date Sep 14 2012
solvent d2o
file exp
ACQUISITION
sw 7997.6
at 1.998
np 31958
fb 4000
bs 1
ss 2
d1 2.000
nt 512
ct 512
TRANSMITTER
tn H1
sfrq 499.856
tof 499.8
tpwr 59
pw 1.833
DECOUPLER
dn C13
dof 0
dm nnn
dmm c
dpwr 43
dmf 22200
SPECIAL
temp 27.0
gain not used
spin 0
hst 0.008
pw90 11.000
alfa 6.600
FLAGS
il n
in n
dp y
hs nn
PROCESSING
lb 0.20
fn 262144
DISPLAY
sp -250.0
wp 5248.4
rfl 999.7
rfp 0
rp -110.4
lp 0.5
PLOT
wc 250
sc 0
vs 2842
th 45
ai ph
(HO)2OPO
AcHN
H
N
O
NH2
5
9
8
7
6
5
4
3
2
1
0 ppm

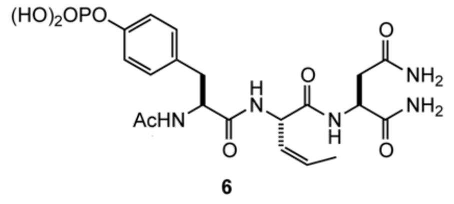


(HO)2OPO
AcHN
H
N
O
O
H
N
O
NH2
NH2
O
5

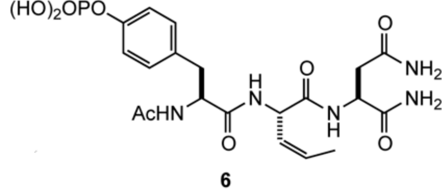


600 MHz nmrox
JMM04 227a-102
exp4 Carbon
SAMPLE
date Apr 19 2011
solvent d2o
file exp
ACQUISITION
sw 40322.6
at 2.000
np 161290
fb 17000
bs 128
d1 2.000
nt 13000
ct 13000
TRANSMITTER
tn C13
sfrq 150.824
tof 2296.3
tpwr 58
pw 2.600
DECOUPLER
dn H1
dof 0
dm yyy
dmm w
dpwr 46
dmf 15337
SPECIAL
temp 27.0
gain 40
spin 20
hst 0.008
pw90 7.800
alfa 10.000
FLAGS
il n
in n
dp y
hs nn
PROCESSING
lb 0.50
fn not used
DISPLAY
sp -754.2
wp 33931.4
rfl 3572.5
rfp 0
rp -57.5
lp 0
PLOT
wc 250
sc 0
vs 405333
th 68
ai cdc ph
200
180
160
140
120
100
80
60
40
20
ppm

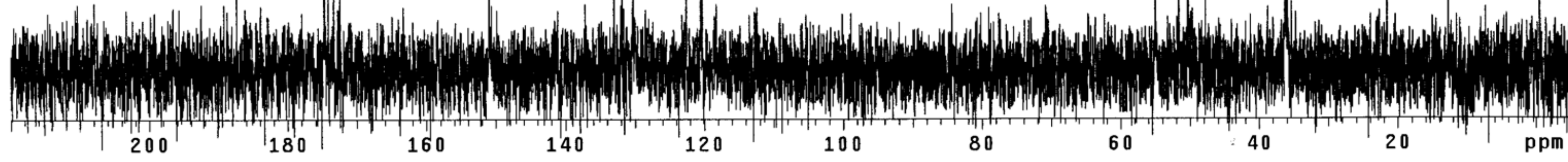
```
500 MHz nmr0

jmm06_185c_h1

exp1  Presat

      SAMPLE                 SPECIAL
date    Aug 23 2012   temp            27.0
solvent         d2o   gain        not used
file            exp   spin               0
   ACQUISITION        hst            0.008
sw           7997.6   pw90          11.000
at            2.000   alfa           6.600
np            31990           FLAGS
fb             4000   il                 n
bs                1   in                 n
ss                2   dp                 y
d1            2.000   hs                nn
nt               64         PROCESSING
ct               64   lb              0.20
   TRANSMITTER        fn            262144
tn               H1           DISPLAY
sfrq        499.856   sp            -250.0
tof           499.8   wp            5248.4
tpwr             59   rfl            999.7
pw            1.833   rfp                0
    DECOUPLER         rp            -106.3
dn              C13   lp             -10.3
dof               0           PLOT
dm              nnn   wc               250
dmm               c   sc                 0
dpwr             43   vs               128
dmf           22200   th                45
                      ai     ph
```



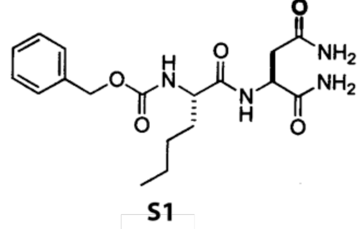
9 8 7 6 5 4 3 2 1 0 ppm

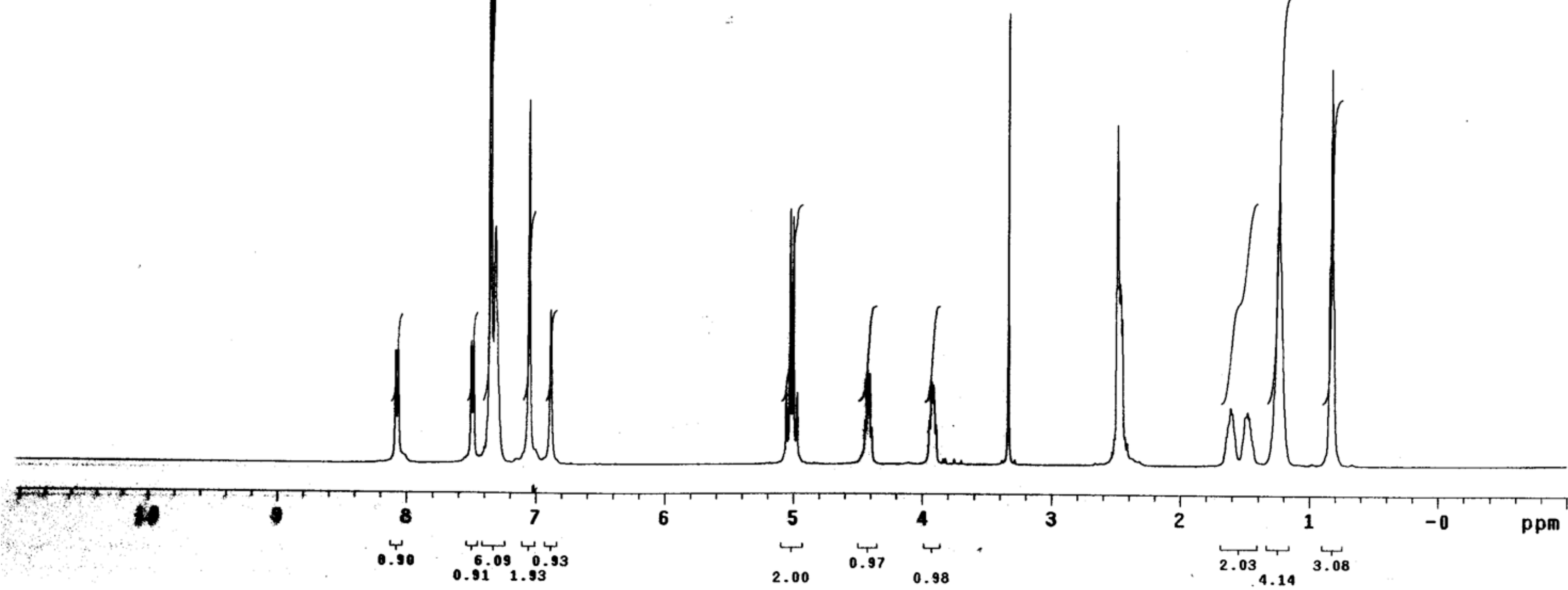


(HO)2OPO
AcHN
NH2
NH2
6



500 MHz nmr0
jmm06_185c_c13
exp4 Carbon
SAMPLE
date Aug 23 2012
solvent d2o
file exp
ACQUISITION
sw 30165.9
at 1.958
np 118154
fb 17000
bs 1
d1 2.000
nt 55000
ct 13610
TRANSMITTER
tn C13
sfrq 125.701
tof 1255.4
tpwr 55
pw 3.862
DECOUPLER
dn H1
dof 0
dm yyy
dmm w
dpwr 38
dmf 11800
SPECIAL
temp 27.0
gain 50
spin 20
hst 0.008
pw90 11.600
alfa 10.000
FLAGS
il n
in n
dp y
hs nn
PROCESSING
lb 2.00
fn not used
DISPLAY
sp -628.5
wp 28279.6
rfl 11595.3
rfp 9678.0
rp -130.0
lp -205.0
PLOT
wc 250
sc 0
vs 85051
th 53
ai ph
200
180
160
140
120
100
80
60
40
20
ppm


S1

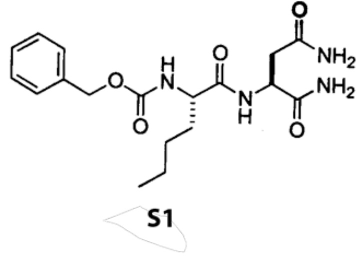

pad=10 run with findz0 before acquisition
pad=10 run with gradshim before acquisition
Archive directory:
Sample directory:
Pulse Sequence: s2pul
Solvent: dmso
Temp. 27.0 C / 300.1 K
File: martin_jed3-78_s2pul_H1
INOVA-500 "nmrfred"
Relax. delay 2.000 sec
Pulse 30.0 degrees
Acq. time 4.049 sec
Width 4807.7 Hz
64 repetitions
OBSERVE H1, 399.8066079 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 65536
Total time 6 min, 39 sec
ppm
0.90
0.91
6.09
1.93
0.93
2.00
0.97
0.98
2.03
4.14
3.08

Archive directory:
Sample directory:

Pulse Sequence: s2pul

Solvent: dmso
Temp. 27.0 C / 300.1 K
User: 1-14-87
File: martin_jed3-78_s2pul_C13
INOVA-500 "nmrfred"

Relax. delay 2.000 sec
Pulse 30.0 degrees
Acq. time 1.300 sec
Width 24509.8 Hz
2000 repetitions
OBSERVE C13, 100.5314974 MHz
DECOUPLE H1, 399.8086099 MHz
Power 44 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
Total time 1 hr, 50 min, 18 sec

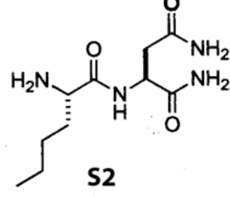


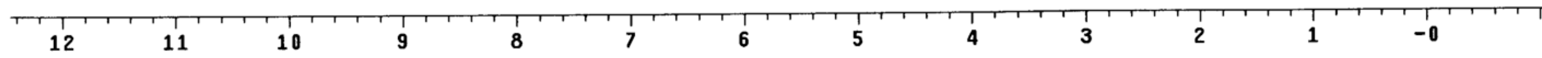
220 200 180 160 140 120 100 80 60 40 20 0 ppm

JEDIII(295)-7/7/07

Pulse Sequence: s2pul
Solvent: Cd3OD
Ambient temperature
Mercury-400BB "nmr6"

