Electronic Supplementary Material for:

Direct and Competitive Fluorescence Anisotropy Assays for the Analysis of Protein Binding to Sulfotyrosine-containing Peptides

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Detailed Procedures for Solid-Phase Peptide Synthesis (SPPS) and (Sulfo)peptide Characterisation

Resin loading (200 μmol scale): Rink amide resin (100-200 mesh) (200 μmol) was washed thoroughly with dry DMF and allowed to swell in dry DMF (5 mL) for 15 min. The resin was filtered and treated with a solution of piperidine/DMF (1:9 v/v, 2 × 2 mL) and gently agitated for 5 min. The resin was filtered and washed with DMF (5 x 2 mL), DCM (5 x 2 mL) and DMF (5 x 2 mL). A mixture of Fmoc-Pro-OH (800 μmol, 4 equiv.), PyBOP (800 μmol, 4 equiv.) and N-methylmorpholine (1600 μmol, NMM, 8 equiv.) was added to the resin and the mixture was gently agitated for 1 h. The resin was filtered and washed with DMF (5 × 2 mL), DCM (5 × 5 mL), and DMF (5 × 2 mL). The resin was subsequently treated with 10vol.% acetic anhydride in pyridine (5 mL) for 3 min to acetylate any unreacted amines on the resin. The resin was filtered and washed with DMF (5 x 2 mL), DCM (5 x 2 mL) and DMF (5 x 2 mL) before being treated with a solution of piperidine/DMF (1:9 v/v, 2 × 2 mL) and gently agitated for 5 min. The efficiency of the initial amino acid loading was quantitatively determined by measurement of the piperidine-fulvene adduct using UV-VIS spectrophotometry (λ = 301 nm) and shown to be quantitative.

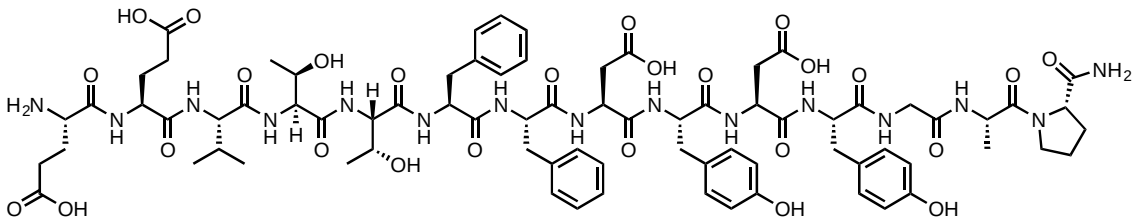
Capping: The resin was treated with a solution of acetic anhydride/pyridine (1:9 v/v, 2 mL) and gently agitated for 3 min. The resin was subsequently washed with DMF (5 × 2 mL), DCM (5 × 5 mL), and DMF (5 × 2 mL).

Fmoc-deprotection: The resin-bound peptide was treated with a solution of piperidine/DMF (1:9 v/v, 2 × 2 mL) and gently agitated for 5 min. The resin was subsequently washed with DMF (5 × 2 mL), DCM (5 × 5 mL), and DMF (5 × 2 mL).

Coupling: A solution of the Fmoc-protected amino acid (4 equiv.), PyBOP (4 equiv.) and NMM (8 equiv.) was added to the N-terminal deprotected peptide and gently agitated for 1 h. The resin was filtered and washed with DMF (5 × 2 mL), DCM (5 × 5 mL), and DMF (5 × 2 mL). *For coupling of neopentyl sulfate ester protected Fmoc-Tyr-OH [Fmoc-Tyr(SO_3np)-OH]):* A solution of Fmoc-Tyr(SO_3np)-OH (1.5 equiv.), HATU (1.5 equiv.) and *N,N*-diisopropylethylamine (DIEA, 3.0 equiv) was prepared and agitated at rt for 10 min. The pre-activated solution was added to the resin bound peptide and allowed to gently agitate for 16 h. The resin was filtered and washed with DMF (5 × 2 mL), DCM (5 × 5 mL), and DMF (5 × 2 mL).

Resin cleavage and ether precipitation: The resin was washed thoroughly with DCM (20 × 2 mL) and treated with a solution of TFA/*i*Pr_3SiH/water (90:5:5 v/v/v, 2 mL) and shaken for 2 h at rt. The resin was filtered and the filtrate was evaporated to dryness. Cold diethyl ether (*ca.* 5 mL) was then added the precipitate suspended and cooled on ice. The suspension was transferred to an eppendorf tube and centrifuged at 3000 rpm for 5 min. The supernatant was carefully removed and the precipitate dried under high-vacuum.

