

Supporting Information

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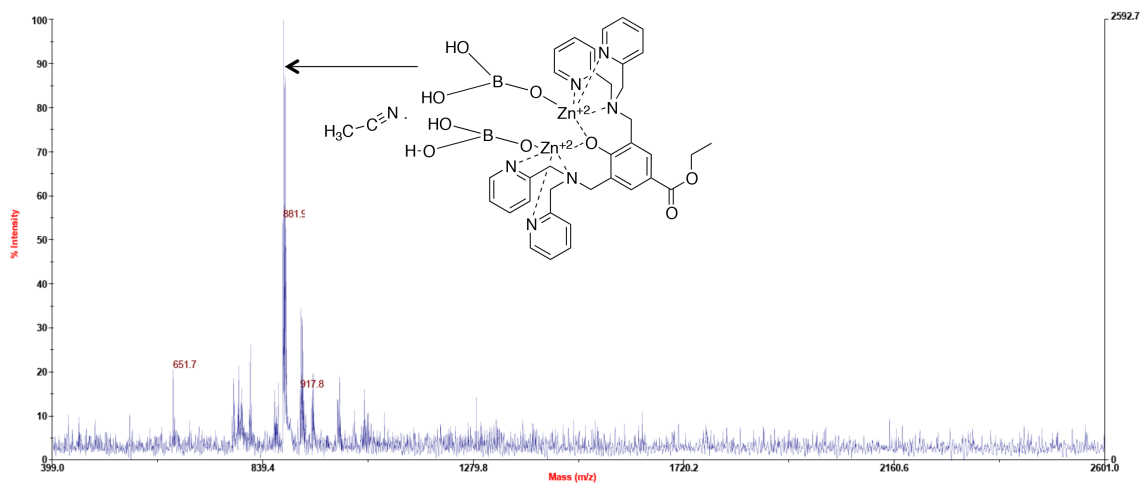
**A Chemical Epitope-Targeting Strategy for Protein Capture Agents:
The Serine 474 Epitope of the Kinase Akt2****

*Arundhati Nag, Samir Das, Mary Beth Yu, Kaycie M. Deyle, Steven W. Millward, and
James R. Heath**

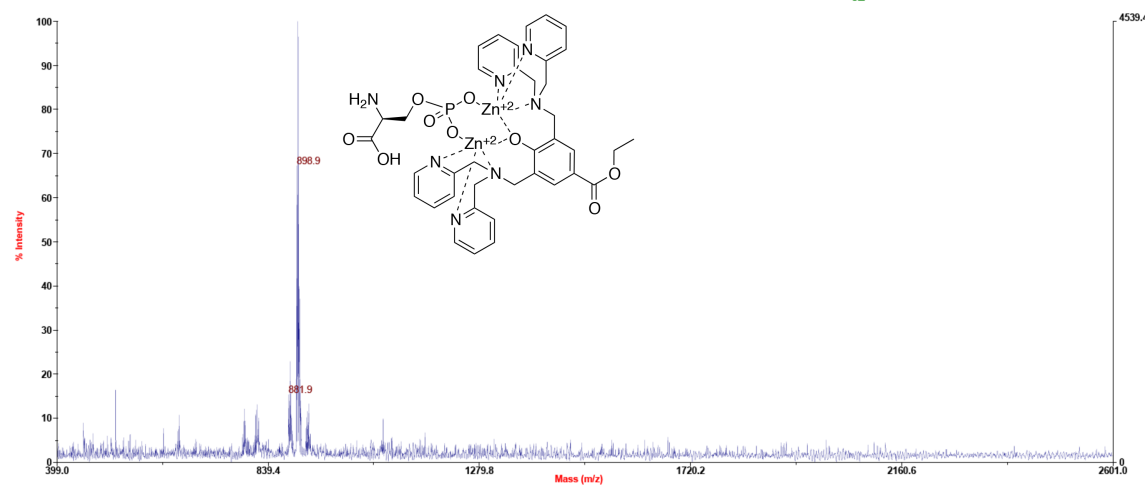
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SUPPLEMENTARY INFORMATION:

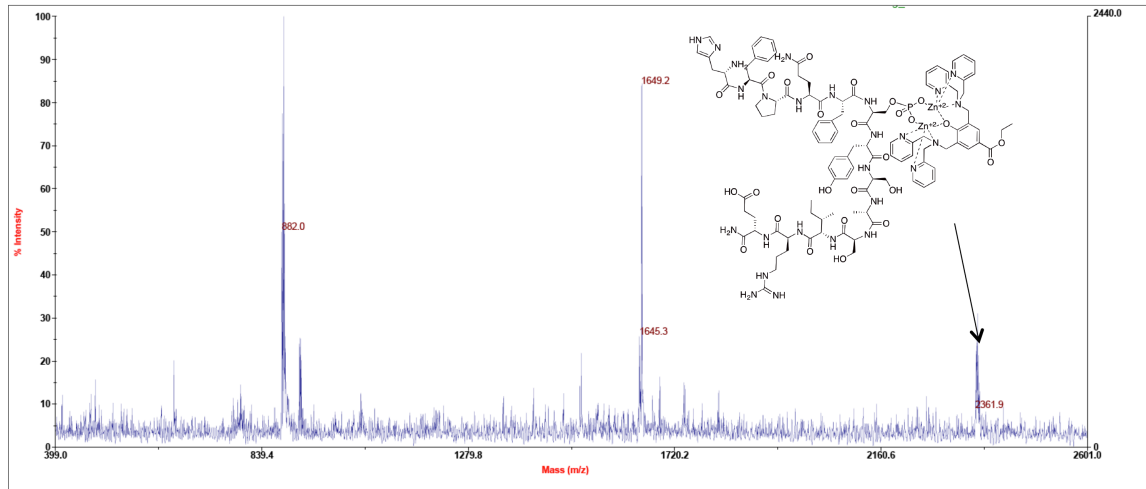
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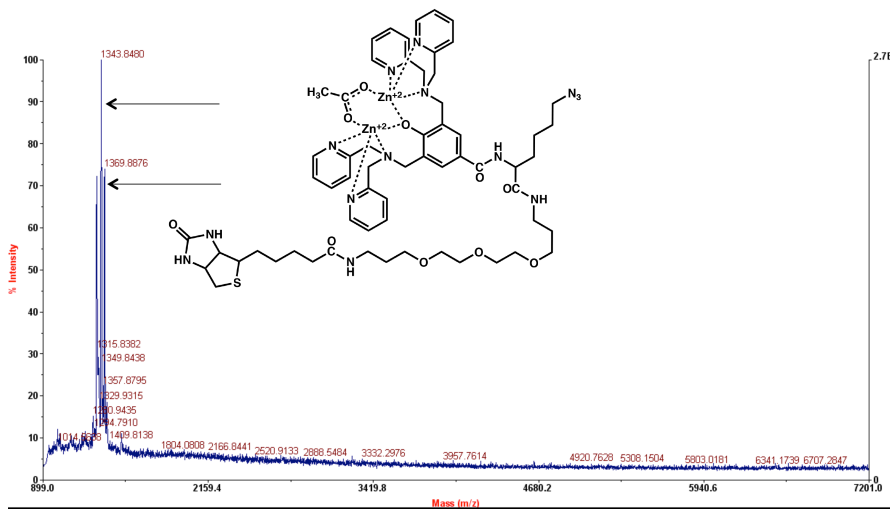
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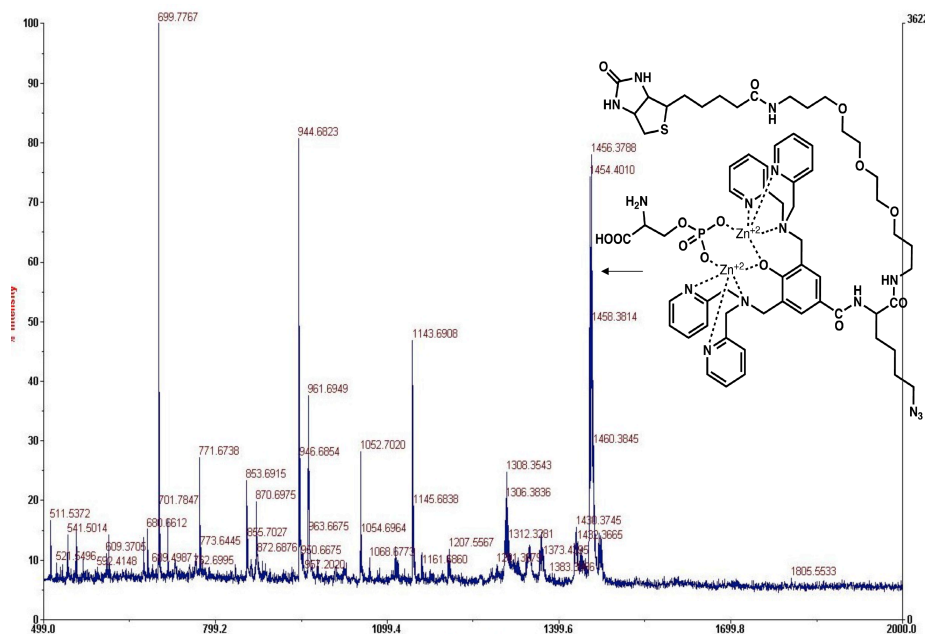
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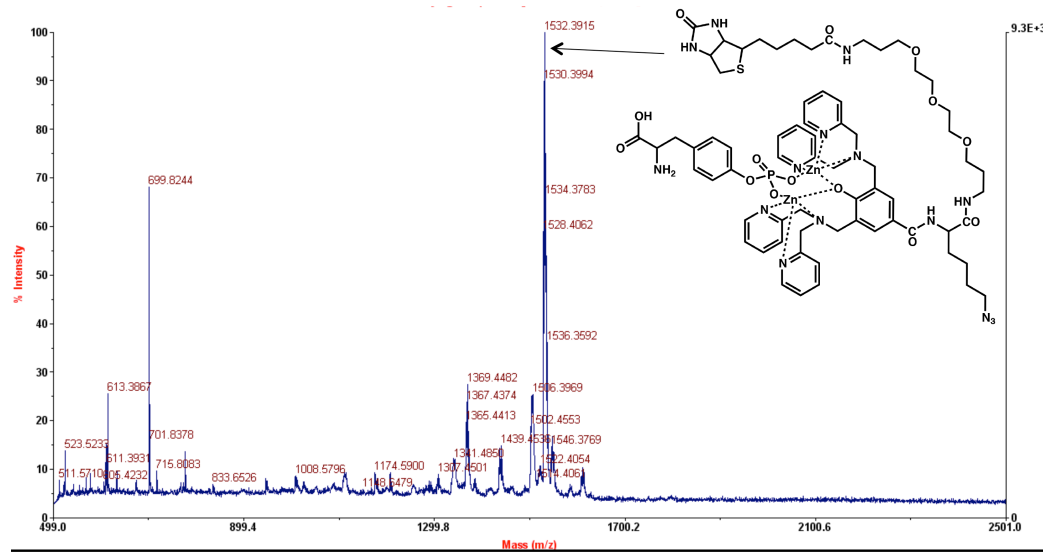
D.