Relax. delay 2.000 sec
Pulse 16.4 degrees
Acq. time 2.856 sec
Width 5602.2 Hz
24 repetitions
OBSERVE H1, 400.2685569 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 0 min, 0 sec

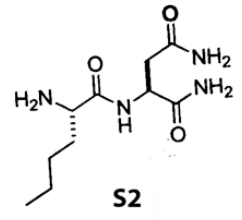


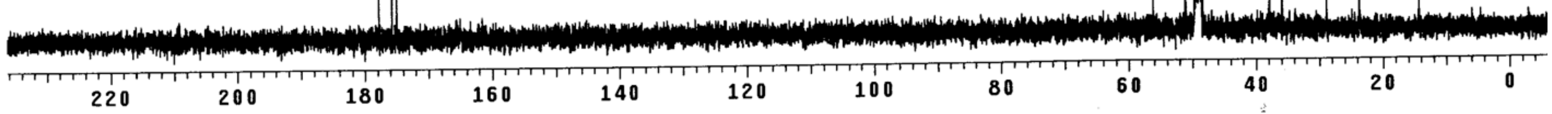


13C OBSERVE

Pulse Sequence: s2pul
 Solvent: CD3OD
 Ambient temperature
Mercury-400BB "nmr6"

 Relax. delay 2.000 sec
 Pulse 22.5 degrees
 Acq. time 1.280 sec
 Width 25188.9 Hz
 684 repetitions
OBSERVE C13, 100.6474659 MHz
DECOUPLE H1, 400.2705726 MHz
 Power 38 dB
 continuously on
 WALTZ-16 modulated
DATA PROCESSING
 Line broadening 1.0 Hz
FT size 65536
Total time 25 hr, 7 min, 34 sec

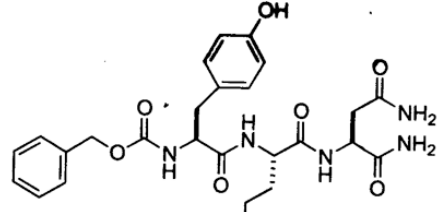




JEDIII(298)-7/11/07

Pulse Sequence: s2pul
Solvent: CD3OD
Ambient temperature
Mercury-400BB "nmr6"

Relax. delay 2.000 sec
Pulse 16.4 degrees
Acq. time 2.856 sec
Width 5602.2 Hz
36 repetitions
OBSERVE H1, 400.2685569 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 0 min, 0 sec



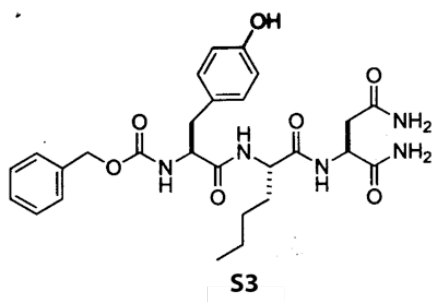
12 11 10 9 8 7 6 5 4 3 2 1 -0

Archive directory:
Sample directory:

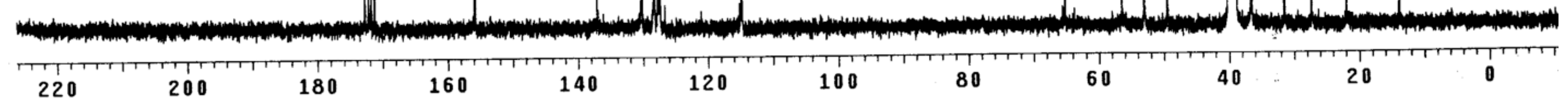
Pulse Sequence: s2pul

Solvent: dmso
Temp. 27.0 C / 300.1 K
User: 1-14-87
File: martin_jed3-298a_s2pul_C13
INOVA-500 "u5b"

Relax. delay 2.000 sec
Pulse 30.0 degrees
Acq. time 1.300 sec
Width 24509.8 Hz
5000 repetitions
OBSERVE C13, 100.5314959 MHz
DECOUPLE H1, 399.8086099 MHz
Power 44 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
Total time 4 hr, 35 min, 47 sec



S3

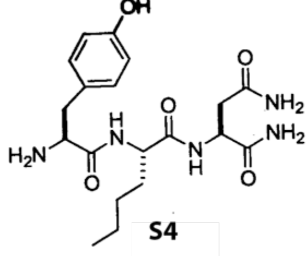


JEDIII(301)-7/12/07

Pulse Sequence: s2pul

Solvent: CD3OD
Ambient temperature
Mercury-400BB "nmr6"

Relax. delay 2.000 sec
Pulse 16.4 degrees
Acq. time 2.856 sec
Width 5602.2 Hz
24 repetitions
OBSERVE H1, 400.2685569 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 0 min, 0 sec



12 11 10 9 8 7 6 5 4 3 2 1 -0

pad=10 run with findz0 before acquisition
pad=10 run with gradshim before acquisition

Archive directory:
Sample directory:

Pulse Sequence: s2pul

Solvent: dmso
Temp. 27.0 C / 300.1 K
User: 1-14-87
File: martin_jed3-301b_s2pul_C13
INOVA-500 "nmrfred"

Relax. delay 2.000 sec
Pulse 30.0 degrees
Acq. time 1.300 sec
Width 24509.8 Hz
2000 repetitions
OBSERVE C13, 100.5314282 MHz
DECOUPLE H1, 399.8086099 MHz
Power 44 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
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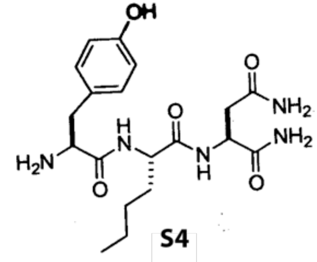

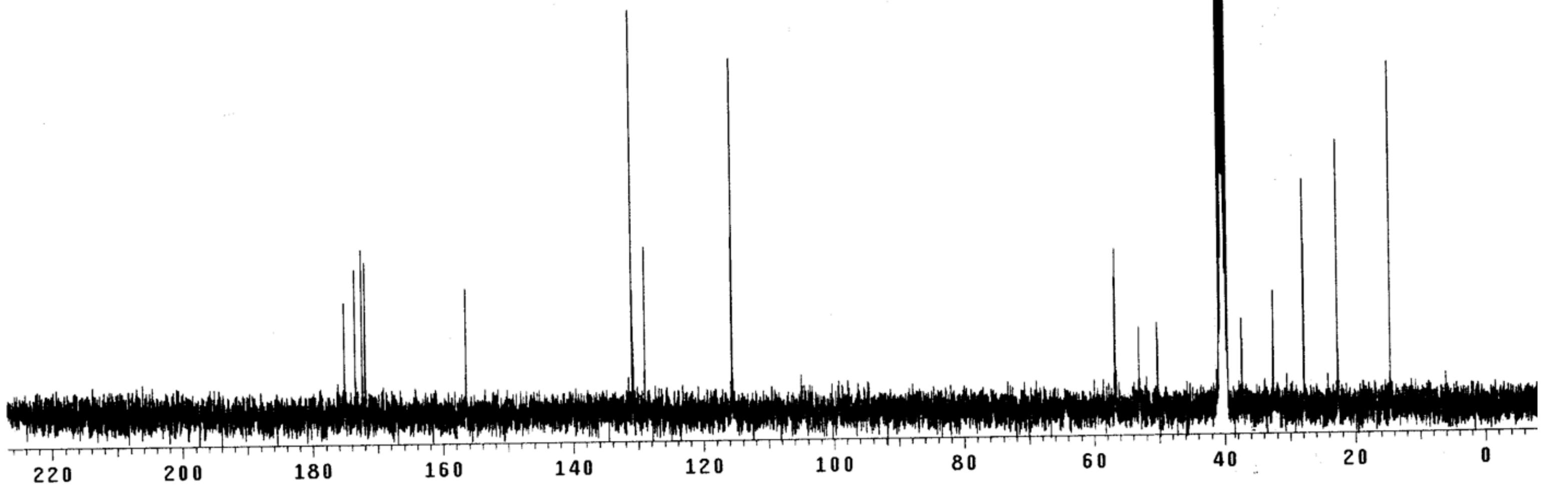

JED7-62

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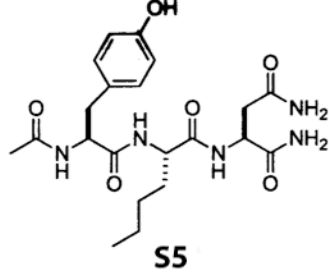

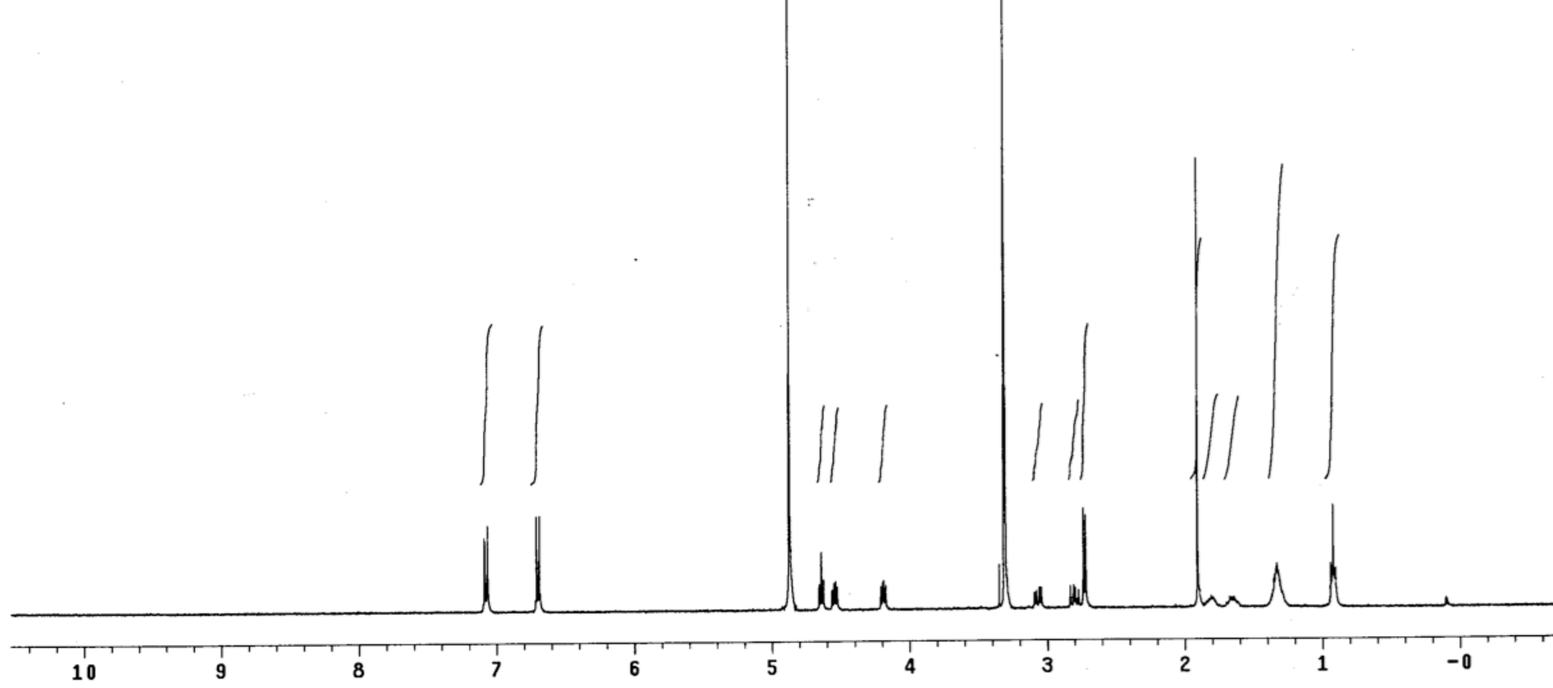

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JED7-62

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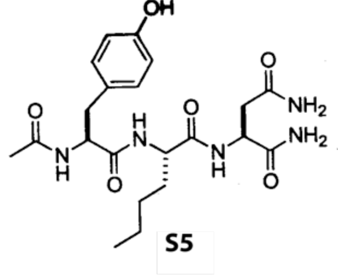
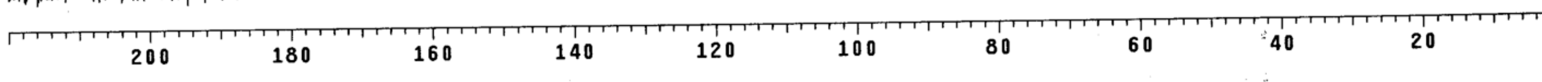