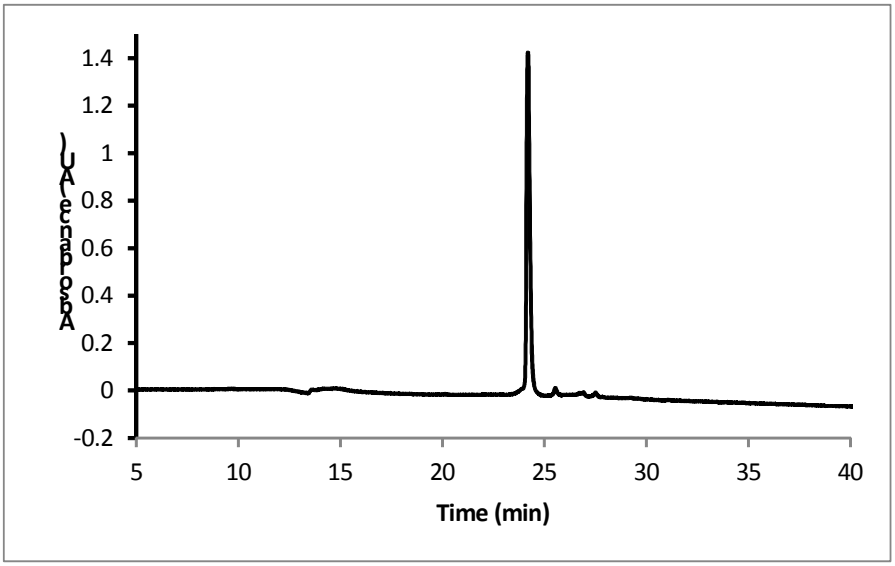
R2A: H-Glu-Glu-Val-Thr-Thr-Phe-Phe-Asp-Tyr-Asp-Tyr-Gly-Ala-Pro-NH_2



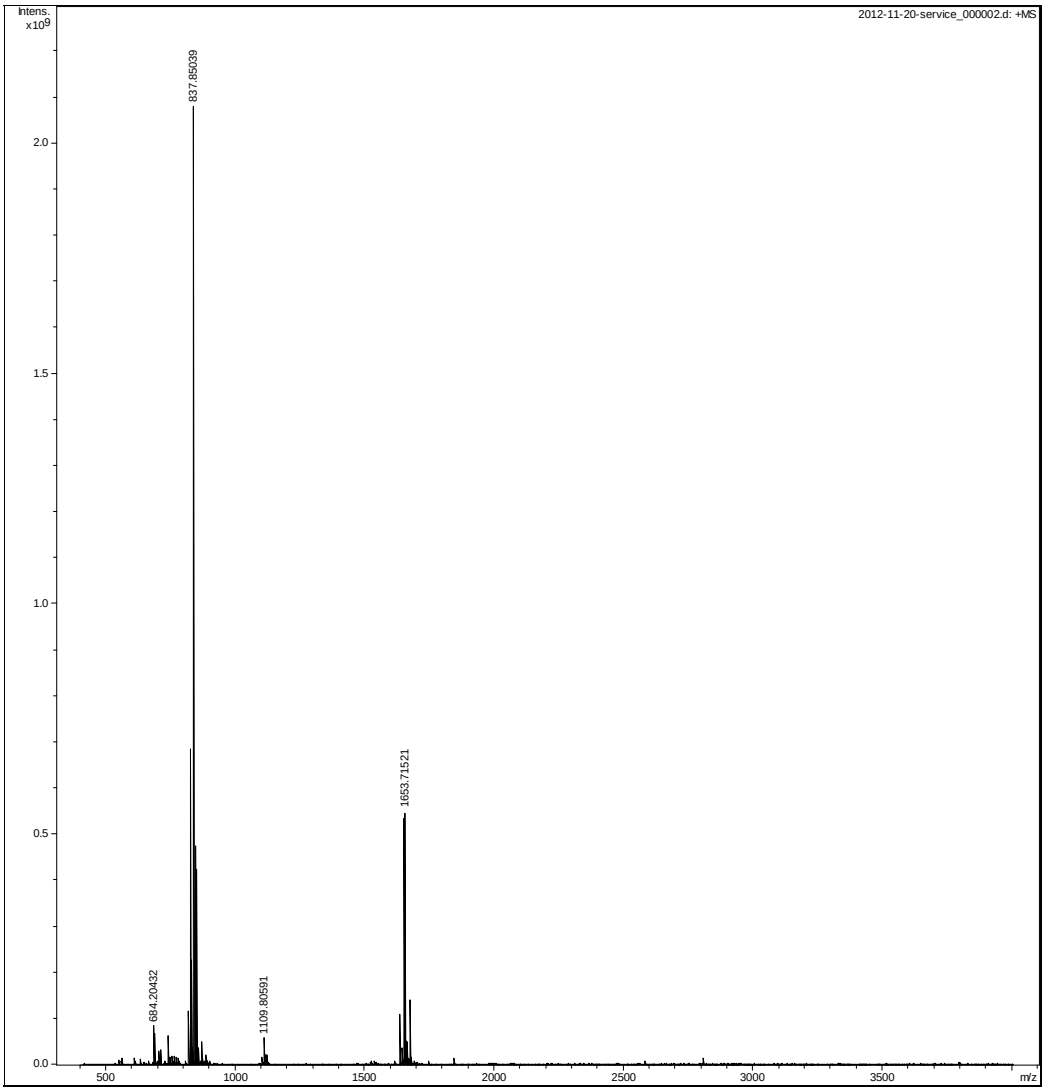
CCR2 peptide **R2A** was prepared as outlined in the Materials and Methods section and purified by preparative C18 HPLC (0 to 40% B over 40 min; R_t 29.5 min) to give a white solid (21 mg, 13 µmol, 26 % based on the original 50 µmol resin loading).

Analytical HPLC: R_t 24.2 min (0-50% B over 40 min, λ = 230 nm); HRMS (ESI^+): Calculated for $C_{77}H_{101}N_{15}O_{26}$: 837.8504 $[M+H+Na]^{2+}$, found 837.8504 $[M+H+Na]^{2+}$.

