Supplementary Information to "A phosphoarginine containing peptide as an artificial SH2 ligand"

Frank T. Hofmann, Claudia Lindemann, Helen Salia, Philipp Adamitzki and Florian P. Seebeck

Abteilung Physikalische Biochemie, Max Planck Institut für molekulare Physiologie, Otto-Hahn Straße 11, 44227 Dortmund, Germany

General methods

Z-Arg-OH was obtained from Bachem (Weil am Rhein, Germany). 5-Carboxyfluorescein *N*-hydroxysuccinimide ester was supplied by Berry&Associates (Dexter, MI, USA). All other amino acids were purchased from Merck Chemicals (Darmstadt, Germany). DCM, DMF and NMP were purchased from Applied Biosystems (Karlsruhe, Germany) all in "peptide synthesis grade". Acetonitrile was supplied by Sigma Aldrich (Munich, Germany) in "HPLC gradient grade". Piperidine was obtained from Alfa Aesar (Karlsruhe, Germany). TFA was supplied in "peptide synthesis grade" by Roth (Karlsruhe, Germany). All other chemicals were obtained from Sigma Aldrich in the highest purity available.
Unless otherwise stated all parts, proportions and percentages are by volume.

RP-HPLC

HPLC was carried out on a Beckmann System Gold (Autosampler 508, Programmable Solvent Module 126, Diode Array Detector 168, Fraction Collector SC 100; Beckmann Coulter Inc., Brea, CA, USA) using columns packed with C18 ProntoSIL AQ with 5 µm particle size and 120 Å pore size (Bischoff, Leonberg, Germany).

Table S1. Eluent systems used for HPLC

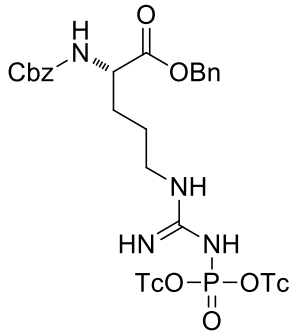
Eluent System	Eluent A	Eluent B
E1	0.1% TFA in H_2O	0.08% TFA in acetonitrile
E2	10 mM ammonium formate in H_2O, pH = 9.2	1 part 100 mM ammonium formate in H_2O, pH = 9.2 diluted with 9 parts acetonitrile
E3	5 mM sodium phosphate in H_2O, pH = 8.0	4 parts 12.5 mM sodium phosphate in H_2O, pH = 8.0 diluted with 6 parts acetonitrile

Analytical HPLC was performed at a flow rate of 1 mL/min on a 250 x 4.6 mm sized column equipped with a 10 x 4.0 mm guard cartridge. Methods A-C: Samples were dissolved in 50% aqueous acetonitrile to a concentration of 0.5-5 mg/mL. After injection 5% eluent B were maintained for 3 min followed by a linear gradient: (A) 5-45% B over 30 min; (B) 5-65% B over 30 min; (C) 5-95% B over 30 min. Method D: Samples were dissolved in water to a concentration of 0.5-5 mg/mL. After injection 1% eluent B was maintained for 5 min followed by a linear gradient 1-26% B over 25 min.

Preparative HPLC was performed on a 250 x 20 mm sized column equipped with a 50 x 20 mm guard column at a flow rate of 10 mL/min using appropriate linear gradients with a slope of 0.2-0.5%/min. Prior to injection peptides were dissolved in a minimal volume of 50% aqueous acetonitrile unless otherwise stated. For acid stable peptides eluent system E1 was used. Peptides containing deprotected phosphoarginine were purified using alkaline eluent systems E2 or E3.

Building block synthesis

Z-Arg(PO_3Tc_2)-OBn



To Z-Arg-OH (10.0 g, 32.4 mmol, 1.0 equiv) NMP (130 mL) was added. Argon was bubbled through the suspension for 15 min. Under argon the stirred suspension was heated in an oil bath at 90°C. After complete dissolution of Z-Arg-OH the solution was allowed to cool to room temperature. Benzyl bromide (4.24 mL, 35.6 mmol, 1.1 equiv) was added dropwise. The reaction was stirred under argon in the dark until TLC analysis showed complete consumption of Z-Arg-OH usually after 5 h. The majority of NMP was removed *in vacuo*.

The resulting yellow oil was dissolved in acetonitrile (486 mL). Triethylamine (18.1 mL, 130 mmol, 4.0 equiv) was added. Bis(trichloroethyl)phosphoryloxychloride (14.7 g, 38.9 mmol, 1.2 equiv) was added in three equal portions after 0, 90 and 180 min. The reaction was stirred until ESI-MS analysis showed complete consumption of Z-Arg-OBn usually after 7 h. The solvent was removed *in vacuo.* Column chromatography on silca gel using gradient elution with 33% - 50% ethyl acetate and 1% formic acid in cyclohexane gave Z-Arg(PO_3Tc_2)-OBn as colorless oil (13.9 g, 18.7 mmol, 58%).

^{1}H-NMR (400 MHz, acetone-d_6, TMS): δ [ppm] = 7.32 (m, 10H, **H^{aryl}**), 6.72 (brs, 1H, N**H**), 6.40 (brm, 1H, N**H**), 5.17 (s, 2H, C**H$_2$**-Ph), 5.08 (m, 2H, C**H$_2$**-Ph), 4.64 (brm, 4H, P-O-C**H$_2$**), 4.30 (m, 1H, C$^\alpha$**H**), 3.33 (brm, 2H, C$^\delta$**H$_2$**), 1.91 (brm, 1H, C$^\beta$**H$_b$**), 1.81 (brm, 1H, C$^\beta$**H$_a$**), 1.71 (brm, 2H, C$^\gamma$**H$_2$**).

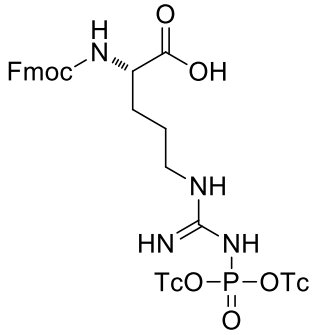
^{13}C-NMR (101 MHz, CD_3CN): δ [ppm] = 173.0, 157.4, 156.7, 138.0, 137.0, 129.5, 129.4, 129.2, 129.0, 128.9, 128.7, 95.4, 77.6, 67.6, 67.2, 54.7, 42.3, 29.4, 25.0.
^{31}P-NMR (162 MHz, acetone-d_6): δ [ppm] = 7.24.

HR-MS (ESI +): *m/z* = 738.99793 $[M+H]^+$, monoisotopic mass calc.: 738.99778 $[C_{25}H_{30}O_7N_4Cl_6P]^+$.

ATR-FT-IR: 3337, 2942, 1715, 1573, 1236, 1098, 1030, 876, 717 cm^{-1}.

R_f (1:1 ethyl acetate / cyclohexane + 1% formic acid) = 0.27.

Fmoc-Arg(PO_3Tc_2)-OH (1)



To a solution of Z-Arg(PO_3Tc_2)-OBn (3.00 g, 4.05 mmol, 1.0 equiv) in acetic acid / trifluoroacetic acid (1:1, 54 mL) palladium on charcoal (0.450 g, 10 wt% Pd) was added under argon. The reaction mixture was vigorously stirred under a hydrogen atmosphere for 3 h. The catalyst was removed by filtration after which the solvent was removed *in vacuo*. Residual acid was removed by coevaporation with ethanol.

The resulting oil was dissolved in water (10 mL) and triethylamine (565 µL, 4.05 mmol, 1.0 equiv) was added. A solution of Fmoc-OSu (1.31 g, 4.05 mmol, 1.0 equiv) in acetonitrile (10 mL) was added dropwise while stirring. Triethylamine was added until the pH of solution stabilized at 9.0 usually after 1 h. Acetic acid was added until a pH of 3.5 was reached. The reaction mixture was mixed with an equal volume of brine and extracted with chloroform (3 x 50 mL). The combined organic layers were dried over anhydrous magnesium sulfate, concentrated *in vacuo* and directly loaded on an equilibrated silica column. Impurities were eluted with ethyl acetate / cyclohexane (1:1) containing 1% formic acid. Pure product was eluted with ethyl acetate containing 1% formic acid. The solvent was removed from product fractions *in vacuo* and the residue dissolved in acetonitrile. After addition of water lyophilization of the suspension gave the product as colorless powder (1.88 g, 2.54 mmol, 63%).

^{1}H-NMR (400 MHz, CD_3CN): δ [ppm] = 7.80 (d, *J* = 7.5 Hz, 2H, **H^{aryl}**), 7.65 (t, *J* = 6.8 Hz, 2H, **H^{aryl}**), 7.40 (t, *J* = 7.5 Hz, 2H, **H^{aryl}**), 7.31 (t, *J* = 6.9 Hz, 2H, **H^{aryl}**), 6.56 (br s, 1H, N**H**), 6.10 (br s, 1H, N**H**), 5.97 (br s, 2H, N**H$_2$**), 4.60 (d, *J* = 4.9 Hz, 4H, C**H$_2$**-CCl_3), 4.32 (d, *J* = 7.0 Hz, 2H, Fmoc-C**H$_2$**), 4.21 (t, *J* = 7.0 Hz, 1H, Fmoc-C**H**), 4.16 (m, 1H, C$^{\alpha}$**H**), 3.23 (m, 2H, C$^{\delta}$**H$_2$**), 1.84 (m, 1H, C$^{\beta}$**H$_b$**), 1.68 (m, 1H, C$^{\beta}$**H$_a$**), 1.59 (m, 2H, C$^{\gamma}$**H$_2$**).