Preparation of Grb2 SH2. Expression and purification of the Grb2 SH2 domain has been described in detail elsewhere.[1] Briefly, the DNA construct containing the QE60 plasmid and residues 53-163 of the Grb2 SH2 domain was obtained from the Schering-Plough Research Institute and expressed in *E. coli* (SG13009, Qiagen). Cultures were grown at 30 °C in LB media containing 0.1 g/L ampicillin (Acros Organics) and 0.035 g/L kanamycin (Sigma-Aldrich) to an OD_{600nm} of 0.5–0.8 at which time expression was induced by isopropyl-β-D-1-thiogalactopyranoside (IPTG, Acros Organics, 1 mM.) After 15 h of incubation followed by sedimentation of the suspension by centrifugation, the cells were resuspended in 25 mM TRIS, 1 mM EDTA, pH 7.5 and lysed using a French Press under a pressure of 500 psi. The lysate was centrifuged and the supernatant purified on a phosphotyrosine affinity column followed by dialysis of the eluent containing the protein in 25 mM TRIS, 1 mM EDTA, pH 7.5. The dialysed protein was further purified on a Q-Sepharose FF column (GE Healthcare).

Preparation of Protein/Ligand Solutions for Crystallization. Prior to the cultivation of crystals of the domain complexed with each ligand, purified protein was dialyzed twice in 3.5 L of distilled water that had been passed through a Nanopure water purification system (Barnstead) to give a resistivity within the range 17.0–18.2 MΩcm. The resulting protein solution was placed in 15 mL Centriplus concentrators (Millipore, MWCO = 2,000) and centrifuged at 3000 g and 4 °C until the protein concentration was within the range 5–15 mg/mL. The ligands were each dissolved in this solution to give a protein-ligand molar ratio of 1:2. These solutions were each heated to 50 °C for 10 min to convert any Grb2 SH2 domain-swapped dimer to the monomeric domain,[2] filtered through a 0.45 micron PVDF filter disk, cooled, and stored at 4 ºC. Crystal Screens I, II, and Lite (Hampton Research) were used to identify initial crystallization conditions; additional screening was performed if necessary. Crystals were cultivated by the hanging-drop, vapor-diffusion method, employing 7 µL drops and 350 µL well solutions in standard 24-well, flat-bottom, polystyrene plates. Specific details pertaining to the growth and diffraction of crystals of Grb2 SH2 complexed with each of the ligands are given:

Grb2 SH2 Complexed with ***1****.* An aqueous solution containing a 2.0 molar ratio of **1** to protein (ca. 12 mg/mL) was prepared. This solution (4.0 µL) was mixed with a precipitant solution containing 0.25 *M* sodium acetate trihydrate, 0.1 *M* TRIS hydrochloride, pH 8.5, and 30% w/v polyethylene glycol, MW 4,000 (3.0 µL) and allowed to equilibrate with the aforementioned precipitant well solution (350 µL) at 298 K. Useable crystals grew after 3 weeks.

Grb2 SH2 Complexed with ***2****.* An aqueous solution containing a 2.0 molar ratio of **2** to protein (ca. 10 mg/mL) was prepared. This solution (4.0 µL) was mixed with a precipitant solution containing 0.2 *M* sodium acetate trihydrate, 0.1 *M* TRIS hydrochloride, pH 8.5, and 30% w/v polyethylene glycol,

MW 4,000 (3.0 μL, Hampton crystal screen I, condition no. 22) and allowed to equilibrate with the aforementioned precipitant well solution (350 μL) at 298 K. Useable crystals grew after 1 month.

Grb2 SH2 Complexed with ***3.*** An aqueous solution containing a 2.0 molar ratio of **1** to protein (ca. 10 mg/mL) was prepared. This solution (4.0 μL) was mixed with a precipitant solution containing 0.3 *M* sodium acetate trihydrate, 0.1 *M* TRIS hydrochloride, pH 8.5, and 25% w/v polyethylene glycol, MW 4,000 (3.0 μL) and allowed to equilibrate with the aforementioned precipitant well solution (350 μL) at 298 K. Useable crystals grew after 3 weeks.

Grb2 SH2 Complexed with ***4.*** An aqueous solution containing a 1.5 molar ratio of **4** to protein (ca. 12 mg/mL) was prepared. This solution (4.0 μL) was mixed with a precipitant solution containing 0.1 *M* HEPES, pH 7.5, and 4.3 M NaCl (3.0 μL, Hampton crystal screen II, condition no. 36) and allowed to equilibrate with the aforementioned precipitant well solution (350 μL) at 298 K. Useable crystals grew after 2 weeks.

Grb2 SH2 Complexed with ***5.*** An aqueous solution containing a 1.5 molar ratio of **5** to protein (ca. 12 mg/mL) was prepared. This solution (3.0 μL) was mixed with a precipitant solution containing 0.1 *M* HEPES, pH 7.5, and 4.3 M NaCl (4.0 μL, Hampton crystal screen II, condition no. 36) and allowed to equilibrate with the aforementioned precipitant well solution (350 μL) at 298 K. Useable crystals grew after 2 weeks.

Grb2 SH2 Complexed with ***6.*** An aqueous solution containing a 2.0 molar ratio of **6** to protein (ca. 10 mg/mL) was prepared. This solution (4.0 μL) was mixed with a precipitant solution containing 0.1 *M* HEPES, pH 7.5, and 4.3 M NaCl (3.0 μL, Hampton crystal screen II, condition no. 36) and allowed to equilibrate with the aforementioned precipitant well solution (350 μL) at 298 K. Useable crystals grew after one week.

Collection of X-ray Diffraction Data. Prior to data collection, crystals were cyroprotected by transferring them to solutions containing salt, buffer and precipitant concentrations equal to their theoretical initial concentrations in the hanging drop experiment, yet containing glycerol in the concentration range 25–35% (v/v). Crystals were allowed to equilibrate in this solution for 0.5-1 min. Once equilibrated, the crystals were removed and flash frozen in liquid nitrogen prior to affixing to the goniometer. X-ray diffraction data were collected at 100 K using a Rigaku RAXIS IV area detector positioned on a Rigaku RU200 rotating angle X-ray generator operated at 40 kV and 70 mA producing CuKα graphite-monochromatic radiation (1.5418 nm). Data frames were collected in 0.5º intervals with exposure times of 120 sec at crystal-to-detector distances of 100-120 mm.

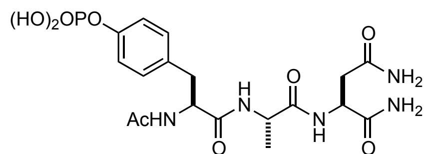
Data Processing and Structure Refinement. Data frames were processed and scaled using HKL2000.[3] CCP4 (Phaser)[4] was used to identify a molecular replacement solution from a published

Grb2 SH2/peptide crystal structure (pdb code 3C7I). Structures were manipulated using the program COOT[5] and refined using CCP4 (Refmac5). Density maps were created using CCP4 (FFT). Ligands containing amino acid replacements were docked into the protein model and the necessary topology files were built using the online program PRODRG.[6]

Normalization of ITC Data. The concentration of ligand in the syringe was determined from the mass of each sample and the volume of buffer into which each sample was diluted. After each titration, the concentration was adjusted such to give an *n* value of 1.00 in accord with the binding event, which is known to occur with 1:1 stoichiometry. Titrations for which the initial concentration yielded *n* values >1.20 were rejected.

Molecular Surface Area Calculations. Changes in nonpolar and polar Connolly surface area[7], ΔCSA_{np} and ΔCSA_{p}, were calculated using Macromodel v. 9.1[8] based on the assumption that no change in the conformation of either the ligand or the protein occurs on complex formation. Specifically, water and other solvent molecules were removed from each X-ray structure and the CSAs of the complexes were calculated. The ligands were removed from each complex and the CSAs of the remaining structures were assumed to be that of the *apo* protein, which varied <0.3% from one structure to another. Likewise, CSAs of the ligands removed from the complexes were assumed to be that of the unbound ligands in solution. Subtraction of the latter two CSAs from the former gave ΔCSA values for the formation of each complex. A probe radius of 1.4 Å was employed.

Representative ITC traces.