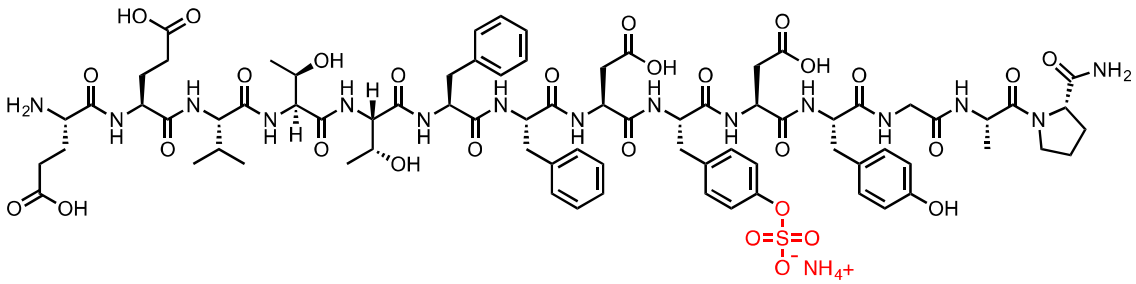
Analytical HPLC



Mass Spectrum



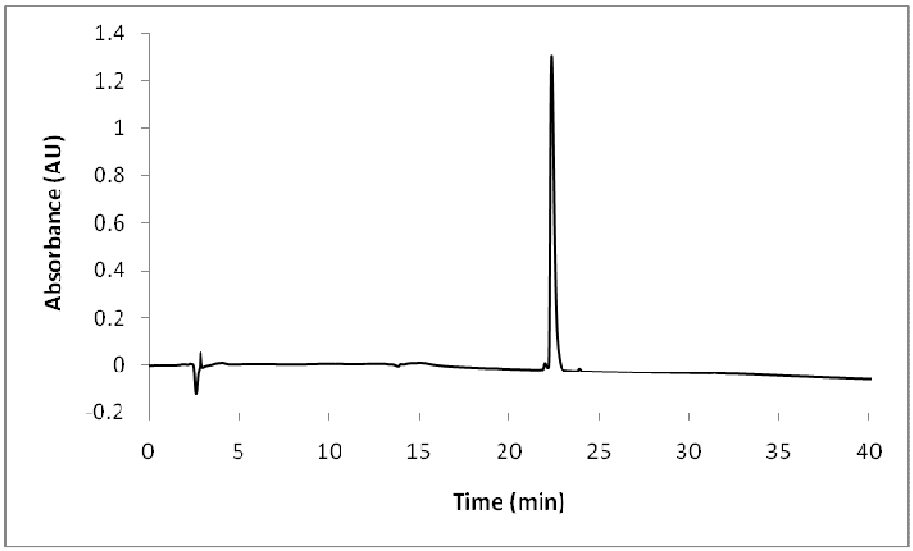
R2B: H-Glu-Glu-Val-Thr-Thr-Phe-Phe-Asp-Tyr($SO_3^-NH_4^+$)-Asp-Tyr-Gly-Ala-Pro-NH_2



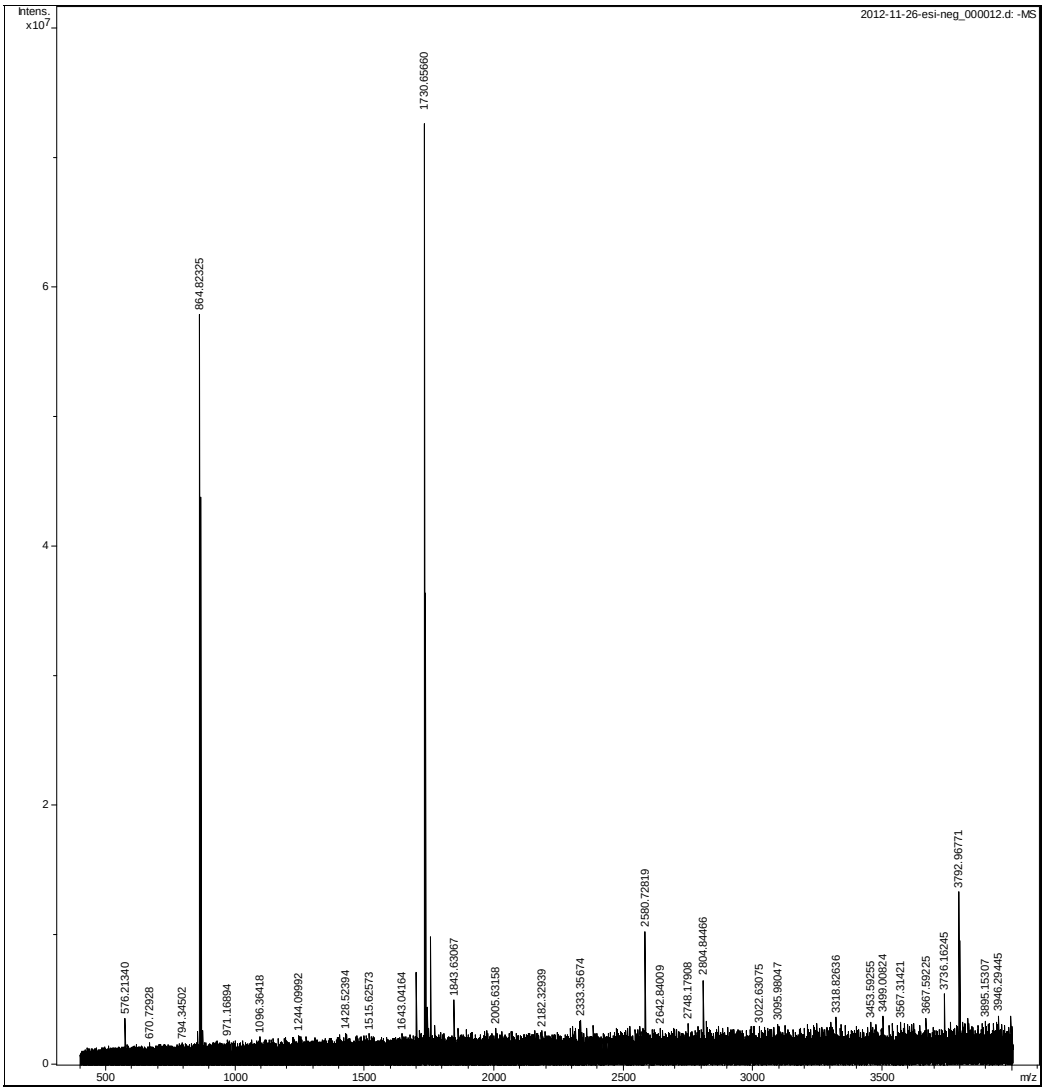
CCR2 sulfopeptide R2B was prepared as outlined in the Materials and Methods section and purified by preparative C18 HPLC (0 to 50% B over 40 min; *Eluent A*, R_t 23.6 min) to give a white solid (12 mg, 8 µmol, 14 % based on the original 50 µmol resin loading).