^{13}C-NMR (101 MHz, CD_3CN): δ [ppm] = 174.0 , 157.5 , 156.4 , 145.0, 144.9, 142.1 , 128.7 , 128.1 , 126.2 , 121.0 , 95.2 (d, *J* = 10.5 Hz), 77.8 (d, *J* = 4.2 Hz), 67.5 , 54.2 , 48.0 , 42.4 , 29.4 , 24.9.

^{31}P-NMR (162 MHz, CD_3CN): δ [ppm] = 5.61.

HR-MS (ESI +): *m/z* = 736.98234 $[M+H]^+$, monoisotopic mass calc.: 736.98213 $[C_{25}H_{28}O_7N_4Cl_6P]^+$.

ATR-FT-IR: 3329, 2941, 1713, 1577, 1222, 1100, 1030, 880, 720 cm^{-1}.

R$_f$ (EtOAc + 1% formic acid) = 0.45.

Peptide Synthesis

Unless otherwise stated peptides were synthesized on a Wang polystyrene resin which was purchased preloaded with Fmoc-Ile (0.59 mmol/g) from Merck Chemicals (Darmstadt, Germany).

Automated peptide synthesis

Automated peptide synthesis was carried out using an ABI 433A peptide synthesizer (Life Technologies, Carlsbad, CA, USA) employing the FastMoc protocol.[1] Protection for amino acids was as follows: Arg(Pbf), Gln(Trt), Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu).

Manual introduction of Arg(PO_3Tc_2)

To a solution of Fmoc-Arg(PO_3Tc_2)-OH (3.00 equiv) and HBTU (2.85 equiv) in a minimal volume of DMF DIPEA (3.60 equiv) was added. The mixture was vigorously shaken and left standing for 2 min after which it was added to the preswollen peptide resin (1.00 equiv) in a PP syringe equipped with a porous PE disc. The reaction mixture was shaken until a negative Kaiser test indicated a complete coupling reaction.[2] The resin was washed with DMF (3x), DCM (3x) and DMF (2x). Fmoc-deprotection was accomplished by two treatments with 20% piperidine in DMF (5 / 10 min) after which the resin was washed with DMF (6x) and DCM (3x).

Manual introduction of amino acid residues

Residues located N-terminally from Arg(PO_3Tc_2) were introduced manually: To a solution of the Fmoc-protected amino acid (5.00 equiv) and HBTU (4.75 equiv) in a minimal volume of DMF DIPEA (6.00 equiv) was added. The mixture was vigorously shaken and left standing for 2 min after which it was added to the preswollen peptide resin (1.00 equiv) in a PP syringe equipped with a porous PE disc. The reaction mixture was shaken until a negative Kaiser test indicated a complete coupling reaction.[2] The resin was washed with DMF (3x), DCM (3x) and DMF (2x). Fmoc-deprotection was accomplished by two treatments with 20% piperidine in DMF (5 / 10 min) after which the resin was washed with DMF (6x) and DCM (3x).

Acetylation

The peptide resin (1.00 equiv) was preswollen in DMF for 30 min in a PP syringe equipped with a porous PE disc. A solution of acetic anhydride (0.5 M, 20 equiv) and DIPEA (0.5 M, 20 equiv) in DMF was prepared and added to the resin. The reaction mixture was shaken until a negative Kaiser test indicated a complete acetylation reaction.[2] The resin was washed with DMF (3x) and DCM (3x).

On resin 5-FAM-labeling

The peptide resin was placed in a PP syringe equipped with a porous PE disc and preswollen in DMF for 30 min. 5-Carboxyfluorescein *N*-hydroxysuccinimide ester (2 equiv) was dissolved in a minimal volume of DMF aided by sonification. The solution was added to the resin. DIPEA (10 equiv) was added and the mixture was agitated. The reaction was left standing in the dark overnight. The resin was washed with DMF (3x) and DCM (3x).

Cleavage

Unless otherwise stated the vacuum-dried resin was agitated for 90 min in 2.5% H_2O and 5% TIS in TFA (15 mL per 1 g resin). The resin was removed by filtration and washed with a small amount of TFA. The filtrate was slowly dropped into stirred Et_2O (10 mL per 1 mL cleavage solution) at -20°C. The resulting precipitate was collected by centrifugation, titurated with ice-cold Et_2O (2x) and dried under vacuum overnight.

Ac-FFREEI-OH (**4c,** Scheme 1)

The peptide was prepared by automated peptide synthesis. After cleavage the peptide was purified by HPLC.

LR-MS (ESI +): *m/z* = 882.8 $[M+H]^+$, average mass calc.: 882.99 $[C_{42}H_{60}N_9O_{12}]^+$.

Ac-FF$_D$REEI-OH (**5,** Scheme 1)

Until Glu^3 the peptide chain was assembled using automated peptide synthesis. The remaining amino acids and N-terminal acetylation were introduced manually. The peptide was cleaved from the resin and used without further purification ($_D$R for D-arginine).

LR-MS (ESI +): *m/z* = 882.9 $[M+H]^+$, average mass calc.: 882.99 $[C_{42}H_{60}N_9O_{12}]^+$.

Ac-FFR(PO$_3$Tc$_2$)EEI-OH

Until Glu^3 the peptide chain was assembled using automated peptide synthesis. The remaining amino acids and N-terminal acetylation were introduced manually. The peptide was cleaved from the resin and used without further purification.

Yield (based on initial resin loading): 72%.

LR-MS (ESI +): *m/z* = 1226.6 $[M+H]^+$, average mass calc.: 1225.80 $[C_{46}H_{63}N_9O_{15}P_1Cl_6]^+$.

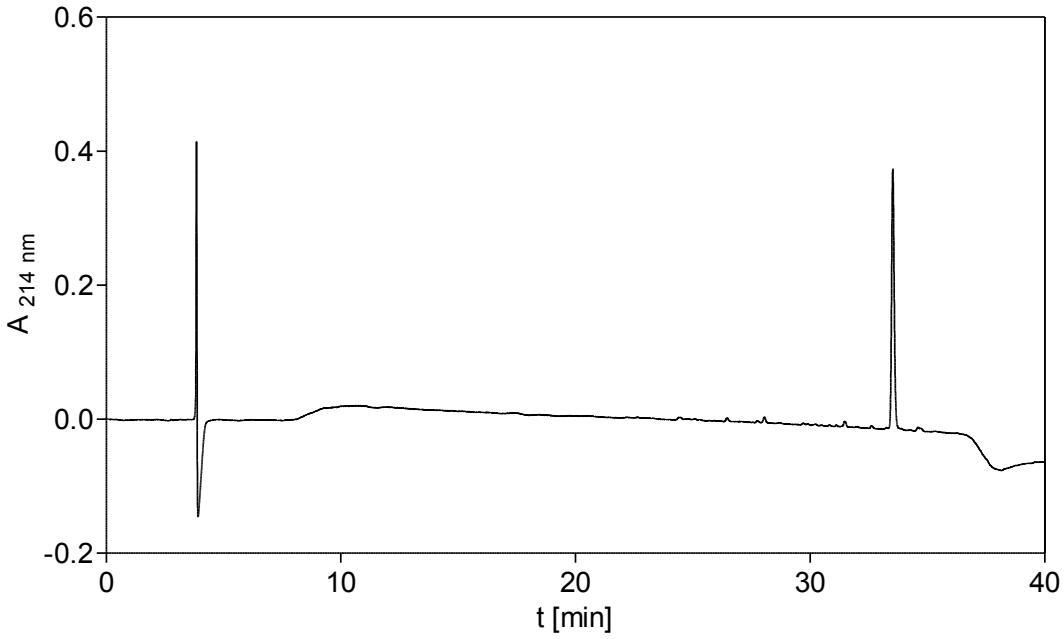


Figure S1. Analytical HPLC chromatogram of crude Ac-FFR(PO$_3$Tc$_2$)EEI-OH (E1, method B)

Ac-FFR(PO_3H_2)EEI-OH (**4a,** Scheme 1)

Ac-FFR(PO_3Tc_2)EEI-OH (10.0 mg, 8.20 µmol) was dissolved in ammonium carbonate buffer (800 µL, 100 mM, pH = 9.2). Ethanol (3.20 mL) and palladium on charcoal (15 mg, 10 wt% Pd) were added. Argon was bubbled through the suspension for 2 min. The reaction mixture was vigorously stirred under a hydrogen atmosphere for 2 h. The catalyst was removed by filtration after which the solvent was removed *in vacuo*. The residue was dissolved in a minimal volume of ammonium carbonate buffer (100 mM, pH = 9.2) and purified by HPLC using eluent system E2.

Yield (based on HPLC chromatogram): 69%.

Yield: 2 mg (2.08 µmol, 25%).

LR-MS (ESI +): *m/z* = 962.7, $[M+H]^+$, average mass calc.: 962.97 $[C_{42}H_{61}N_9O_{15}P_1]^+$.