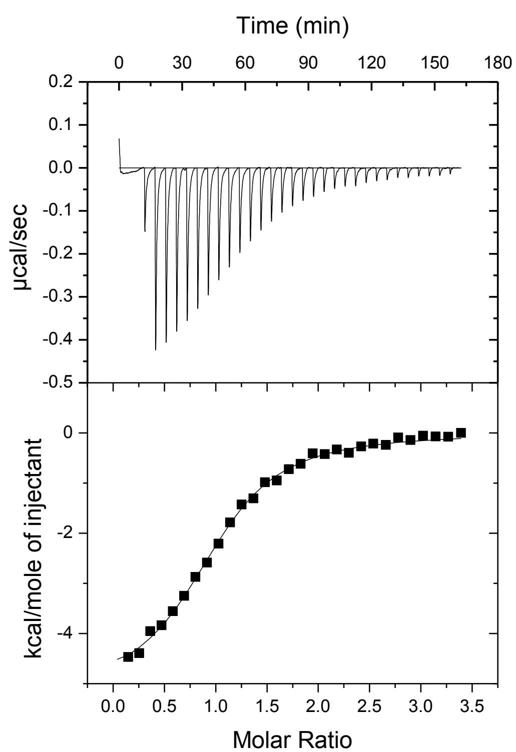


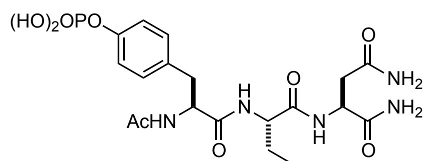
$K_a = (2.2 \pm 0.1) \times 10^5 \ M^{-1}$

$\Delta G^o = -7.3 \pm 0.1$ kcal mol^{-1}

$\Delta H^o = -4.9 \pm 0.3$ kcal mol^{-1}

$-T\Delta S^o = -2.4 \pm 0.1$ kcal mol^{-1}



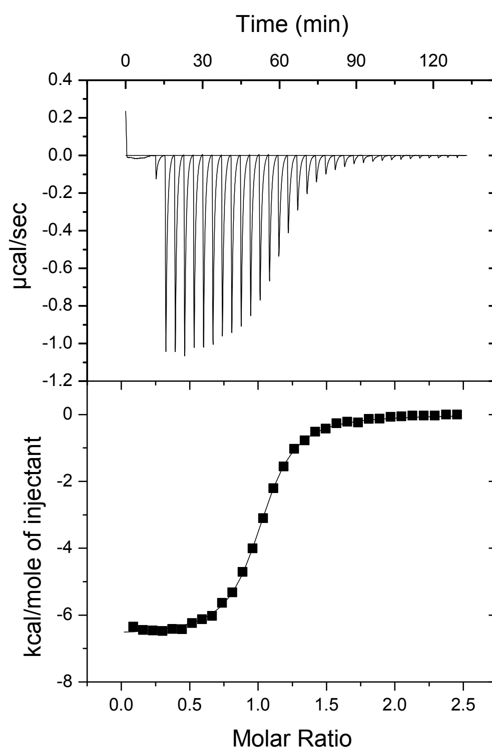


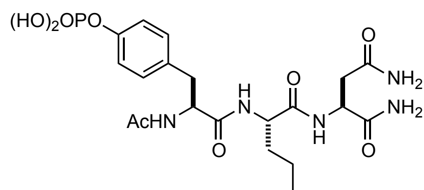
$K_a = (8.6 \pm 0.2) \times 10^5\ M^{-1}$

$\Delta G^o = -8.1 \pm 0.1$ kcal mol^{-1}

$\Delta H^o = -6.8 \pm 0.5$ kcal mol^{-1}

$-T\Delta S^o = -1.3 \pm 0.1$ kcal mol^{-1}



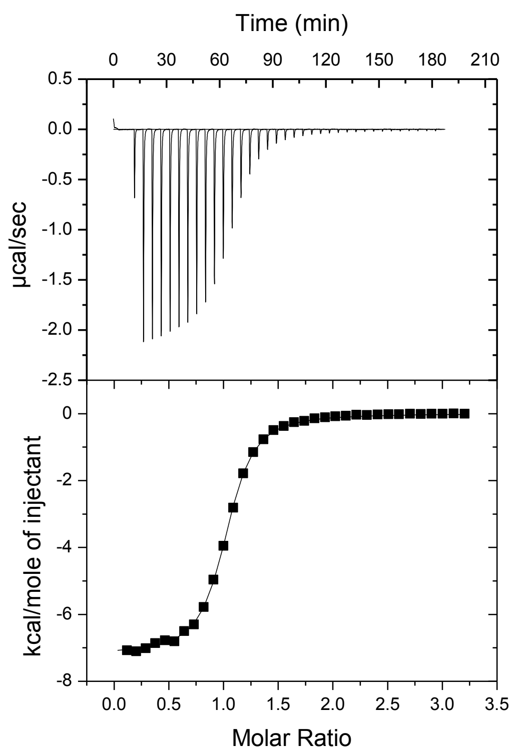


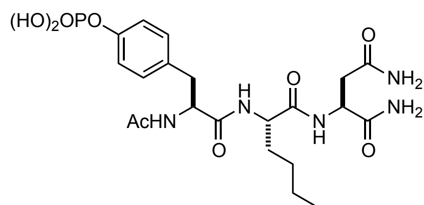
$$K_a = (7.6 \pm 1.0) \times 10^5\ M^{-1}$$

$$\Delta G^o = -8.0 \pm 0.1\ \text{kcal mol}^{-1}$$

$$\Delta H^o = -6.7 \pm 0.5\ \text{kcal mol}^{-1}$$

$$-T\Delta S^o = -1.3 \pm 0.3\ \text{kcal mol}^{-1}$$



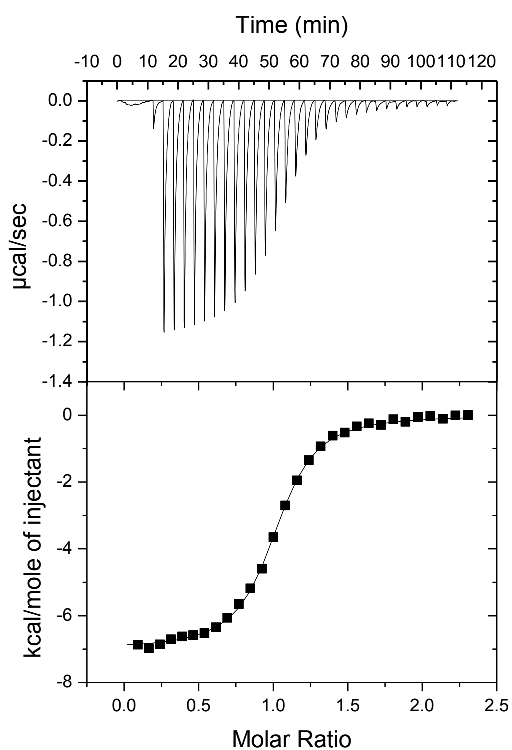


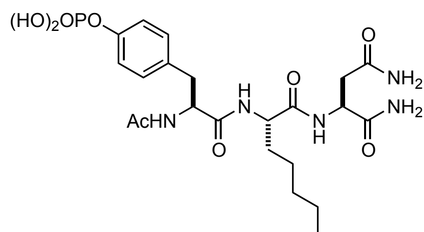
$K_a = (8.4 \pm 0.6) \times 10^5\ M^{-1}$

$\Delta G^o = -8.1 \pm 0.1$ kcal mol^{-1}

$\Delta H^o = -7.3 \pm 0.3$ kcal mol^{-1}

$-T\Delta S^o = -0.8 \pm 0.2$ kcal mol^{-1}



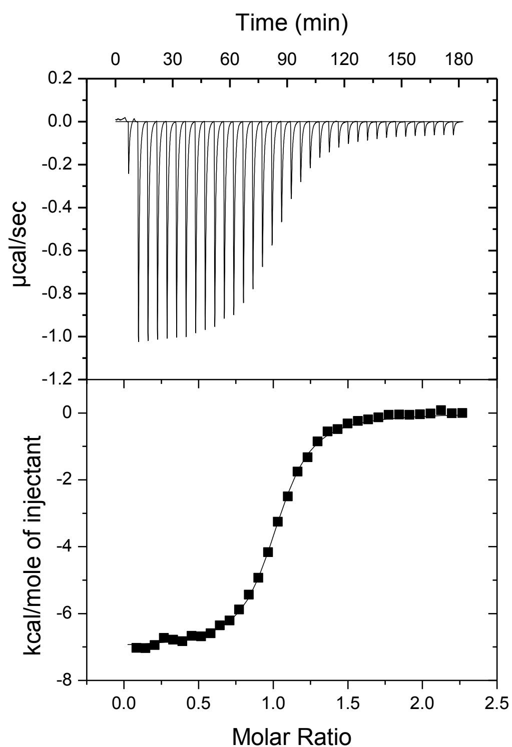


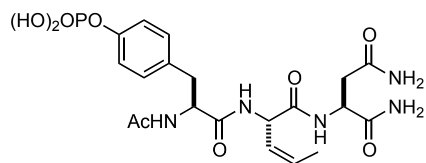
$K_a = (7.8 \pm 0.6) \times 10^5\ M^{-1}$

$\Delta G^o = -8.0 \pm 0.1$ kcal mol^{-1}

$\Delta H^o = -7.2 \pm 0.3$ kcal mol^{-1}

$-T\Delta S^o = -0.8 \pm 0.2$ kcal mol^{-1}





$K_a = (6.1 \pm 0.2) \times 10^5\ M^{-1}$

$\Delta G^o = -7.9 \pm 0.1$ kcal mol^{-1}

$\Delta H^o = -7.0 \pm 0.2$ kcal mol^{-1}

$-T\Delta S^o = -0.9 \pm 0.1$ kcal mol^{-1}

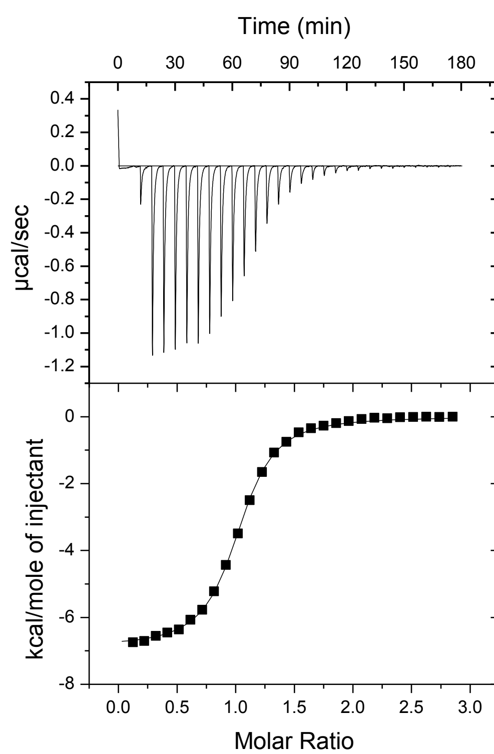


Table S1. X-ray diffraction data and refinement statistics for crystals of the Grb2 SH2 domain complexed with ligands **1**, **2**, and **3**. Numbers in parenthesis refer to the outermost resolution shell.

Data Set	Grb2 SH2/ **1**	Grb2 SH2/ **2**	Grb2 SH2 / **3**
Data Collection			
Total reflections	36674	165553	93375
Unique reflections[a]	9007 / 8515	12350 / 12269	11405 / 11102
Resolution range (Å)	50.00-1.78 (1.81-1.78)	50-1.64 (1.70-1.64)	50.00-1.71 (1.77-1.71)
Completeness, %	89.4 (89.0)	97.6 (99.9)	99.9 (100.0)
Data redundancy	4.1 (3.6)	13.4 (13.8)	8.2 (7.9)
R_{merge}	0.40 (0.176)	0.049 (0.147)	0.044 (0.183)
I/ σ(I)	24.8 (6.3)	42.3 (20.6)	45.0 (13.6)
Refinement			
No. reflections[b]	8942 / 8521	12262 / 11666	11304 / 10761
R_{cryst}[c] / R_{free}, %	15.6 / 20.7	17.1 / 23.0	15.9 / 20.8
RMS dev. from ideality			
Bond lengths (Å)	0.022	0.024	0.027
Bond angles (deg)	2.14	2.06	2.120
B-factor restraints (Å^2)			
Backbone bonds (RMS / σ)		1.4 / 1.5	1.4 / 1.5
Side-chain bonds (RMS / σ)		3.0 / 3.0	2.9 / 3.0
Backbone angles (RMS / σ)		2.2 / 2.0	2.1 / 2.0
Side-chain angles (RMS / σ)		4.4 / 4.5	4.4 / 4.5
Crystal			
Space group	$P4_32_12$	$P4_32_12$	$P4_32_12$
Cell dimensions			
a, b, c (Å)	42.08, 42.08, 109.08	41.96, 41.96, 108.96	42.18, 42.18, 109.60
α, β, γ (deg.)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Complexes in asym. unit	1	1	1
Solvent content, %	34.2	33.3	34.4
Matthews Coef; Vm (Å^3/Da)	1.87	1.84	1.87
Final Model			
Protein residues	100	101	100
Protein atoms	825	835	825
Ligand atoms	33	34	35
Water molecules	70	96	100
Solvent molecules	3	0	2
Res. in alt. conformations	6	11	9

[a] no. reflections / no. for which I/σ(I) ≥ 1.0

[b] no. reflections used in refinement; working set / free R set

[c] $R_{cryst} = \sum ||F_{calc}| - |F_{obs}|| / \sum |F_{obs}|$, where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively.