Analytical HPLC: R_t 23.2 min (0-50% B over 40 min, λ = 230 nm); HRMS (ESI^-): Calculated for $C_{77}H_{104}N_{16}O_{29}S$: 1730.6538 $[M\text{-}NH_3\text{-}H]^-$, found 1730.6587 $[M\text{-}NH_3\text{-}H]^-$.

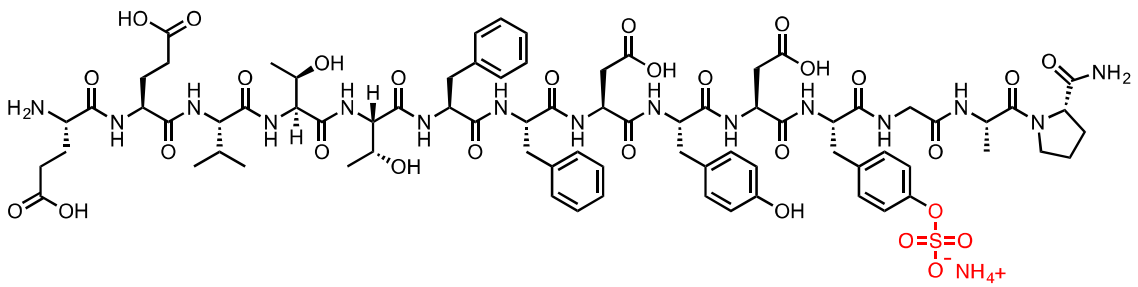
Analytical HPLC



Mass Spectrum



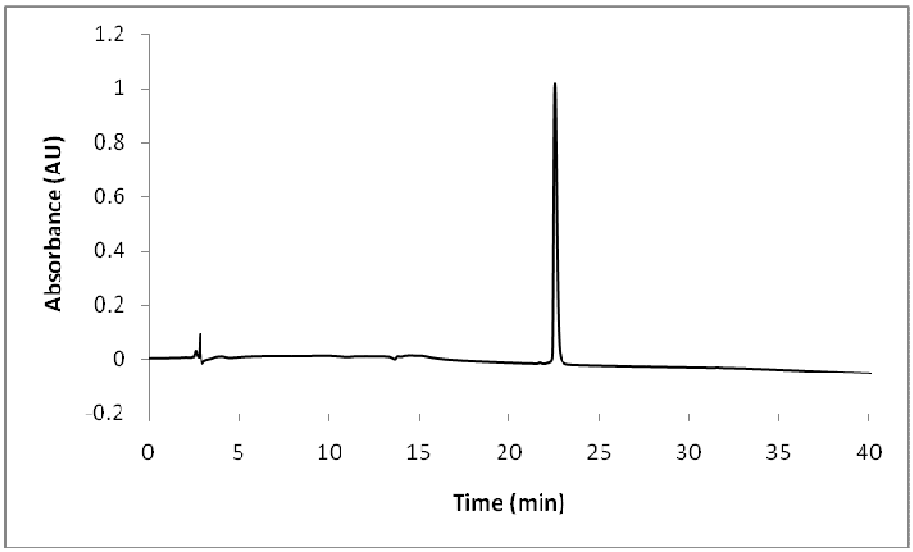
R2C: H-Glu-Glu-Val-Thr-Thr-Phe-Phe-Asp-Tyr-Asp-Tyr($SO_3^-NH_4^+$)-Gly-Ala-Pro-NH_2