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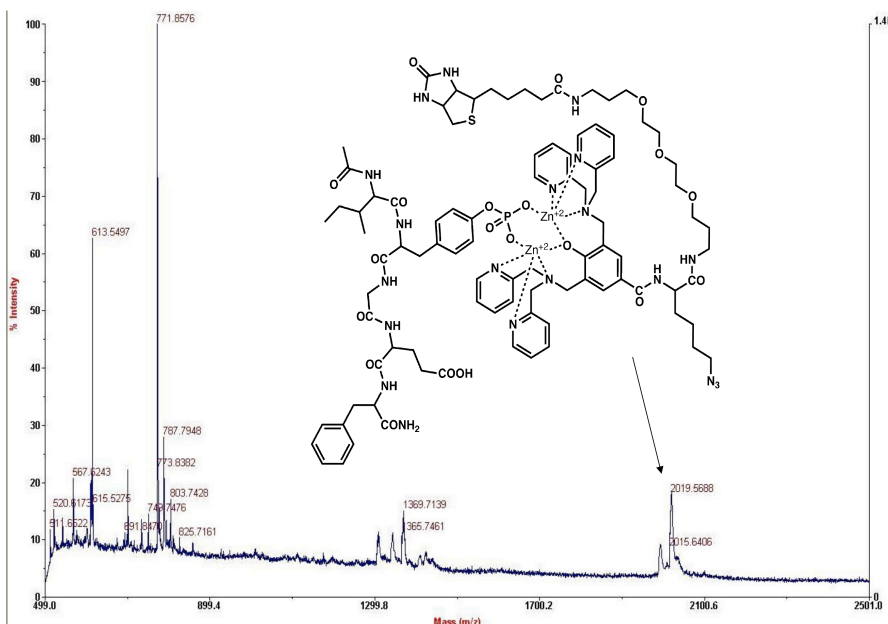