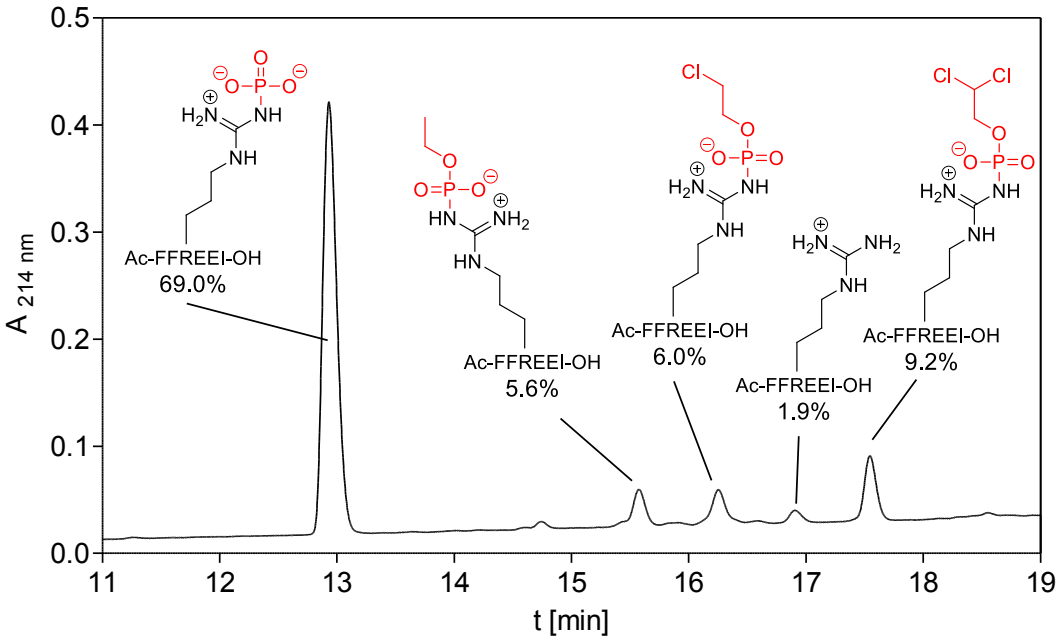


Figure S2. Analytical HPLC chromatogram of crude Ac-FFR(PO_3H_2)EEI-OH after hydrogenolytic deprotection (E2, method B)

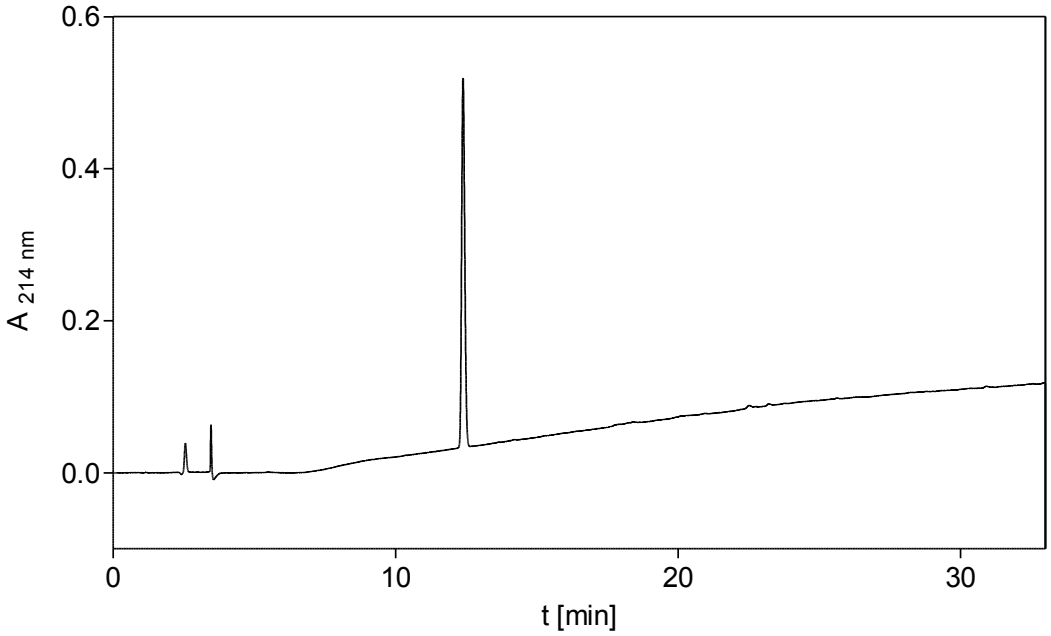


Figure S3. Analytical HPLC chromatogram of Ac-FFR(PO_3H_2)EEI-OH after HPLC purification (E2, method B)

Ac-FFY(PO_3H_2)EEI-OH (**4b,** Scheme 1)

Until Glu[3] the peptide chain was assembled using automated peptide synthesis. Tyr(PO_3) was introduced manually via the Fmoc-Tyr($PO(NMe_2)_2$)-OH building block. The remaining amino acids and N-terminal acetylation were introduced manually. After cleavage from the resin the peptide was purified by HPLC.

LR-MS (ESI +): *m/z* = 969.4 $[M+H]^+$, average mass calc.: 969.96 $[C_{45}H_{58}N_6O_{16}P]^+$.

5-FAM-Y(PO_3H_2)EEI-OH (**6a,** Scheme 1)

Until Glu[3] the peptide chain was assembled using automated peptide synthesis. Tyr(PO_3) was introduced manually via the Fmoc-Tyr($PO(NMe_2)_2$)-OH building block. N-terminal 5-Carboxyfluorescein was introduced manually. The peptide was cleaved from the resin according to the procedure published by Chao et al. and used without further purification.[3]

LR-MS (ESI +): *m/z* = 991.5 $[M+H]^+$, average mass calc.: 991.88 $[C_{46}H_{48}N_4O_{19}P]^+$.

5-FAM-Y(SO_3H)EEI-OH (**6b,** Scheme 1)

Until Glu[3] the peptide chain was assembled using automated peptide synthesis on 2-chlorotrityl resin which was purchased preloaded with H-Ile (0.53 mmol/g) from Merck Chemicals (Darmstadt, Germany). Tyr(SO_3H) was introduced manually via the

Fmoc-Tyr(SO_3H)-OH building block. N-terminal 5-Carboxyfluorescein was introduced manually. The peptide was cleaved from the resin using 90% aqueous TFA at 0°C.[4] The crude peptide was purified by HPLC.

LR-MS (ESI -): *m/z* = 988.6 $[M-H]^-$, average mass calc.: 989.95 $[C_{46}H_{45}N_4O_{19}S]^-$.

Ac-LAR(PO_3Tc_2)RIRGERK-OH

Until Arg[4] the peptide chain was assembled using automated peptide synthesis on Wang polystyrene resin which was purchased preloaded with Fmoc-Lys(Boc) (0.67 mmol/g) from Merck Chemicals (Darmstadt, Germany). The remaining amino acids and N-terminal acetylation were introduced manually. The crude peptide was purified by HPLC.

LR-MS (ESI +): *m/z* = 1640.6 $[M+H]^+$, average mass calc.: 1640.36 $[C_{58}H_{105}N_{23}O_{17}P_1Cl_6]^+$.
Yield (based on HPLC chromatogram): 55%.
Yield (based on initial resin loading): 194 mg (118 µmol, 59%).

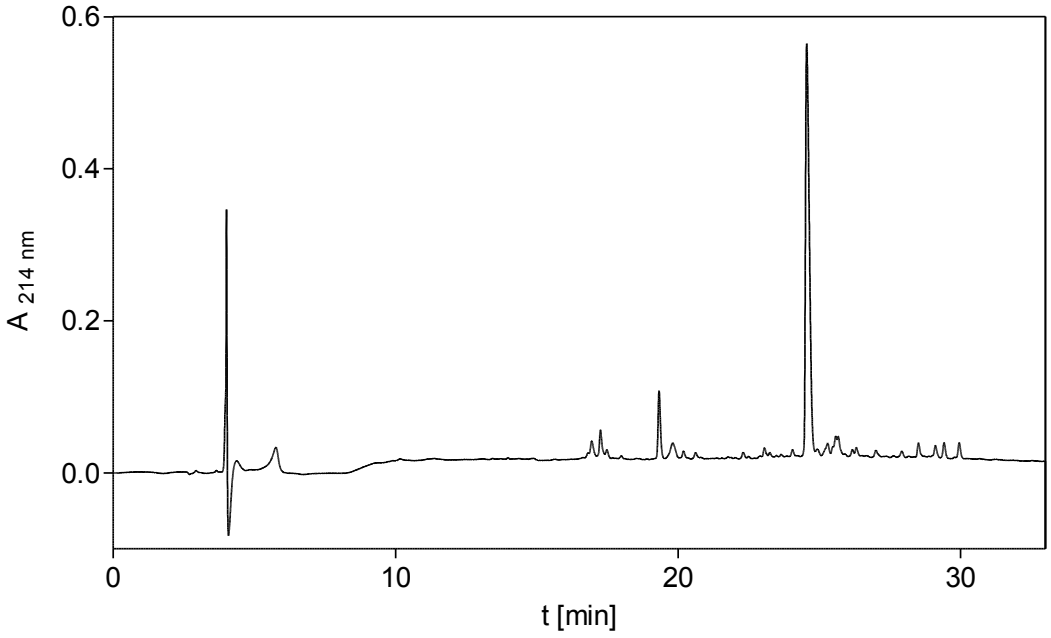


Figure S4. Analytical HPLC chromatogram of crude Ac-LAR(PO_3Tc_2)RIRGERK-OH (E1, method B)

Ac-LAR(PO_3H_2)RIRGERK-OH (**2,** Scheme 1)

Ac-LAR(PO_3Tc_2)RIRGERK-OH (71.0 mg, 43.3 µmol) was dissolved in ammonium carbonate buffer (5.68 mL, 100 mM, pH = 9.2). Ethanol (22.7 mL) and palladium on charcoal (107 mg, 10 wt% Pd) were added. Argon was bubbled through the suspension for 5 min. The reaction mixture was vigorously stirred under a hydrogen atmosphere for 3 h. The catalyst was removed by centrifugation and washed with 50% aqueous acetonitrile (5 x 8 mL). The combined supernatants were filtered and sodium phosphate buffer (1 mL, 100 mM, pH = 8) was added. The solvent was removed *in vacuo*. The residue was dissolved in a minimal volume of water and purified by HPLC using eluent system E3.