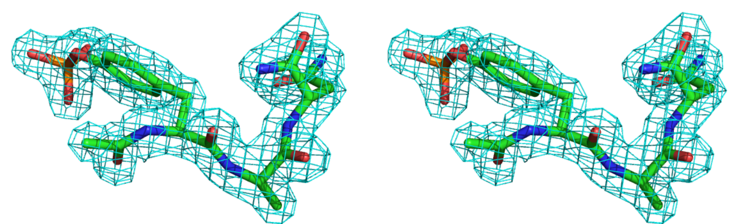
Table S2. X-ray diffraction data and refinement statistics for crystals of the Grb2 SH2 domain complexed with ligands **4**, **5**, and **6**. Numbers in parenthesis refer to the outermost resolution shell.

Data Set	Grb2 SH2/ **4**	Grb2 SH2/ **5**	Grb2 SH2 / **6**
Data Collection			
Total reflections	93255	120858	82827
Unique reflections[a]	12709 / 12359	11541 / 10389	11594 / 10298
Resolution range (Å)	50.00-1.64 (1.70-1.64)	50.00-1.67 (1.73-1.67)	50.00-1.64 (1.70-1.64)
Completeness, %	99.1 (99.8)	93.0 (73.8)	91.1 (62.8)
Data redundancy	7.3 (6.7)	10.5 (6.1)	7.1 (3.6)
R_{merge}	0.058 (0.332)	0.092 (0.497)	0.075 (0.750)
I/ σ(I)	25.4 (7.3)	26.8 (3.2)	23.4 (1.5)
Refinement			
No. reflections[b]	12595 / 11983	7942 / 7576	8317 / 7913
R_{cryst}[c] / R_{free}, %	17.3 / 24.1	15.5 / 19.5	19.1 / 25.7
RMS dev. from ideality			
Bond lengths (Å)	0.024	0.023	0.017
Bond angles (deg)	2.13	1.80	1.82
B-factor restraints ($Å^2$)			
Backbone bonds (RMS / σ)	1.4 / 1.5	1.2 / 1.5	
Side-chain bonds (RMS / σ)	3.1 / 3.0	3.0 / 3.0	
Backbone angles (RMS / σ)	2.2 / 2.0	1.9 / 2.0	
Side-chain angles (RMS / σ)	4.5 / 4.5	4.3 / 4.5	
Crystal			
Space group	$P4_32_12$	$P4_32_12$	$P4_32_12$
Cell dimensions			
a, b, c (Å)	42.16, 42.16, 109.19	42.30, 42.30, 110.45	42.05, 42.05, 109.13
α, β, γ (deg.)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Complexes in asym. unit	1	1	1
Solvent content, %	34.0	35.2	33.7
Matthews Coef; V_m ($Å^3$/Da)	1.86	1.90	1.85
Final Model			
Protein residues	101	100	101
Protein atoms	833	826	819
Ligand atoms	36	37	35
Water molecules	97	97	54
Solvent molecules	5	3	2
Res. in alt. conformations	9	9	1

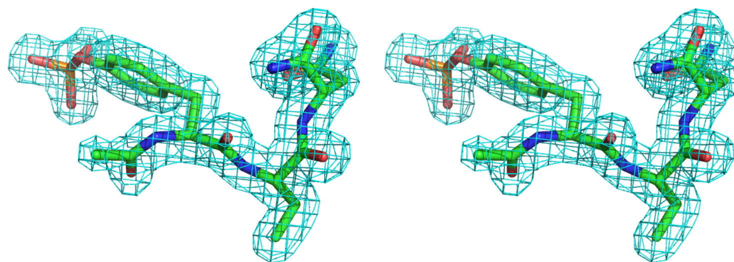
[a] no. reflections / no. for which I/σ(I) ≥ 1.0

[b] no. reflections used in refinement; working set / free R set

[c] $R_{cryst} = \sum ||F_{calc}| - |F_{obs}|| / \sum |F_{obs}|$, where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively.



(a)



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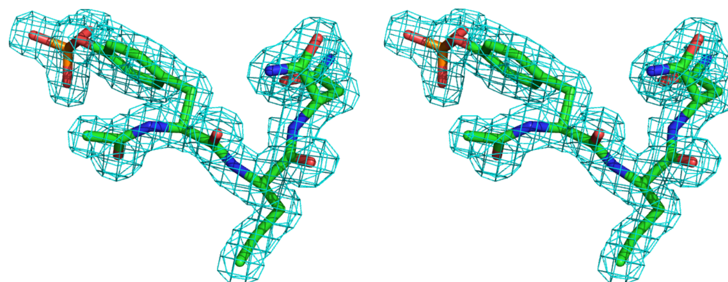
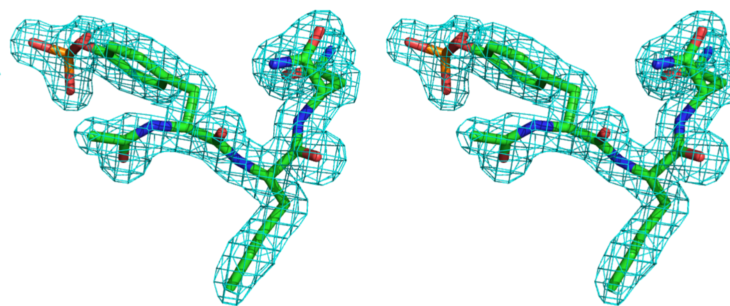
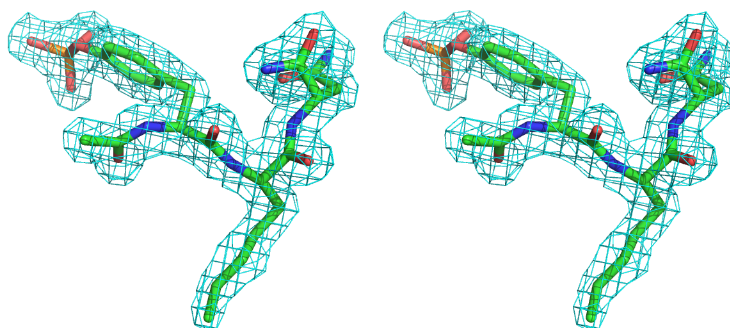


(c)

Figure S1. Electron density difference maps showing the bound structures of ligands **1**–**3** in cross-eyed stereo. The maps, indicated by the cyan wire mesh, are weighted $2F_o$-F_c maps contoured at +1 σ, showing only the portion within 1.0–1.5 Å of each ligand atom in the complexes for clarity. a) Complex of the domain with **1**. b) Complex of the domain with **2**. c) Complex of the domain with **3**.



(a)



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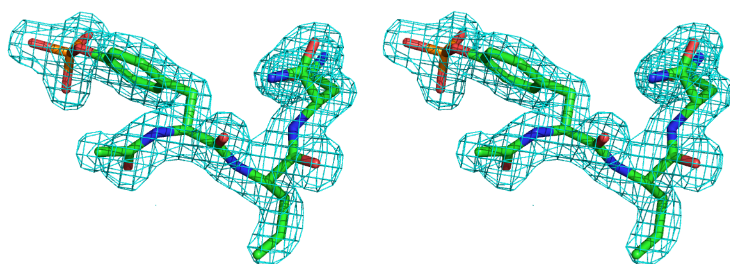
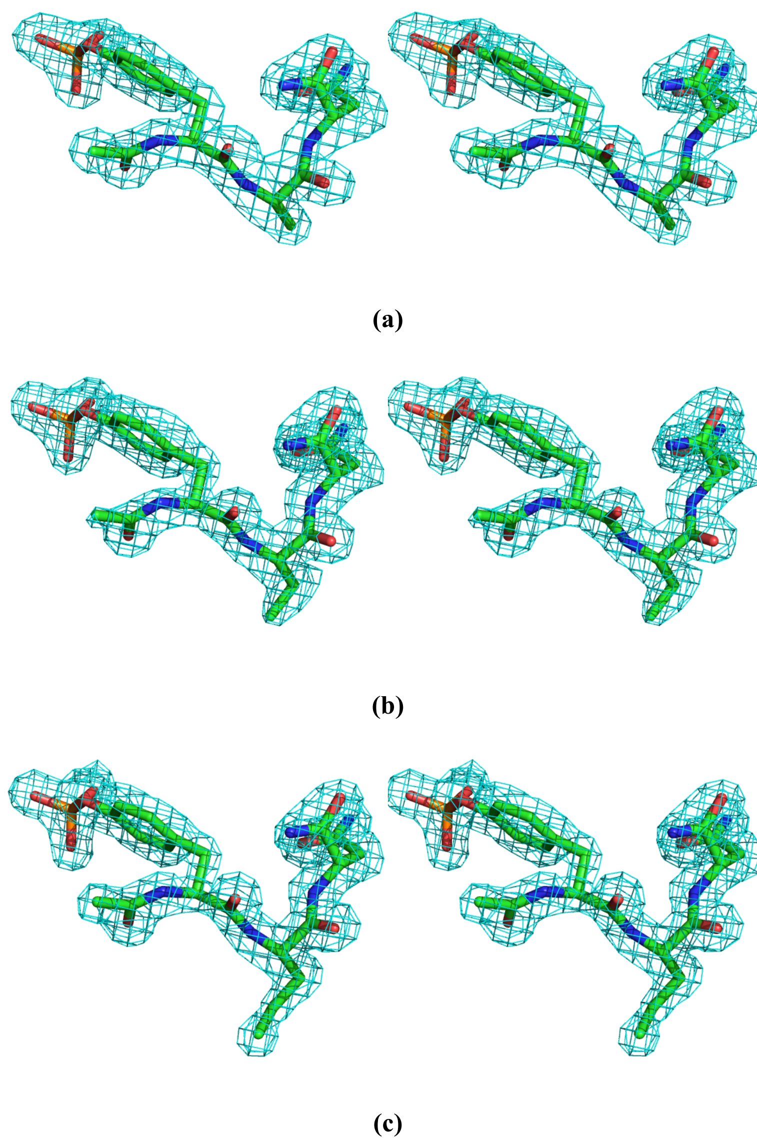


(c)

Figure S2. Electron density difference maps showing the bound structures of ligands **4-6** in cross-eyed stereo. The maps, indicated by the cyan wire mesh, are weighted $2F_o$-F_c maps contoured at +1 σ, showing only the portion within 1.0–1.5 Å of each ligand atom in the complexes for clarity. a) Complex of the domain with **4**. b) Complex of the domain with **5**. c) Complex of the domain with **6**.