Figure S1: Structure and Maldi TOF spectra of dinuclear zinc complexes with phosphorylated amino acids and phosphorylated peptides. **A.** Mass spectrum and structure of $Zn_2(EtL_1) \cdot 2H_2BO_3 \cdot CH_3CN$ (calculated mass 881.15; observed mass = 881.9) **B.** Mass spectrum and structure of $Zn_2(EtL_1)$ - phosphoserine (calculated mass = 901.5; observed mass = 898.9) **C.** Mass spectrum and structure of Zn_2EtL_1 complexed to 13 amino acid long C terminal phosphorylated Akt peptide, **phospho-Akt2-13mer** (calculated mass = 2364.1; observed mass = 2361.9). This 13-mer contains the hydrophobic motif of the C-terminus, as well as pSer474, and so is the targeted epitope. **D.** Mass spectrum and structure of Zn_2L_1 -Az4-PEG₂-Biotin (calculated mass $M \cdot 2H_2O$ = 1370.2, $(M-N_2) \cdot 2H_2O$ = 1342.2; observed mass $M \cdot 2H_2O$ = 1369, $(M-N_2) \cdot 2H_2O$ = 1343.8) (PEG = polyethylene glycol). **E.** Mass spectrum and structure of Zn_2L_1 -Az4-PEG₂-Biotin - phosphoserine (calculated mass = 1457.22; observed mass = 1454.4). **F.** Mass spectrum and structure of Zn_2L_1 -Az4-PEG₂-Biotin - phosphotyrosine (calculated mass = 1532.31; observed mass = 1532.39). **G.** Mass spectrum and structure of Zn_2L_1 -Az4-PEG₂-Biotin complexed to the peptide substrate for the pSrc protein Ac-I-pY-GEF (calculated mass = 2019.86; observed mass = 2019.57).