LR-MS (ESI +): *m/z* = 1377.5 $[M+H]^+$, average mass calc.: 1377.53 $[C_{54}H_{103}N_{23}O_{17}P_1]^+$.
Yield (based on HPLC chromatogram): 75%.
Yield (corrected for sodium phosphate content): 27.4 mg (19.9 µmol, 46%).

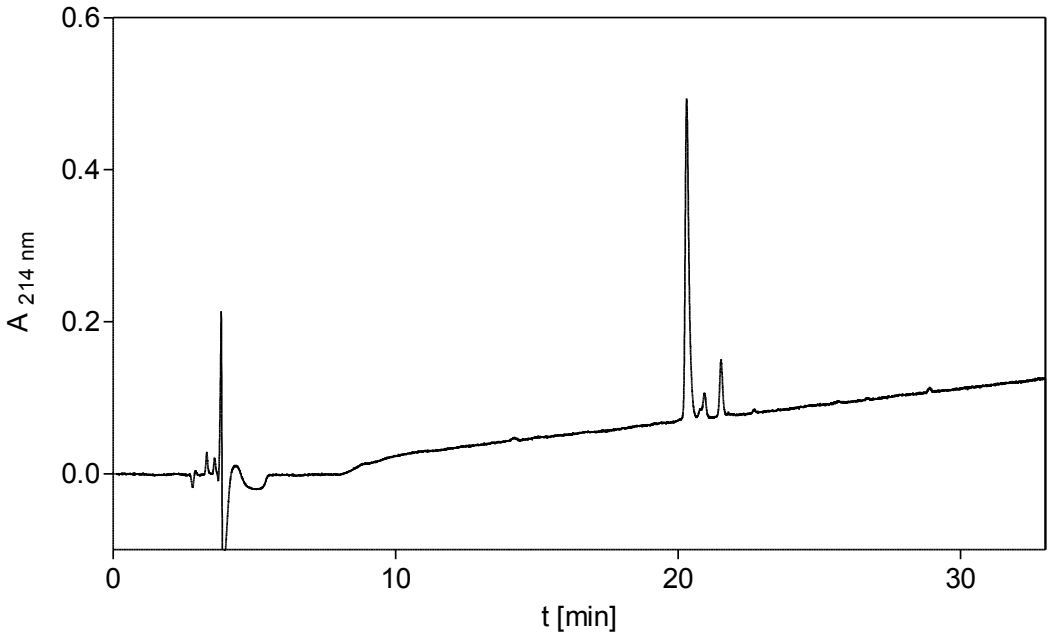


Figure S5. Analytical HPLC chromatogram of crude Ac-LAR(PO_3Tc_2)RIRGERK-OH after hydrogenolytic deprotection (E1, method A)

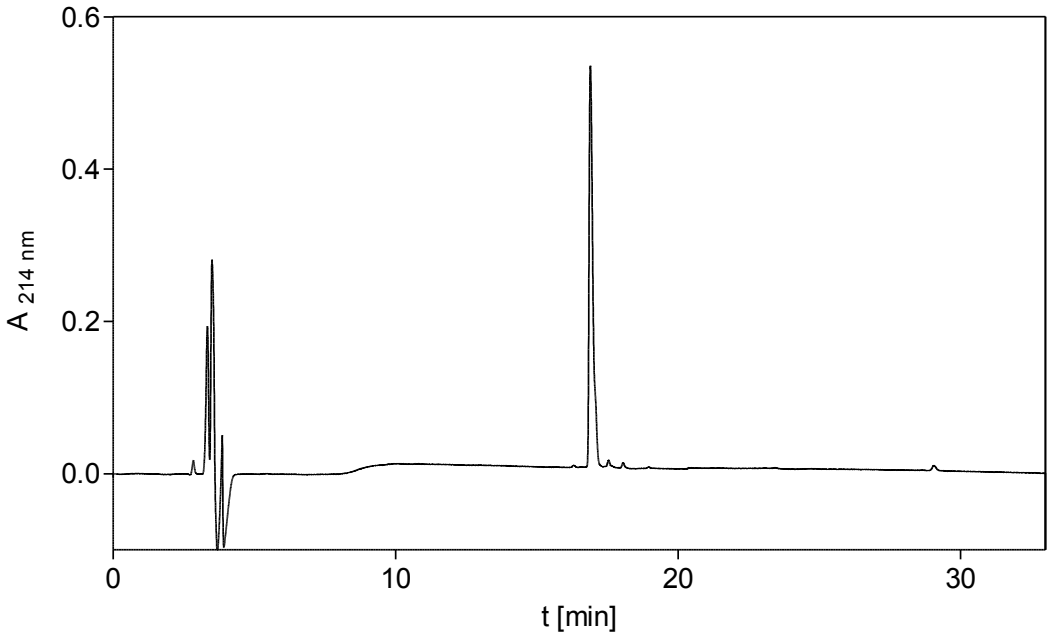


Figure S6. Analytical HPLC chromatogram of Ac-LAR(PO_3H_2)RIRGERK-OH after HPLC purification (E1, method B)

Ac-AR(PO_3Tc_2)TKQTA-OH

Until Thr[3] the peptide chain was assembled using automated peptide synthesis on Tentagel R PHB resin which was purchased preloaded with Fmoc-Ala (0.20 mmol/g) from Rapp Polymere GmbH (Tübingen, Germany). The remaining amino acids and N-terminal acetylation were introduced manually. The crude peptide was purified by HPLC.

LR-MS (ESI +): *m/z* = 1161.3 $[M+H]^+$, average mass calc.: 1160.73 $[C_{37}H_{64}N_{12}O_{15}P_1Cl_6]^+$.
Yield (based on HPLC chromatogram): 76%.
Yield (based on initial resin loading): 51.3 mg (44.2 µmol, 44%).

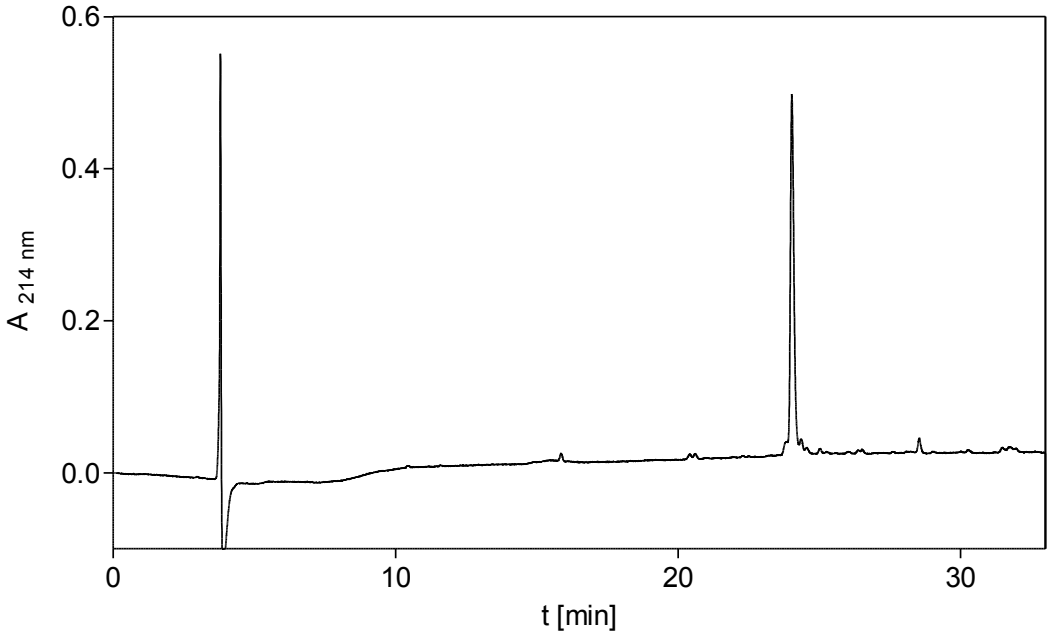


Figure S7. Analytical HPLC chromatogram of crude Ac-AR(PO_3Tc_2)TKQTA (E1, method A)

Ac-AR(PO_3H_2)TKQTA-OH (**3,** Scheme 1)

Ac-AR(PO_3Tc_2)TKQTA-OH (37.0 mg, 41.2 µmol) was dissolved in ammonium carbonate buffer (5.92 mL, 100 mM, pH = 9.2). Ethanol (11.8 mL) and palladium on charcoal (55.7 mg, 10 wt% Pd) were added. Argon was bubbled through the suspension for 5 min. The reaction mixture was vigorously stirred under a hydrogen atmosphere for 1 h. The catalyst was removed by centrifugation and washed with 50% aqueous acetonitrile (5 x 3 mL). The combined supernatants were filtered and sodium phosphate buffer (1 mL, 100 mM, pH = 8) was added. The solvent was removed *in vacuo*. The residue was dissolved in a minimal volume of water and purified by HPLC using eluent system E3.