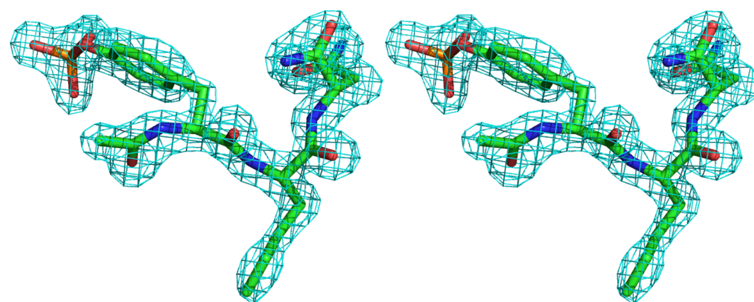


(a)

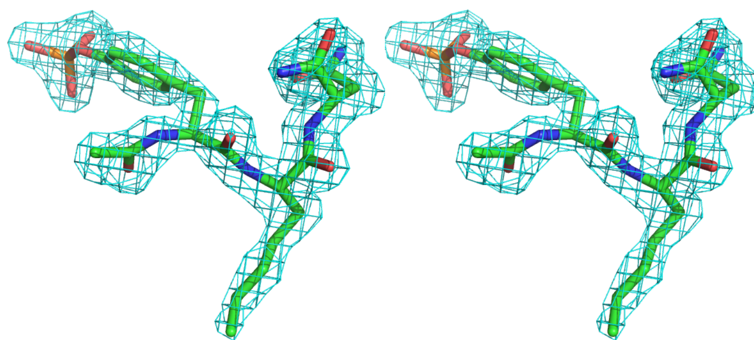
(b)

(c)

Figure S3. Electron density difference omit maps showing the bound structures of ligands **1**–**3** in cross-eyed stereo. The omit maps, indicated by the cyan wire mesh, are weighted F_o-F_c maps contoured at +3 σ, showing only the portion within 1.0–1.5 Å of each ligand atom in the complexes for clarity. a) Complex of the domain with **1**. b) Complex of the domain with **2**. c) Complex of the domain with **3**.



(a)



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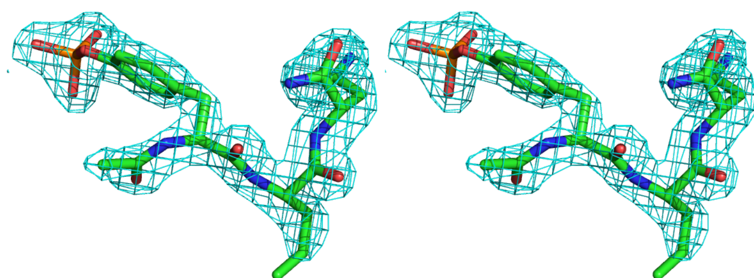
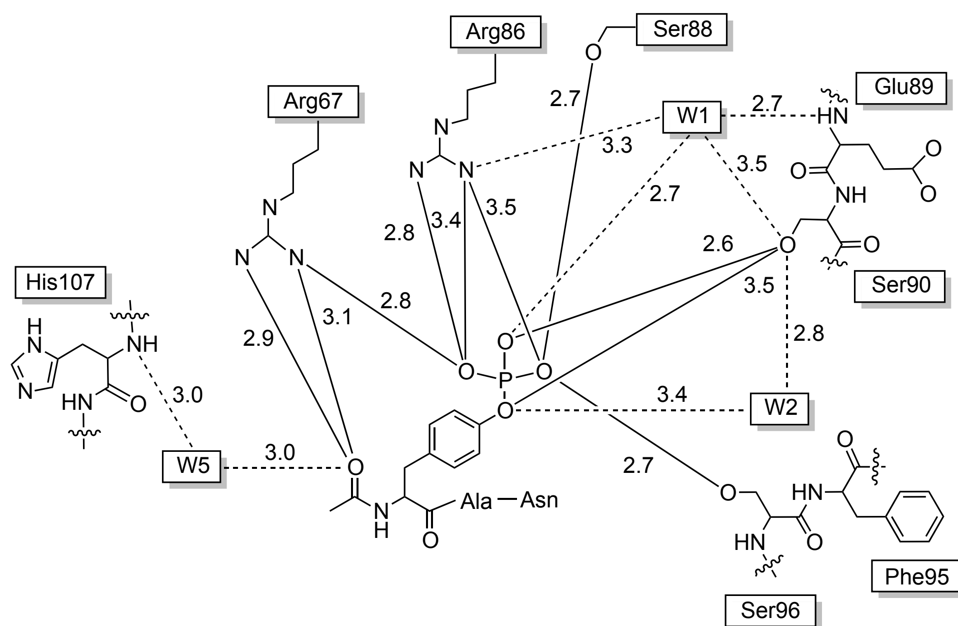
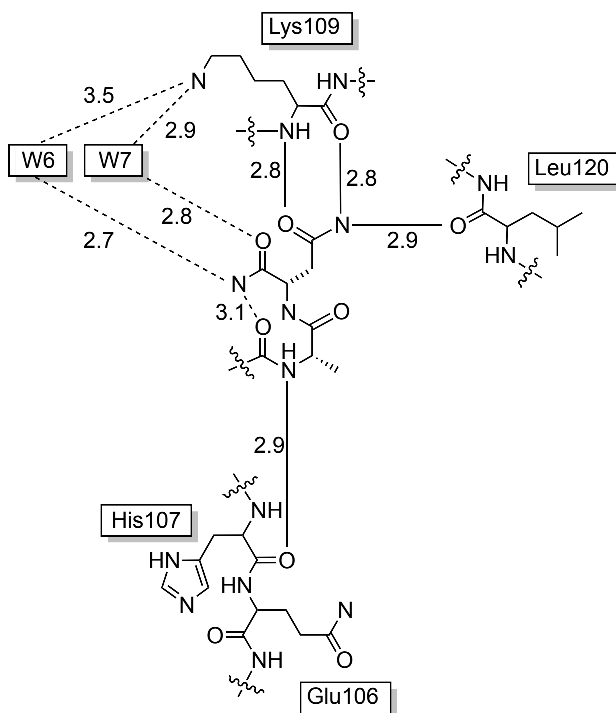


(c)

Figure S4. Electron density difference omit maps showing the bound structures of ligands **4-6** in cross-eyed stereo. The omit maps, indicated by the cyan wire mesh, are weighted F_o-F_c maps contoured at +3 σ, showing only the portion within 1.0–1.5 Å of each ligand atom in the complexes for clarity. a) Complex of the domain with **4**. b) Complex of the domain with **5**. c) Complex of the domain with **6**.

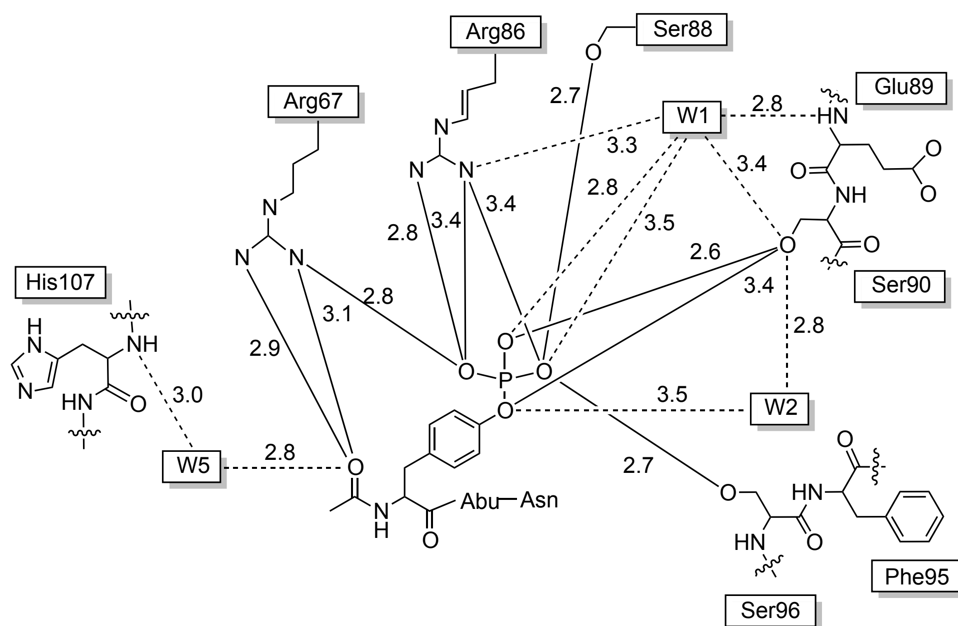


(a)

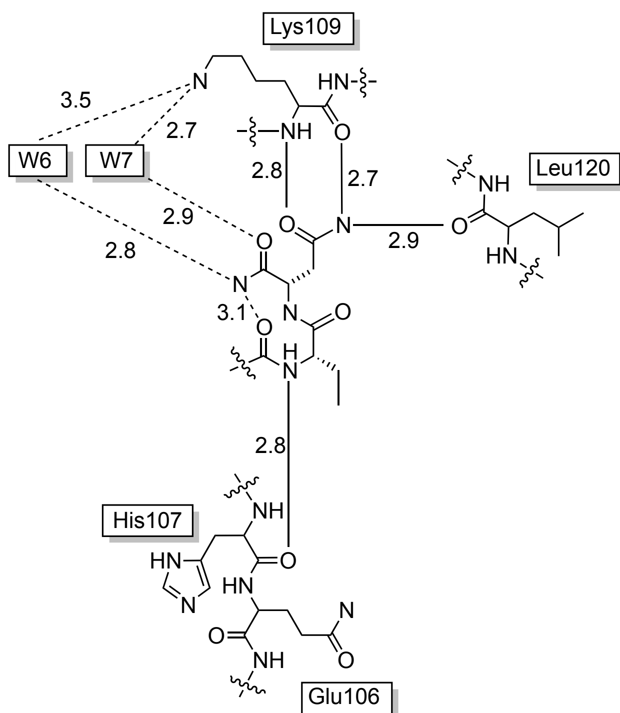


(b)

Figure S5. Polar direct (solid lines) and water-mediated (dashed lines) interactions within the range 2.5–3.5 Å in the complex of **1**. All labile hydrogen atoms have been omitted for clarity except those on protein backbone nitrogen atoms. Only those ordered water molecules that mediate a single protein-ligand interaction are shown, and these are numbered so that water molecules that are conserved in at least two complexes have the same number. (a) Interactions between the domain and the Ac–pTyr replacement. (b) Interactions between the domain and the Xaa–Asn region of the ligand.