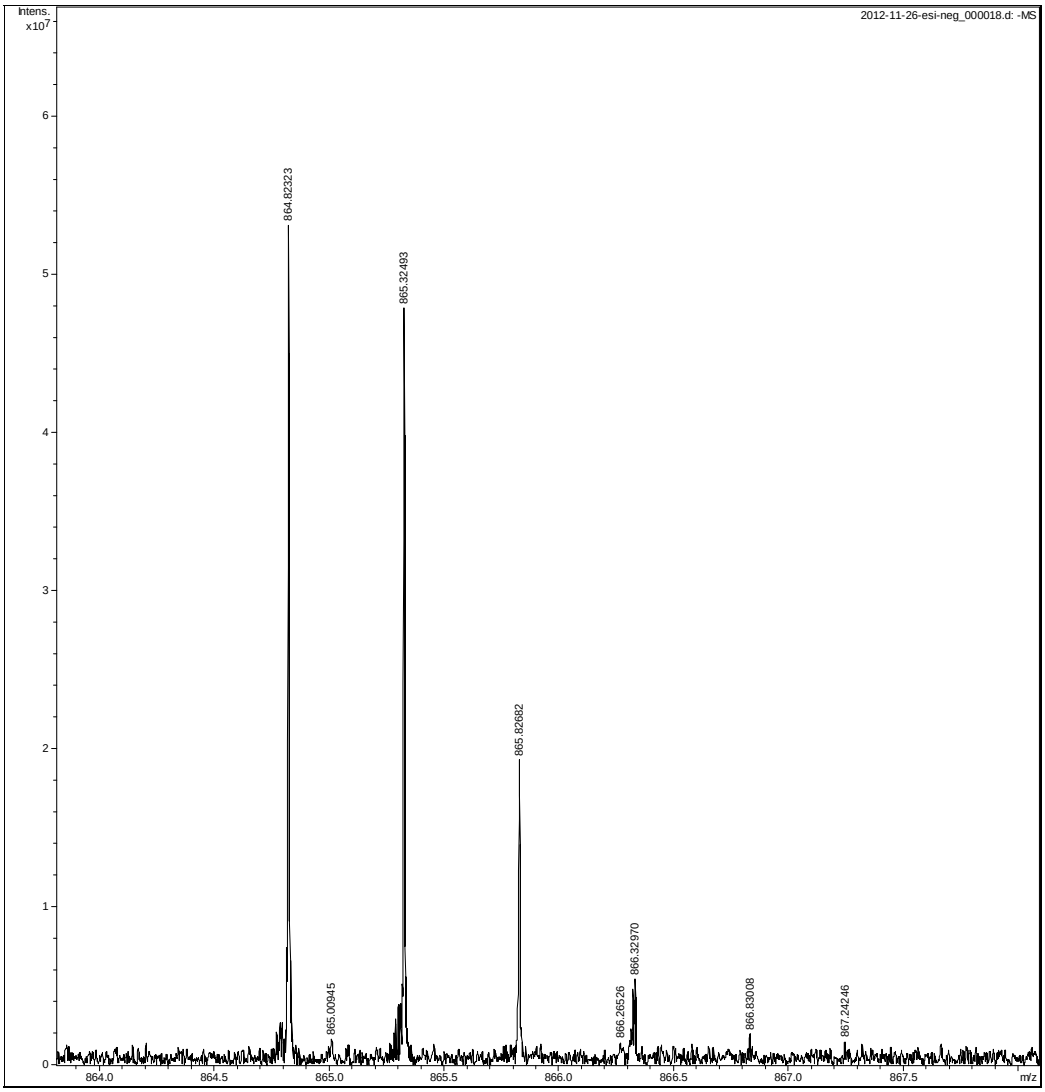
CCR2 sulfopeptide R2C was prepared as outlined in the Materials and Methods section and purified by preparative C18 HPLC (0 to 50% B over 40 min; *Eluent A*, R_t 22.9 min) to give a white solid (13 mg, 9 µmol, 15 % based on the original 50 µmol resin loading).

Analytical HPLC: R_t 22.8 min (0-50% B over 40 min, λ = 230 nm); HRMS (ESI^-): Calculated for $C_{77}H_{104}N_{16}O_{29}S$: 864.8232 $[M-2NH_3-2H]^{2-}$, found 864.8232 $[M-2NH_3-2H]^{2-}$.