LR-MS (ESI +): *m/z* = 897.47 $[M+H]^+$, average mass calc.: 897.90 $[C_{33}H_{62}N_{12}O_{15}P_1]^+$.
Yield (based on HPLC chromatogram): 73%.
Yield (corrected for sodium phosphate content): 17.0 mg (18.9 µmol, 46%).

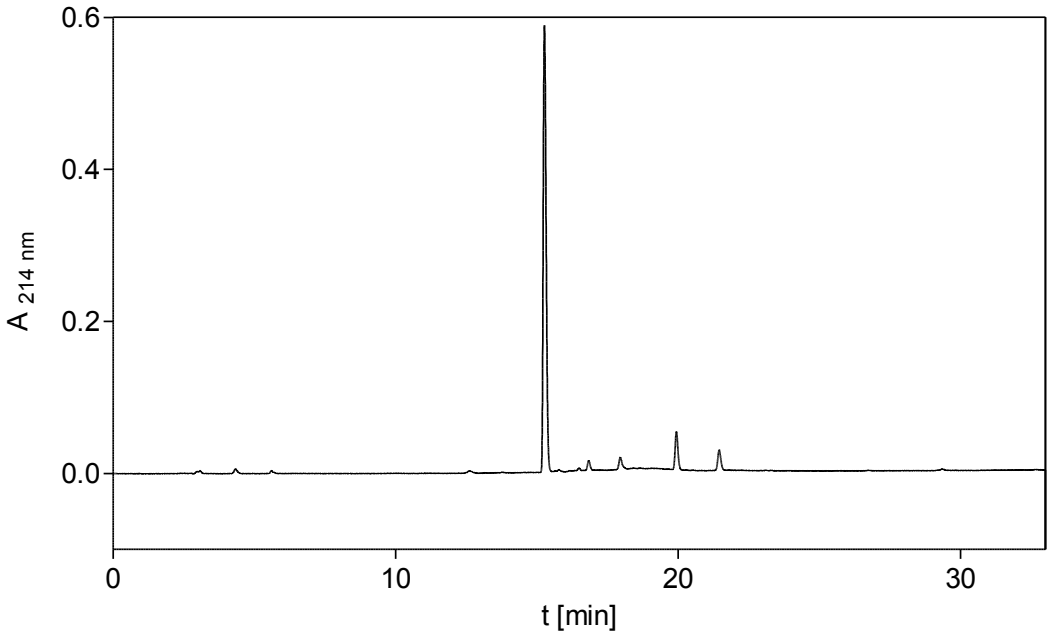


Figure S8. Analytical HPLC chromatogram of crude Ac-AR(PO_3Tc_2)TKQTA-OH after hydrogenolytic deprotection (E3, method D)

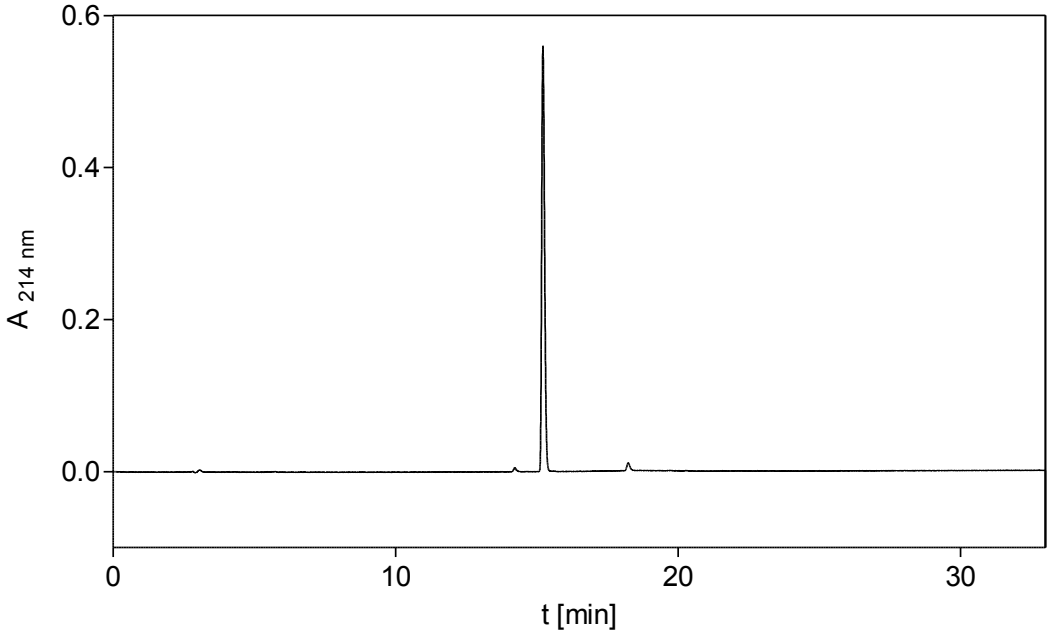


Figure S9. Analytical HPLC chromatogram of Ac-AR(PO_3H_2)TKQTA-OH after HPLC purification (E3, method D)

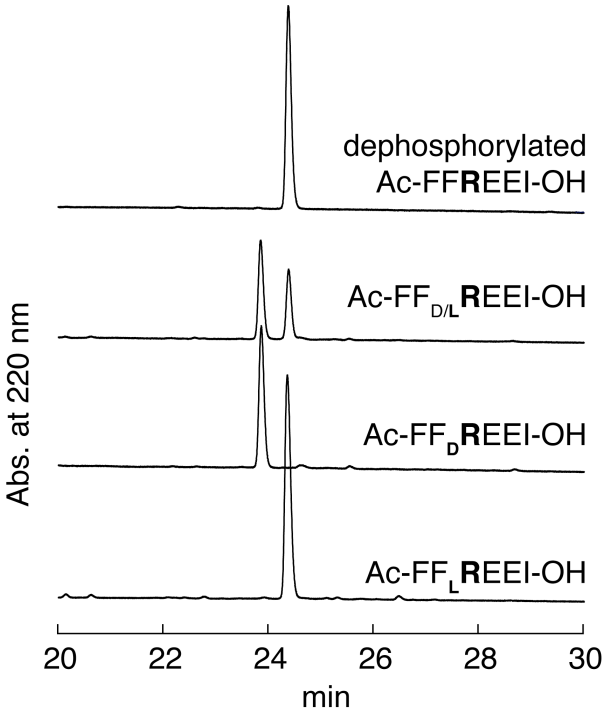


Figure S10. The stereochemical homogeneity of compound **1** and peptide **4a** was analyzed indirectly by comparison of dephosphorylated **4a** with authentic peptides containing either L- or D-arginine in place of phosphoarginine (**4c** and **5**). **4a** was dephosphorylated by incubation in 0.05 M HCl for 1.5 h at 40°C. The dephosphorylated **4a** co-elutes with the L-arginine containing standard with less than 1% signal co-eluting with the D-arginine containing standard (**Figure S10**). We therefore conclude that the preparation of **1** was at least 99% enantiopure and that peptide synthesis did not result in significant epimerization.

Cloning and production of SH2 protein (SH2)

The SH2 gene was assembled from synthetic oligonucleotides (Table S2). The amplified DNA-fragment was Nco1/Xho1 digested and ligated into a pET28a vector. The corresponding protein was produced in Bl21 cells upon IPTG induction at 37°C and purified on NTA-agarose. The purified protein was dialyzed into binding buffer (50 mM TRIS, 100 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH = 7.5). 1 L of culture yielded 20 mg purified SH2 (ε_{280} = 14440 $M^{-1}\,cm^{-1}$) with *m/z* = 12889 (average mass calc.: 12888) as inferred by ESI MS.

Table S2. Synthetic SH2 template (Nco1 and Xho1 sites in bold)

SH21	TATTAA**CCATGG**GCTGGTATTTTGGCAAAATTACCCGCCGCGAAAG
SH22	GGTGCCGCGCGGGTTTTCCGGGTTCAGCAGCAGGCGTTCGCTTTCGCGGCGGGTAA
SH23	ACCCGCGCGGCACCTTTCTGGTGCGCGAAAGCGAAACCACCAAAGGCGCGTATT
SH24	TTCAGGCCTTTCGCGTTATCAAAATCGCTCACGCTCAGGCAATACGCGCCTTTGGTG
SH25	AACGCGAAAGGCCTGAACGTGAAACATTATAAAATTCGCAAACTGGATAGCGGCGGC
SH26	GCTGCAGGCTGCTAAACTGGGTGCGGCTGGTAATATAAAAGCCGCCGCTATCCAG
SH27	GTTTAGCAGCCTGCAGCAGCTGGTGGCGTATTATAGCAAACATGCGGATGGCCTGTG
SH28	GTGACCTACGCTAGTCGGGCACACGTTGGTCAGGCGATGGCACAGGCCATCCGCAT
SH29	CGACTAGCGTAGGTCACCACCACCATCACCATCGGCTATAGGTAGCTAGCTCGAGTATTAA
SH20	TTAATA**CTCGAG**CTAGCTA

Fluorescence polarization measurements

Assays were set up in black, flat bottom 384-well plates (Greiner Bio-One, Frickenhausen, Germany). Fluorescence polarisation measurements were carried out on a Safire 2 microplate reader (Tecan, Männedorf, Switzerland). Excitation was performed at 470 nm while emission was monitored at 525 nm with a bandwidth of 5 nm. Gain and Z-position were optimized automatically. For each well 50 measurements were averaged.