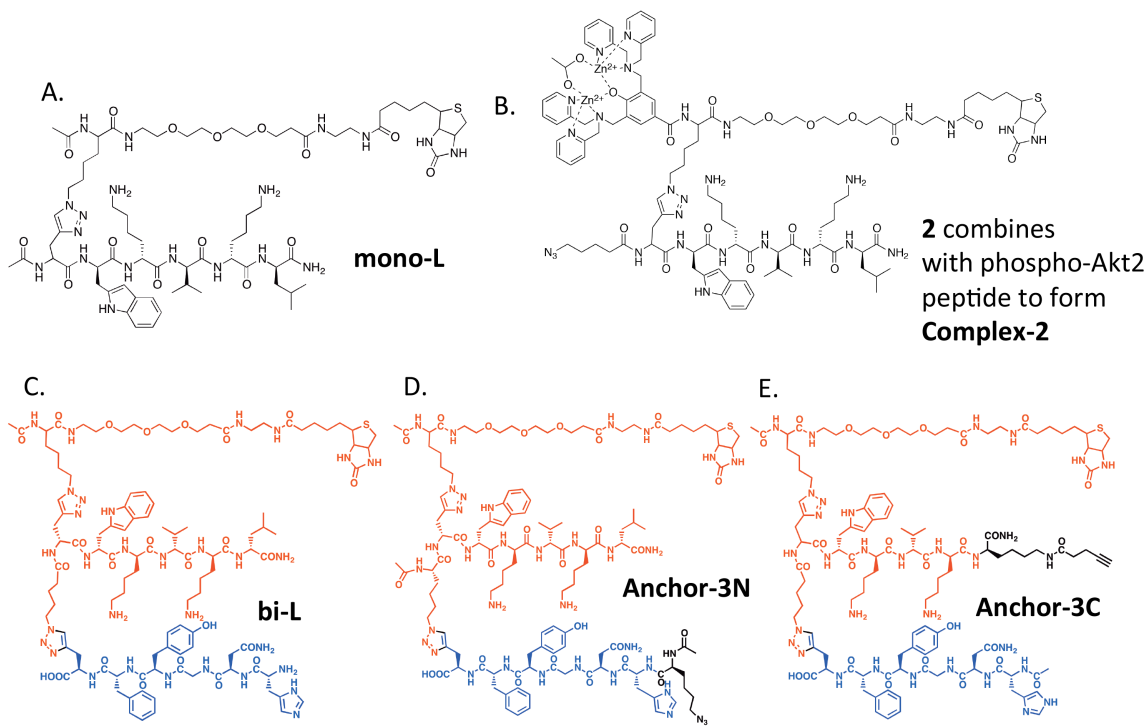


Figure S2: Molecular structure of various PCC agents. A. Structure of the monoligand peptide **mono-L** developed against the target epitope. B. 1^o ligand is linked to the Zn chelator using the Cu(I) catalyzed click reaction. The synthesized molecule **2** binds to the Akt2 fragment containing pS474 to form **Complex-2**, which is used as the target in the biligand screen. C. Molecular structure of the biligand **bi-L** isolated in the screen against the target peptide. 1^o ligand (in red) is linked, through Cu catalyzed Alkyne Azide Cycloaddition (CuAAC) reaction, to the 2^o ligand (in blue). D. An azide is appended at the N terminus of the 2^o peptide to synthesize peptide **anchor-3N**, which is used in screening for 3^o ligands that click to the N-terminus of the biligand. E. An alkyne is appended to the C terminus of the 1^o peptide to synthesize peptide **anchor-3C**, which is used in screening for 3^o ligands that click to the C-terminus of the biligand.