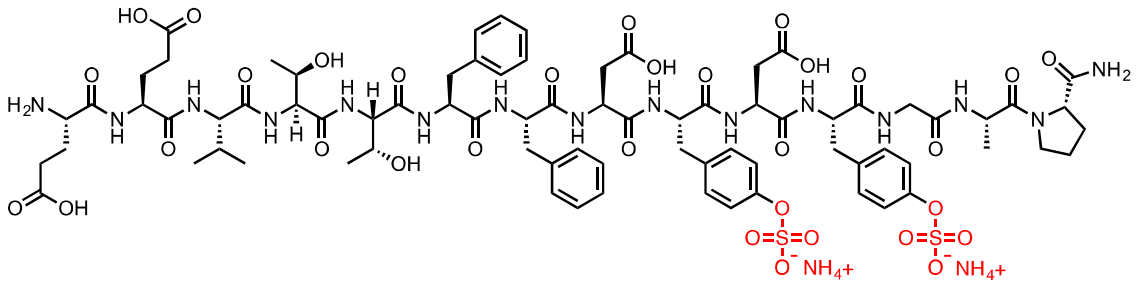
Analytical HPLC



Mass Spectrum



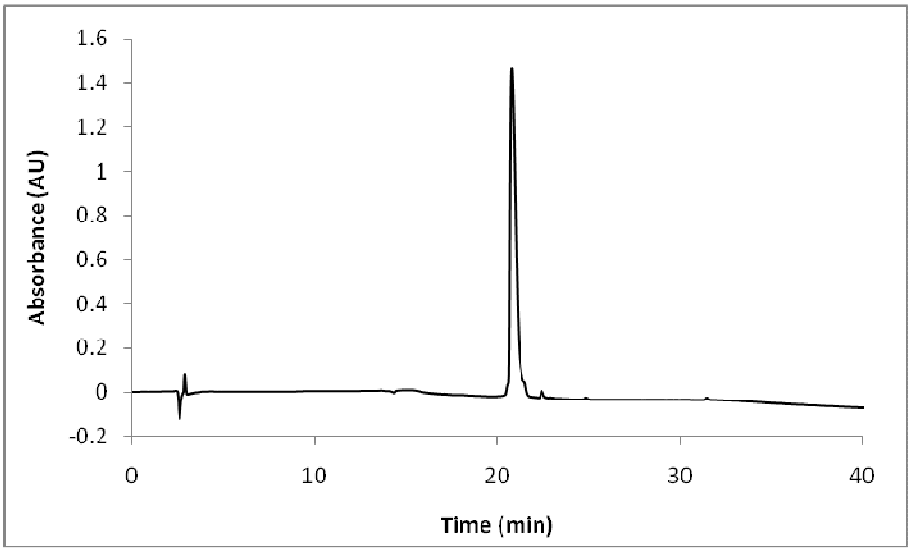
R2D: H-Glu-Glu-Val-Thr-Thr-Phe-Phe-Asp-Tyr($SO_3^-NH_4^+$)-Asp-Tyr($SO_3^-NH_4^+$)-Gly-Ala-Pro-NH_2



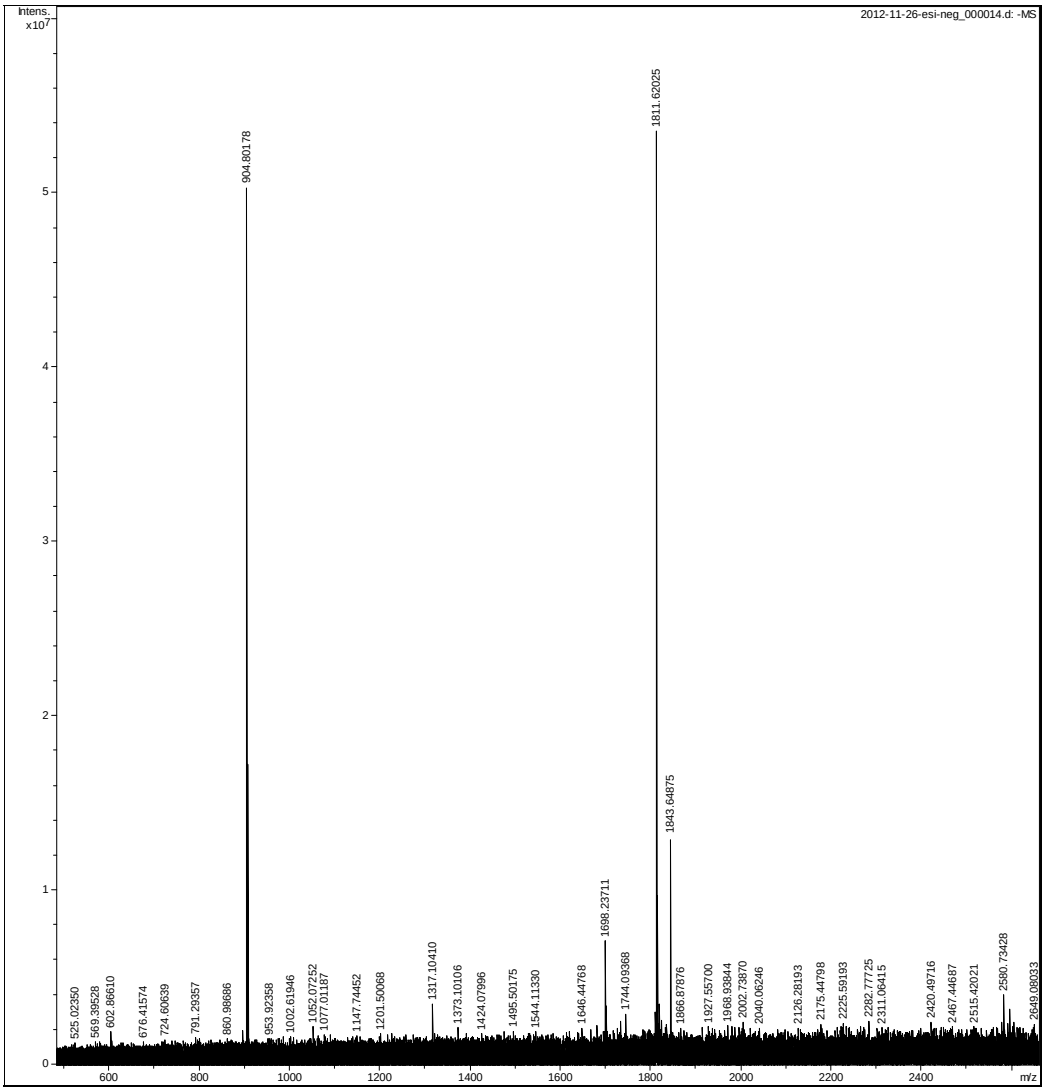
CCR2 sulfopeptide R2D was prepared as outlined in the Materials and Methods section and purified by preparative C18 HPLC (0 to 40% B over 40 min; *Eluent A*, R_t 24.5 min) to give a white solid (10 mg, 6 µmol, 11 % based on the original 50 µmol resin loading).