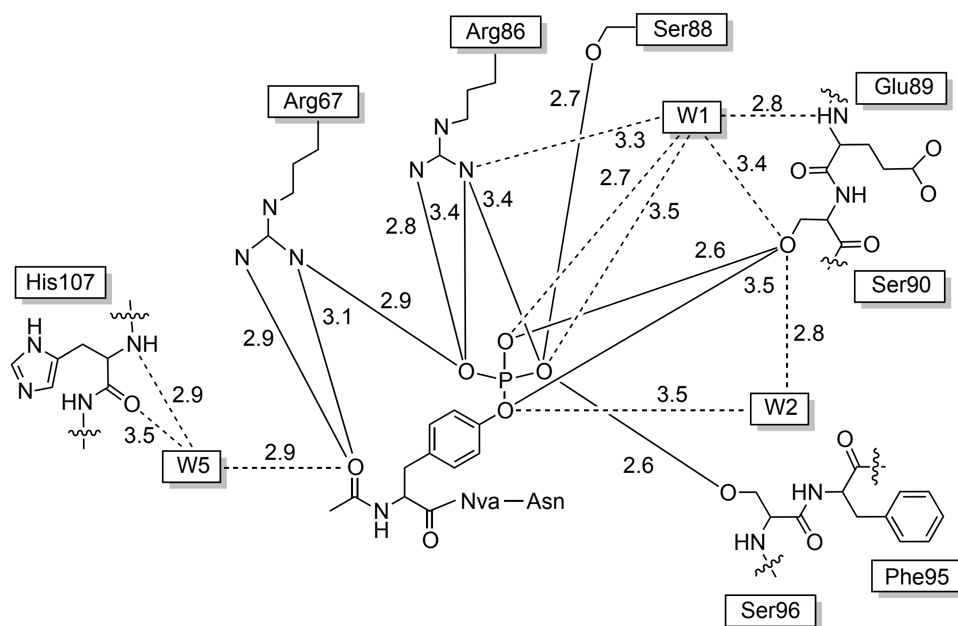


(a)

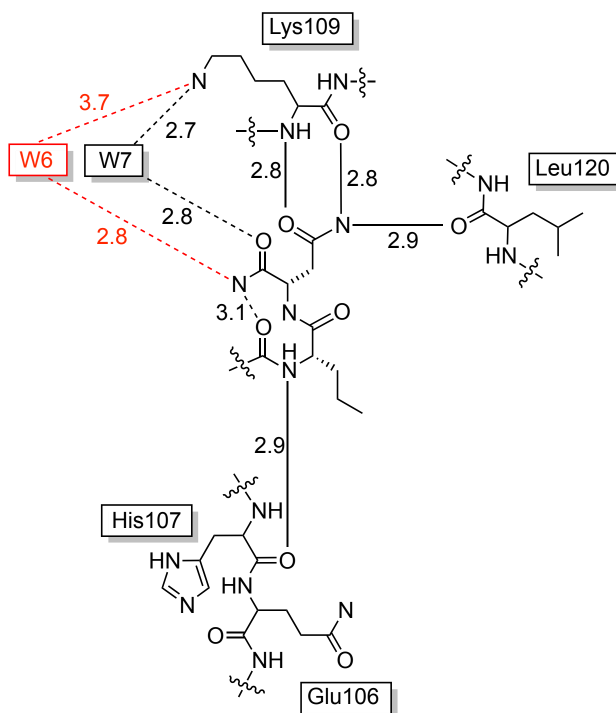


(b)

Figure S6. Polar direct (solid lines) and water-mediated (dashed lines) interactions within the range 2.5–3.5 Å in the complex of **2**. All labile hydrogen atoms have been omitted for clarity except those on protein backbone nitrogen atoms. Only those ordered water molecules that mediate a single protein-ligand interaction are shown, and these are numbered so that water molecules that are conserved in at least two complexes have the same number. (a) Interactions between the domain and the Ac–pTyr replacement. (b) Interactions between the domain and the Xaa–Asn region of the ligand.

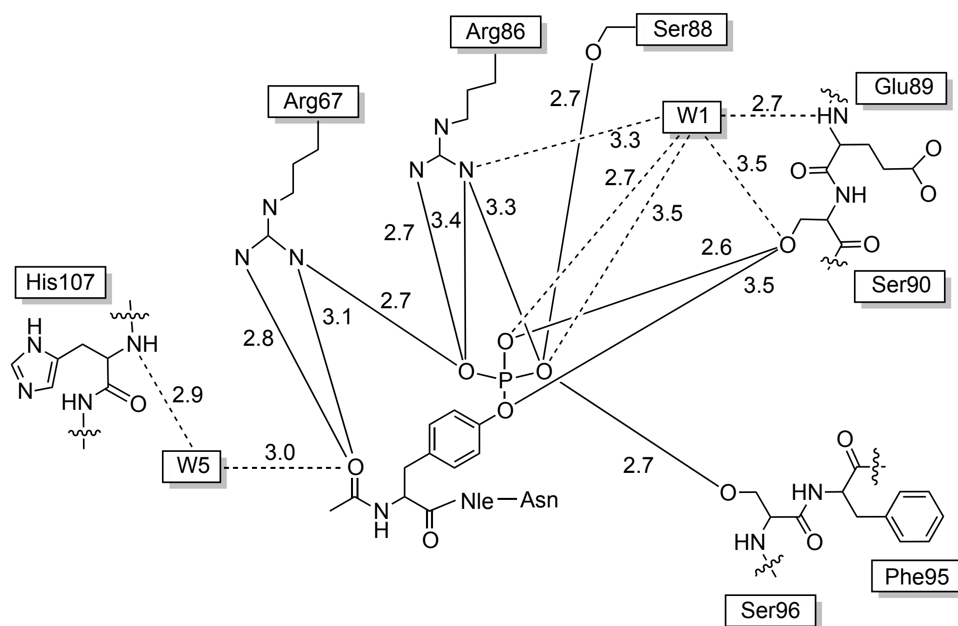


(a)

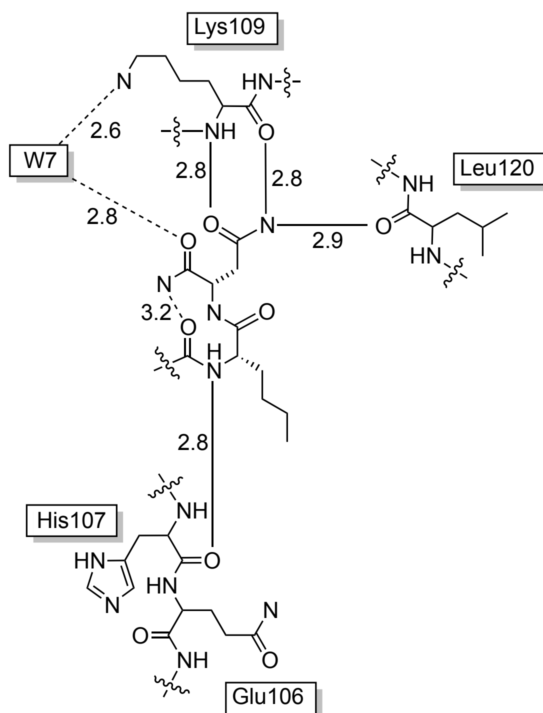


(b)

Figure S7. Polar direct (solid lines) and water-mediated (dashed lines) interactions within the range 2.5–3.5 Å in the complex of **3**. All labile hydrogen atoms have been omitted for clarity except those on protein backbone nitrogen atoms. Only those ordered water molecules that mediate a single protein-ligand interaction are shown, and these are numbered so that water molecules that are conserved in at least two complexes have the same number. Contacts in red are longer than 3.5 Å. (a) Interactions between the domain and the Ac–pTyr replacement. (b) Interactions between the domain and the Xaa–Asn region of the ligand.

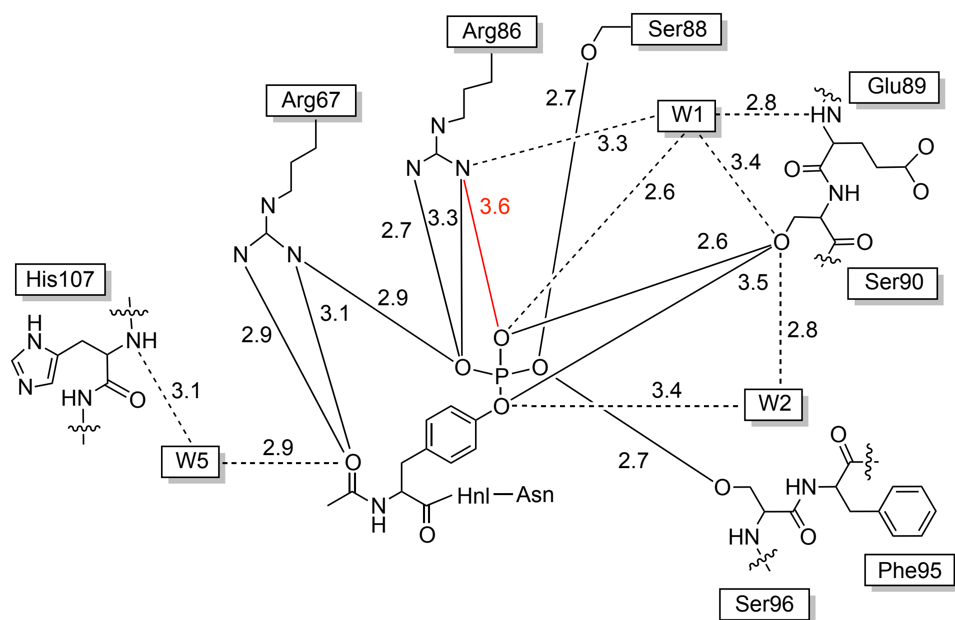


(a)

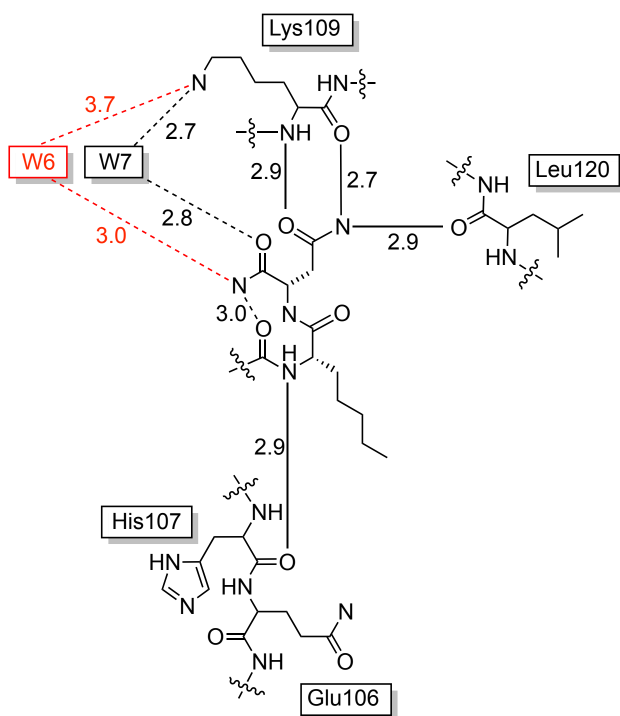


(b)

Figure S8. Polar direct (solid lines) and water-mediated (dashed lines) interactions within the range 2.5–3.5 Å in the complex of **4**. All labile hydrogen atoms have been omitted for clarity except those on protein backbone nitrogen atoms. Only those ordered water molecules that mediate a single protein-ligand interaction are shown, and these are numbered so that water molecules that are conserved in at least two complexes have the same number. (a) Interactions between the domain and the Ac–pTyr replacement. (b) Interactions between the domain and the Xaa–Asn region of the ligand.