Direct titration

To determine binding affinities of fluorescently labeled peptides (**6a** and **6b**, Scheme 1) to SH2 a direct titration of the peptides with SH2 was performed. Serial 1:1 dilution series with each 24 dilutions of 97.5 µM SH2 in binding buffer (50 mM TRIS, 100 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH = 7.5) were prepared in 3 rows of a 384-well plate so that each well contained a volume of 45 µL. To each well 5 µL of a 100 nM or 1µM solution of the 5-Carboxyfluoresceine labeled peptide in binding buffer were added. As a control **6a** and **6b** were titrated to binding buffer containing no SH2. The 384-well plate was sealed with adhesive PCR film (Eppendorf, Hamburg, Germany). Fluorescence polarization was measured after 2 h incubation in the dark at 4°C. Equation (1) was fitted to the experimental data:[5]

$$P = P_{\min} + \left(\left([\mathrm{L}]_t + [\mathrm{R}]_t + K_d \right) - \sqrt{\left([\mathrm{L}]_t + [\mathrm{R}]_t + K_d \right)^2 - 4[\mathrm{L}]_t[\mathrm{R}]_t} \right) \frac{P_{\max} - P_{\min}}{2[\mathrm{R}]_t} \qquad (1)$$

$P \equiv$ fluorescence polarization
$P_{\min} \equiv$ minimal fluorescence polarization
$P_{\max} \equiv$ maximal fluorescence polarization
$[\mathrm{L}]_t \equiv$ total peptide concentration
$[\mathrm{R}]_t \equiv$ total SH2 concentration
$K_d \equiv$ dissociation constant

Ligand displacement titration

To measure binding affinities of non-fluorescent compounds (**4a-c** and **7**, Scheme 1) to SH2 we established a fluorescence polarization based displacement assay. The complex between SH2 and a fluorescent sensor (**6a**, Scheme 1) was titrated with unlabeled compounds which causes displacement of **6a** and therefore a decrease in fluorescence polarization.[6,7] Using equation (2) we adjusted the SH2 concentration so 92% of **6a** peptide binds to SH2 in absence of a competing ligand.

$$K_d = \frac{(1-a)\cdot[L]_t[R]}{a\cdot[L]_t} \text{ with } [R]=[R]_t - a\cdot[L]_t \Leftrightarrow$$

$$[R]_t = \frac{a}{1-a}\cdot K_d + a\cdot[L]_t \quad (2)$$

$K_d \equiv$ dissociation constant (SH2:sensor)
$a \equiv$ fraction of the sensor in complex with SH2
$[R] \equiv$ concentration of unbound SH2
$[L]_t \equiv$ total sensor concentration
$[R]_t \equiv$ total SH2 concentration

Experimental data was fitted to an inhibitory dose-response curve. The resulting IC_{50} values were then converted to K_d using equation (3).[5,8]

$$K_d = \frac{0.5\cdot IC_{50}[RL]K_{d,\,sensor}}{[L]_t[R]_t + 0.5\cdot[RL]\left(-[R]_t - [L]_t + 0.5\cdot[RL] - K_{d,\,sensor}\right)} \quad (3)$$

$K_{d,\,sensor} \equiv$ dissociation constant (5-FAM-Y(PO_3H_2)EEI-OH:SH2)
$[RL] \equiv$ complex concentration (5-FAM-Y(PO_3H_2)EEI-OH:SH2)
$[R]_t \equiv$ total SH2 concentration
$[L]_t \equiv$ total 5-FAM-Y(PO_3H_2)EEI-OH concentration

The experimental setup was as follows. Serial 1:1 dilution series with each 24 dilutions of unlabeled compound in binding buffer (50 mM TRIS, 100 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH = 7.5) containing 600 nM SH2 and 10 nM **6a** were prepared in 3 rows of a 384-well plate so that each well contained a final volume of 40 µL. As control the SH2 complex with the fluorescent sensor was titrated with binding buffer. The 384-well plate was sealed with adhesive PCR film (Eppendorf, Hamburg, Germany). Fluorescence polarization was measured after 2 h incubation in the dark at 4°C.

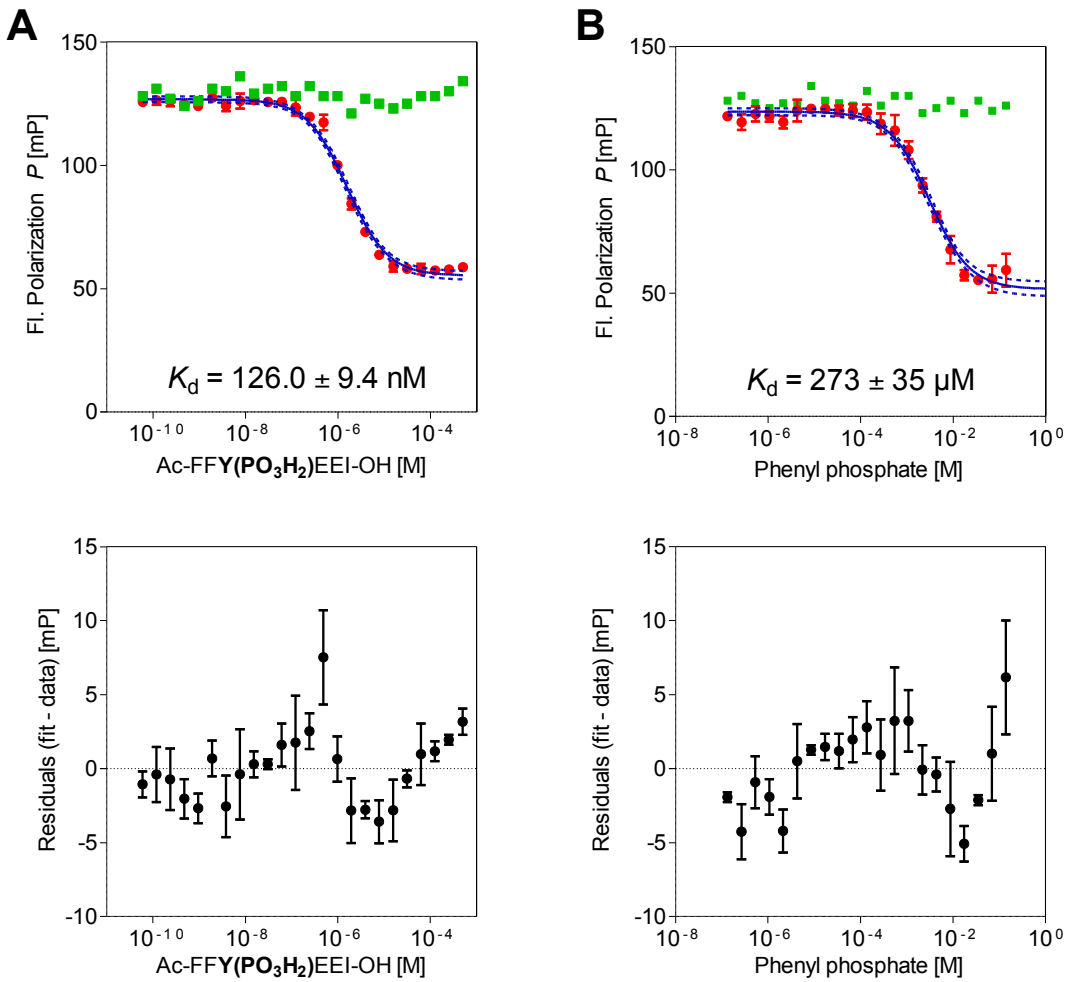


Figure S11. 5-FAM-Y(PO_3H_2)EEI-OH (10 nM, **6a,** Scheme 1) in complex with SH2 (600 nM) was titrated with (A) Ac-FFY(PO_3H_2)EEI-OH (**4b**) or (B) phenyl phosphate (**7**). Data points (●) are averages of 3 replicates with error bars indicating standard deviations. Data points were fitted to eq. (3). Fits (—) are shown with their 95% confidence interval (---). Controls are shown as green squares (■).