Analytical HPLC: R_t 20.8 min (0-50% B over 40 min, λ = 230 nm); HRMS (ESI^-): Calculated for $C_{77}H_{107}N_{17}O_{32}S_2$: 904.8017 $[M-2NH_3-2H]^{2-}$, found 904.8018 $[M-2NH_3-2H]^{2-}$.

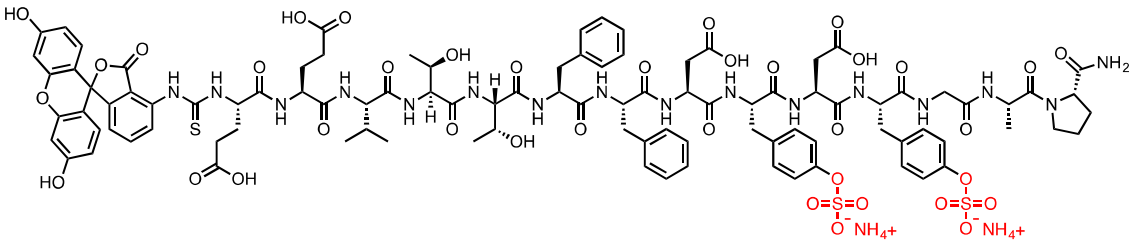
Analytical HPLC



Mass Spectrum



FL-R2D: FITC-Glu-Glu-Val-Thr-Thr-Phe-Phe-Asp-Tyr($SO_3^-NH_4^+$)-Asp-Tyr($SO_3^-NH_4^+$)-Gly-Ala-Pro-NH_2

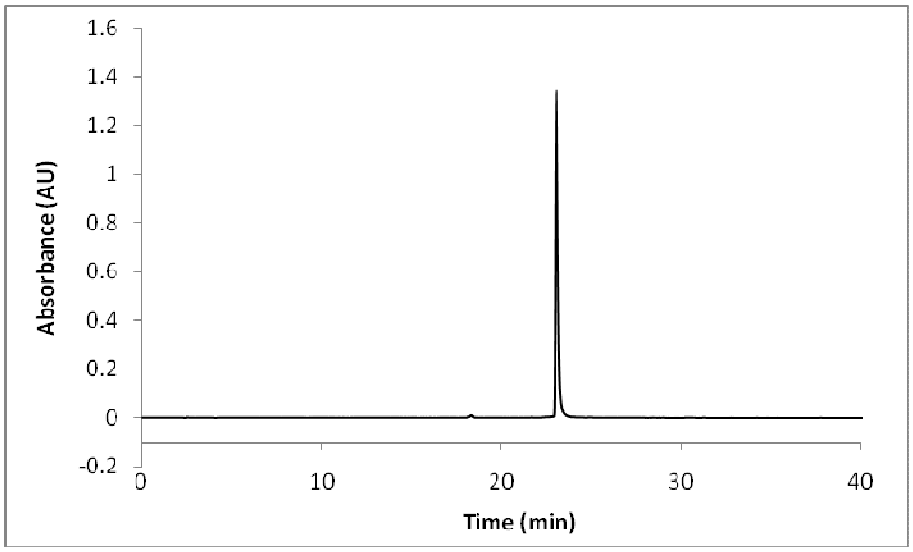


Chemical Formula: $C_{98}H_{118}N_{18}O_{37}S_3$
Exact Mass: 2234.7067

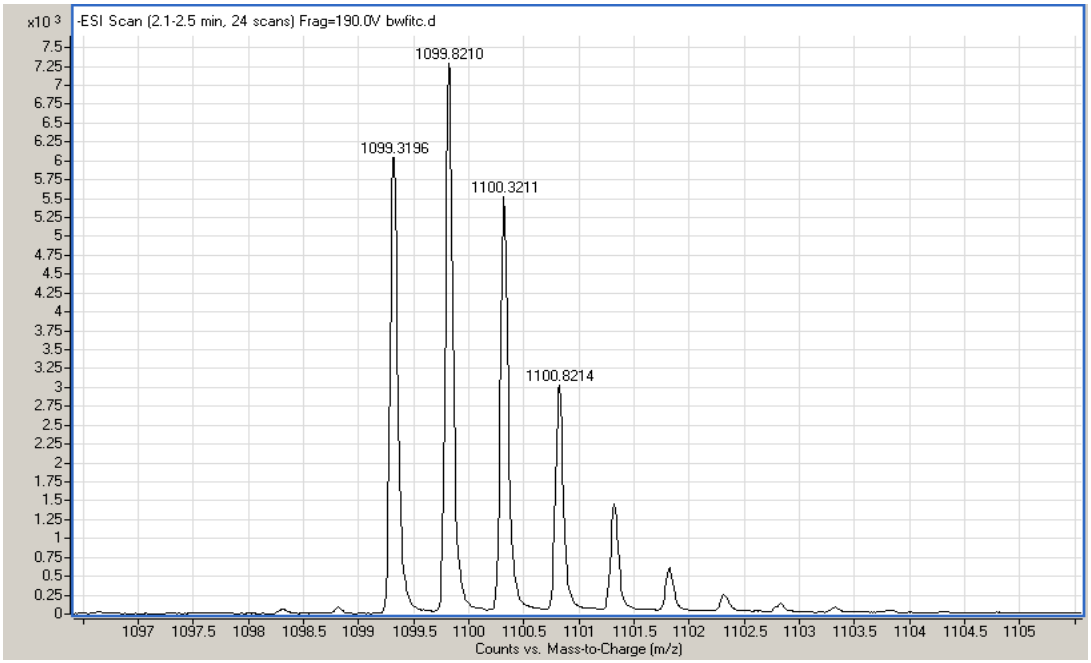
Fluorescein-labelled CCR2 sulfopeptide FL-R2D was prepared and purified as outlined in the Materials and Methods section.