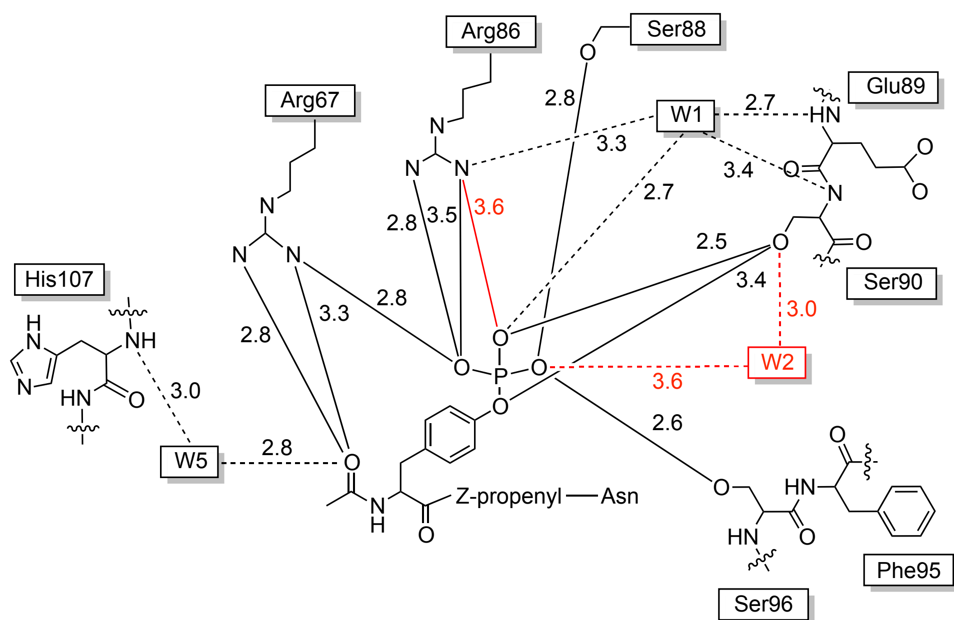


(a)

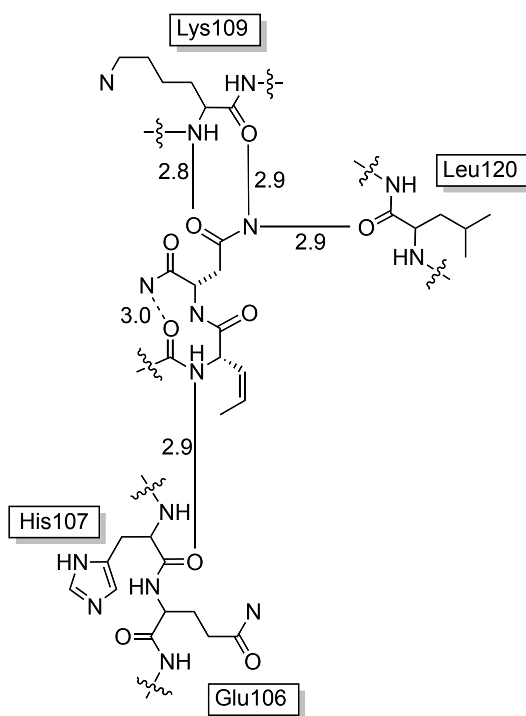


(b)

Figure S9. Polar direct (solid lines) and water-mediated (dashed lines) interactions within the range 2.5–3.5 Å in the complex of **5**. All labile hydrogen atoms have been omitted for clarity except those on protein backbone nitrogen atoms. Only those ordered water molecules that mediate a single protein-ligand interaction are shown, and these are numbered so that water molecules that are conserved in at least two complexes have the same number. Contacts in red are longer than 3.5 Å. (a) Interactions between the domain and the Ac–pTyr replacement. (b) Interactions between the domain and the Xaa–Asn region of the ligand.



(a)



(b)

Figure S10. Polar direct (solid lines) and water-mediated (dashed lines) interactions within the range 2.5–3.5 Å in the complex of **6**. All labile hydrogen atoms have been omitted for clarity except those on protein backbone nitrogen atoms. Only those ordered water molecules that mediate a single protein-ligand interaction are shown, and these are numbered so that water molecules that are conserved in at least two complexes have the same number. Contacts in red as longer than 3.5 Å. (a) Interactions between the domain and the Ac–pTyr replacement. (b) Interactions between the domain and the Xaa–Asn region of the ligand.

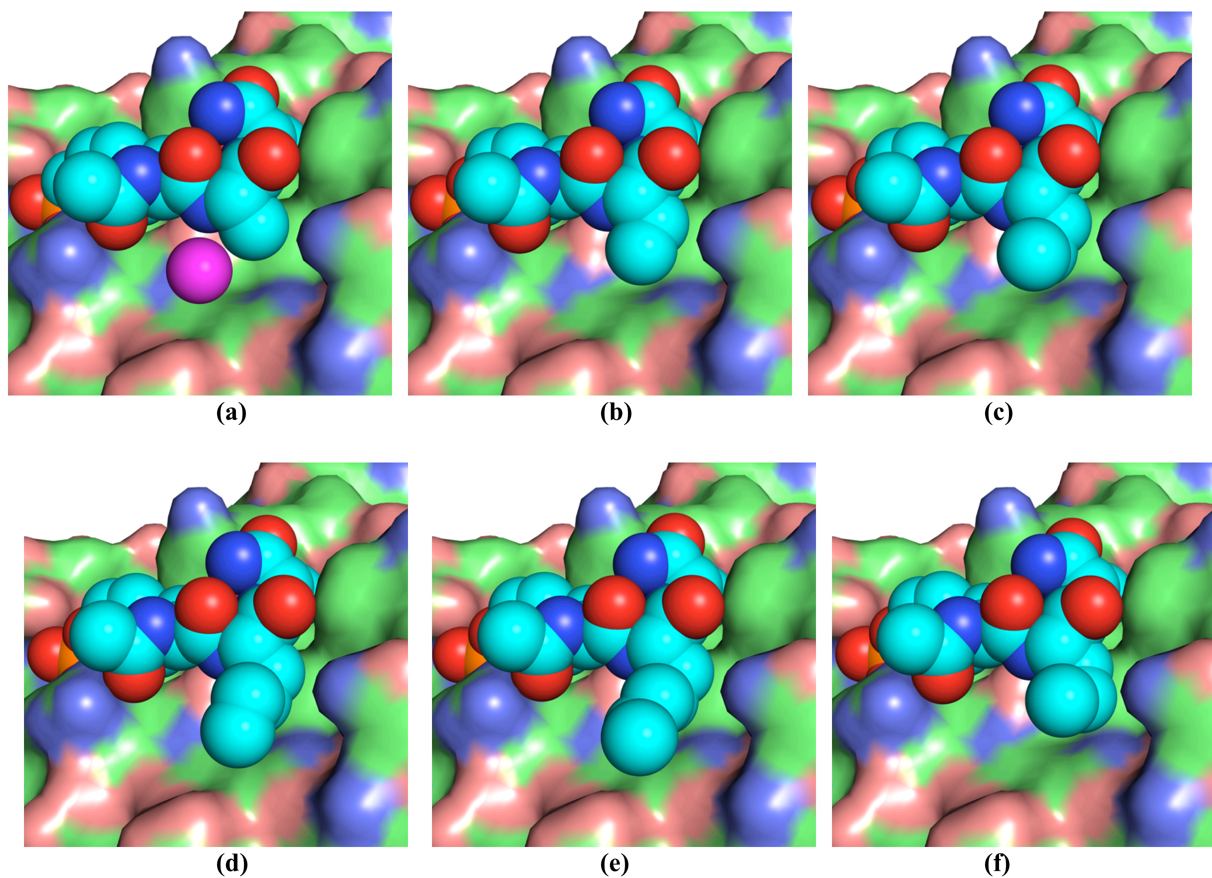


Figure S11. Compounds **1-6** (panels (a)-(f), spheres with atomic vdW radii) bound to the Grb2 SH2 domain (vdW surface) showing the extent to which each fills the pY+1 sub-pocket. Oxygen and nitrogen atoms are colored red and blue, respectively, with those belonging to the ligand colored a darker hue than those belonging to the domain. Carbon atoms belonging to the ligand are colored cyan while those belonging to the domain are colored green. The oxygen atom of an ordered water molecule present in the complex with **1** yet absent in the complexes of **2**-**6** is shown as a purple sphere in panel (a).

Table S3. Thermodynamic and ΔCSA data.

Ligand	ΔG° (kcal•mol^{-1})	ΔH° (kcal•mol^{-1})	$-T\Delta S^{\circ}$ (kcal•mol^{-1})	ΔC_p (cal•mol^{-1}K^{-1})	CSA_p (Å^2)	CSA_{np} (Å^2)	$\Delta G^{\circ}/\Delta CSA_{np}$ (cal•mol^{-1}Å^{-2})
1	–7.3 ± 0.1	–4.9 ± 0.3	–2.4 ± 0.1	–123 ± 9	54.6	79.6	–40.4
2	–8.1 ± 0.1	–6.8 ± 0.5	–1.3 ± 0.1	–170 ±15	56.3	89.4	–42.7
3	–8.0 ± 0.1	–6.7 ± 0.5	–1.3 ± 0.3	–173 ± 13	58.3	01.7	–39.7
4	–8.1 ± 0.1	–7.3 ± 0.3	–0.8 ± 0.2	–138 ± 12	51.3	19.9	–36.7
5	–8.0 ± 0.1	–7.2 ± 0.3	–0.8 ± 0.2	–148 ± 7	51.6	17.3	–36.9
6	–7.9 ± 0.1	–7.0 ± 0.2	–0.9 ± 0.1	–176 ± 7	65.5	04.2	–38.7

Table S4. Thermodynamic and average atomic B-factor data.

Ligand	ΔG° (kcal•mol^{-1})	ΔH° (kcal•mol^{-1})	$-T\Delta S^{\circ}$ (kcal•mol^{-1})	B-factor (Å^2)[a]		
				Domain[b]	BC-loop[c]	Residues 106-108[d]
1	–7.3 ± 0.1	–4.9 ± 0.3	–2.4 ± 0.1	24.6	24.3	23.9
2	–8.1 ± 0.1	–6.8 ± 0.5	–1.3 ± 0.1	25.1	25.3	23.1
3	–8.0 ± 0.1	–6.7 ± 0.5	–1.3 ± 0.3	22.3	22.6	20.6
4	–8.1 ± 0.1	–7.3 ± 0.3	–0.8 ± 0.2	33.1	33.9	31.3
5	–8.0 ± 0.1	–7.2 ± 0.3	–0.8 ± 0.2	24.1	24.8	22.3
6	–7.9 ± 0.1	–7.0 ± 0.2	–0.9 ± 0.1	24.5	25.2	24.2

[a] Average B-factor data adjusted according to the method recommended by Ringe *et al.*[9] for comparing data obtained from different crystals.
[b] All atoms of residues Pro59-Ile151.
[c] All atoms of residues Ser88-Pro92 of the pY sub-pocket. [d] All atoms of residues Gln106-Phe108 of the pY+1 sub-pocket.

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