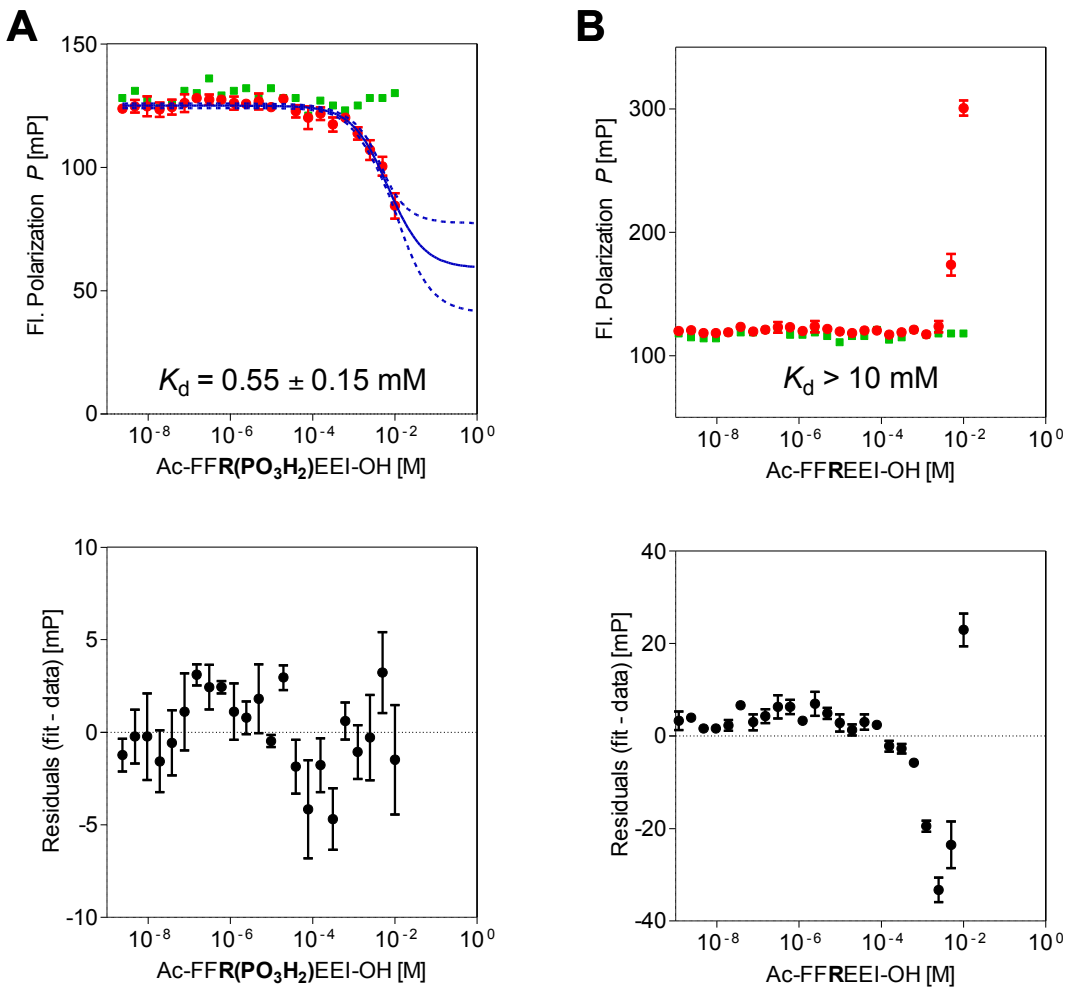


Figure S12. 5-FAM-Y(PO_3H_2)EEI-OH (10 nM, **6a,** Scheme 1) in complex with SH2 (600 nM) was titrated with (A) Ac-FFR(PO_3H_2)EEI-OH (**4a**), (B) Ac-FFREEI-OH (**4c**). Data points (●) are averages of 3 replicates with error bars indicating standard deviations. For (A) data points were fitted to eq. (3). The fit (—) is shown with its 95% confidence interval (---). Controls are shown as green squares (■).

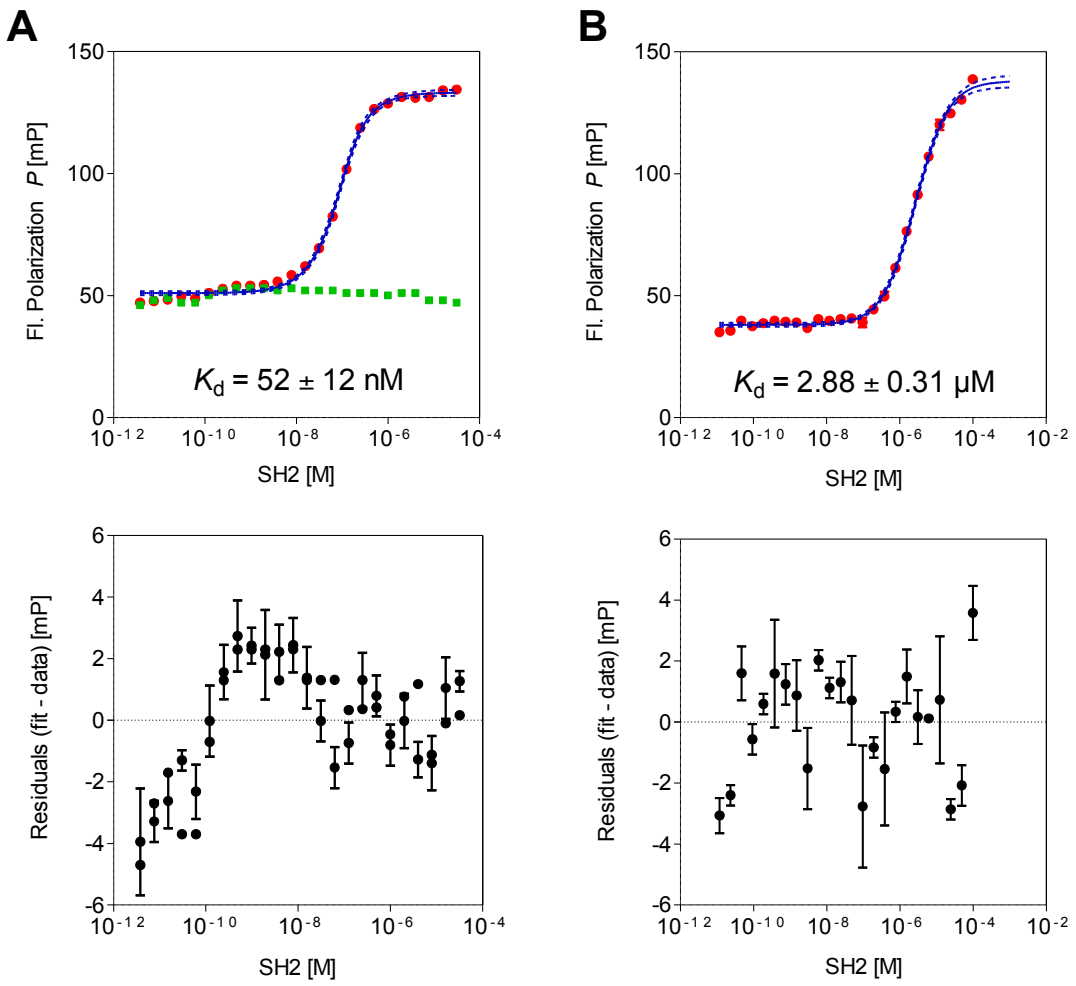


Figure S13. Direct titration of (A) 100 nM 5-FAM-Y(PO_3H_2)EEI-OH (**6a,** Scheme 1) and (B) 10 nM 5-FAM-Y(SO_3H)EEI-OH (**6b,** Scheme 1) with SH2 (●) or buffer as control (■). Data points (●) are averages of 3 replicates with error bars indicating standard deviations. Data points were fitted to eq. (1). Fits (—) are shown with their 95% confidence interval (---).

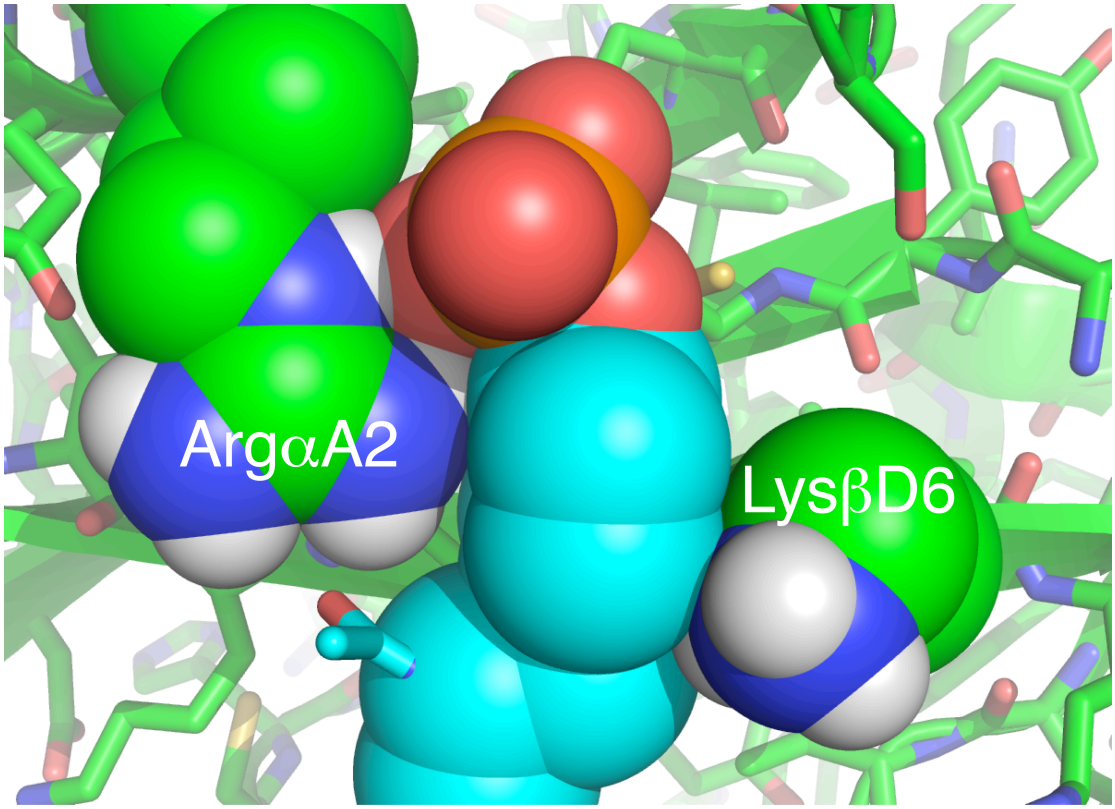


Figure S14. The Src SH2 domain in complex with a pTyr containing peptide. ArgαA2 and LysβD6 shown in space filling representation.