Analytical HPLC: R_t 23.1 min (0-50% B over 40 min, λ = 280 nm); HRMS (ESI^-): Calculated for $C_{98}H_{118}N_{18}O_{37}S_3$: 732.5462 $[M-2NH_3-3H]^{3-}$, found 732.5453. $[M-2NH_3-3H]^{3-}$.

Analytical HPLC



Mass Spectrum



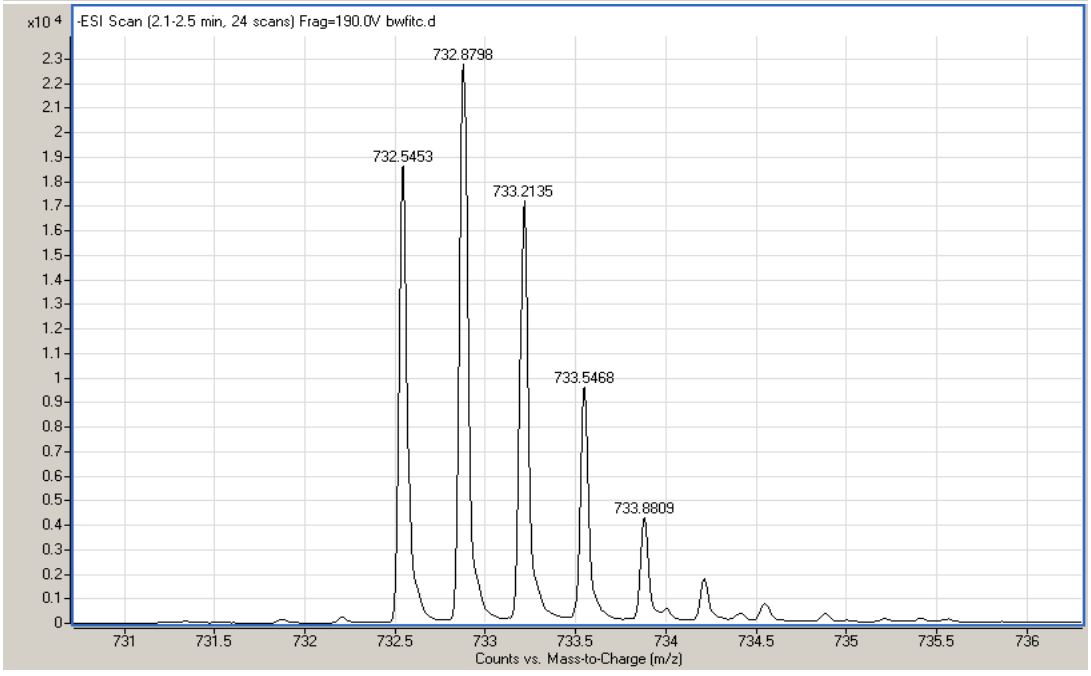
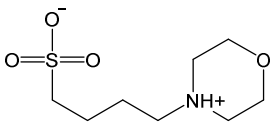
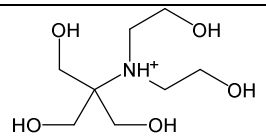
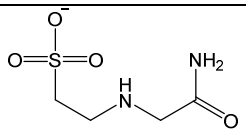
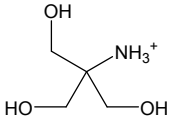
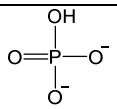
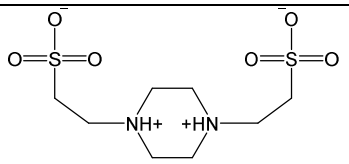
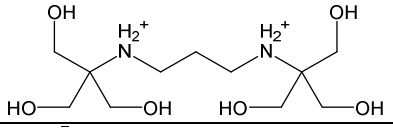
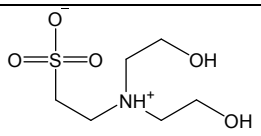


Table S1. Predicted charges at pH 7 of buffers used in the direct binding assay[1]

Buffer	Buffer Structure	Negative Charges (pH 7)	Positive Charges (pH 7)	Net Charge (pH 7)
MOPS[1] **Average**		1.00	0.60	-0.40
Bis-Tris		0.77	1.00	0.23
ACES		1.00	0.41	-0.59
Acetate		0.99	0.00	-0.99
Tris		0.08	1.00	0.92
Phosphate		1.39	0.00	-1.39
PIPES		2.00	1.16	-0.84
Bis-Tris Propane		1.38	2.00	0.62
BES		1.00	0.61	-0.39

[1]Predictions are based on the pK_a values reported by R.N. Goldberg et al. Thermodynamic quantities for the ionization reactions of buffers, *J Phys Chem Ref Data*, **31** (2002) 231-370.

Fig. S1. Chemical shift changes for eight NH groups of MCP-1(P8A) as a function of sodium phosphate (filled circles and solid lines) or sodium chloride (open squares and dashed lines) concentration.