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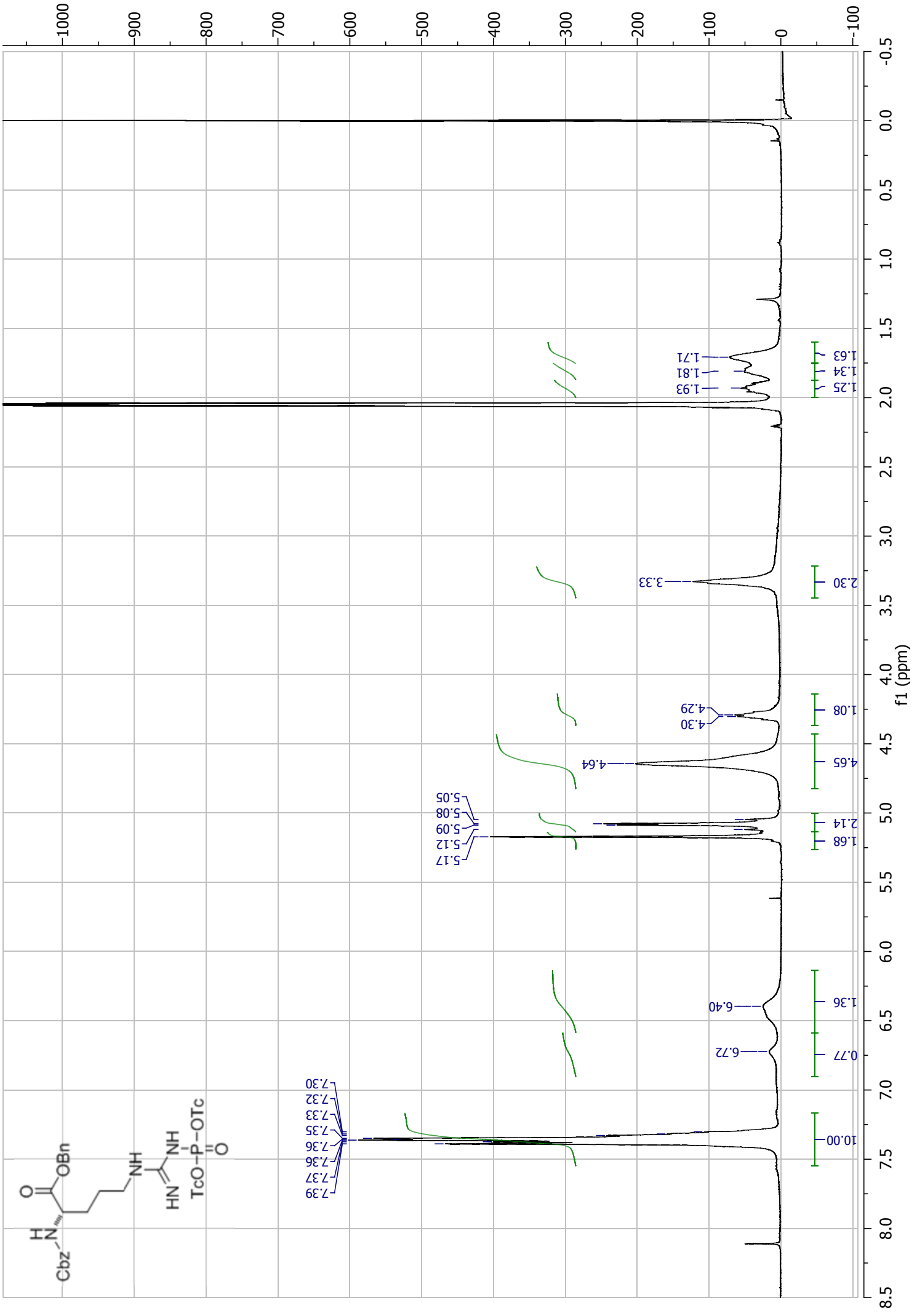
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7.35
7.33
7.32
7.30
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6.40
5.17
5.12
5.09
5.08
5.05
4.64
4.30
4.29
3.33
1.93
1.81
1.71
10.00
0.77
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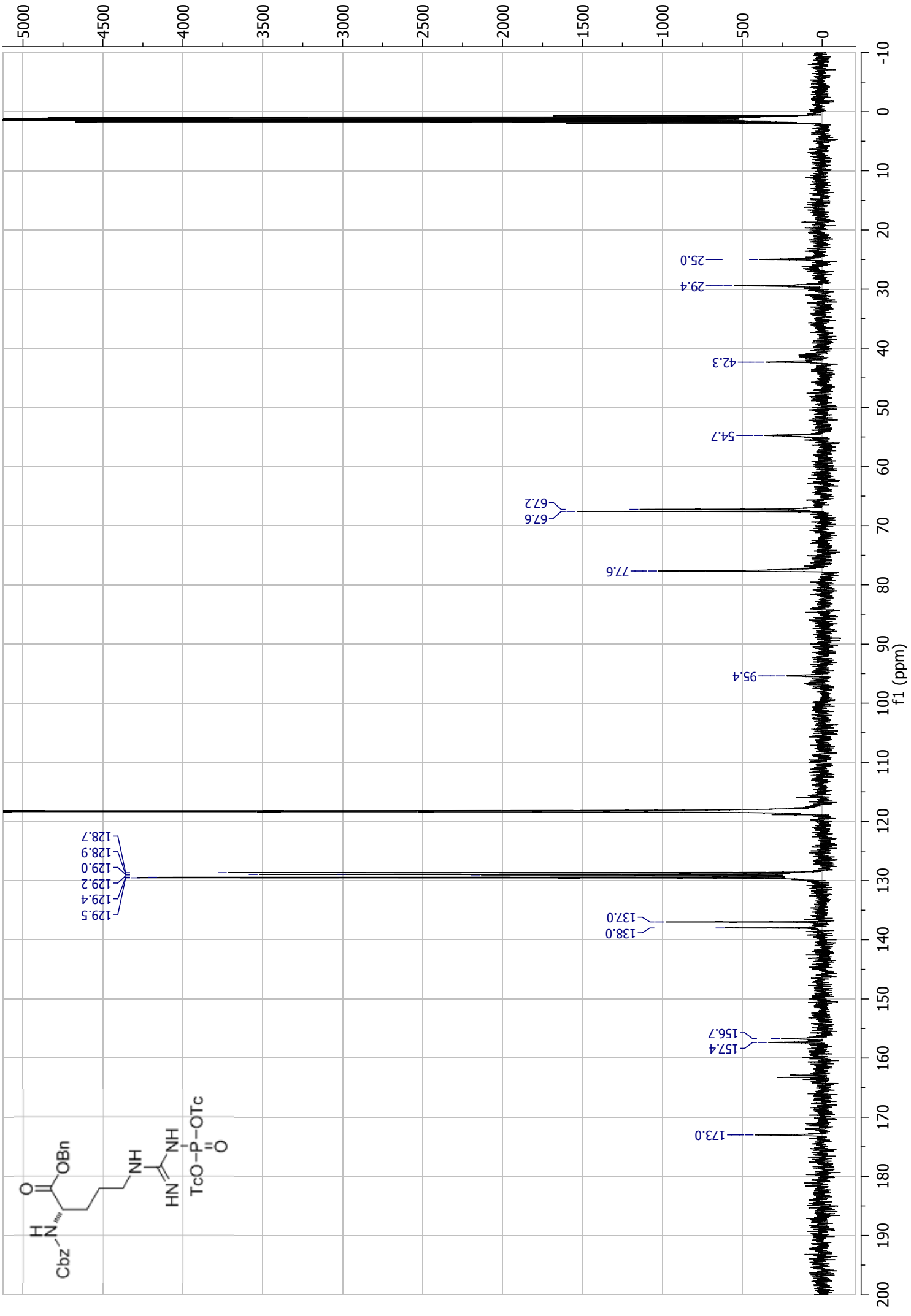
2.14
1.68
4.65
1.08
2.30
1.63
1.34
1.25
f1 (ppm)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67.2
77.6
95.4
129.5
129.4
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128.7
137.0
138.0
157.4
156.7
173.0
f1 (ppm)