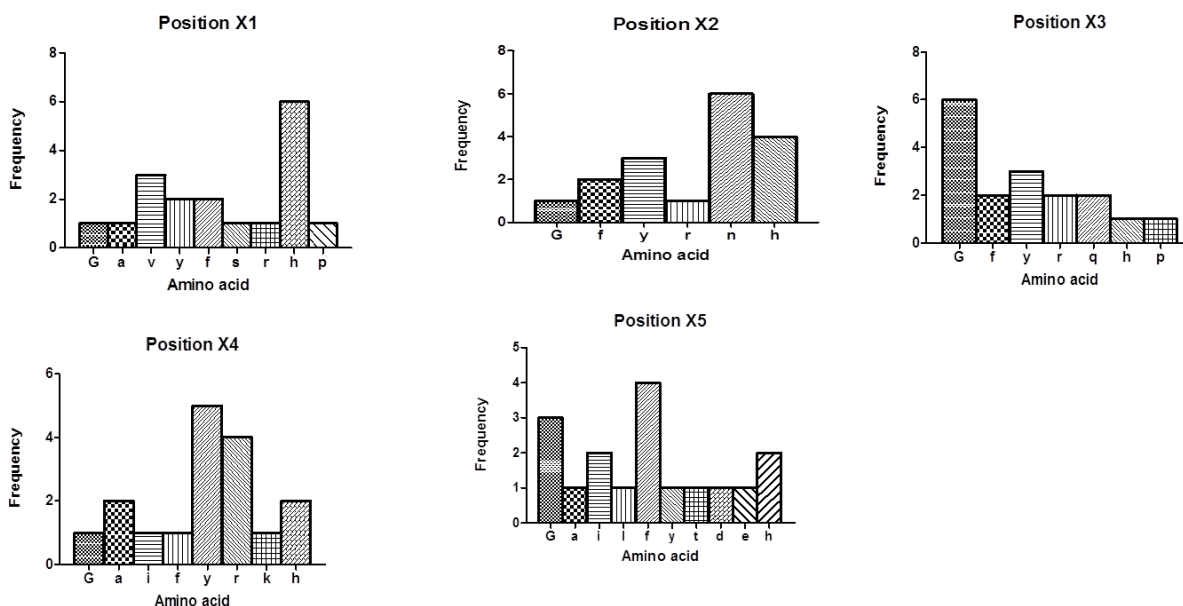


Figure S3: Histogram of biligand hit sequences from the in situ click screen. Amino acid frequencies in the hit sequences X1X2X3X4X5-D-Pra from X1 to X5 are plotted as histograms. Note that on the basis of the frequencies, hnGyf-D-Pra is the consensus sequence.

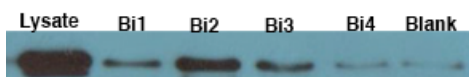


Figure S4: Biligand selection on the basis of the efficiency to immunoprecipitate Akt from OVCAR3 cells. Biligands with hnGyG - D-Pra (**Bi1**), hnGyf - D-Pra (**Bi2**), hnGre -D-Pra (**Bi3**), hnGai - D-Pra (**Bi4**) as the 2^o peptide arm are used to immunoprecipitate Akt2 from OVCAR3 cells following the described procedure. The best candidate **Bi2** is chosen as the biligand **bi-L** and used in further stages of ligand development. That candidate contained the 2^o peptide sequence (hnGyf) which, remarkably, is the sequence that best reflects the statistic illustrated in the positional histograms of Fig S3.

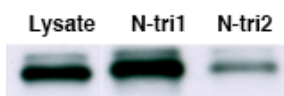


Figure S5: N-terminal triligand selection on the basis of the efficiency to immunoprecipitate Akt from OVCAR3 cells. N-terminal triligands synthesized with D-Pra-yyrfG (**N-tri1**) and D-Pra-ssGry (**N-tri2**) immunoprecipitate Akt2 from OVCAR3 cells. **N-tri1** is chosen as the final N terminal triligand **N-tL**.

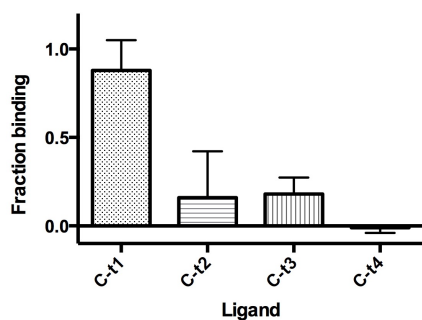
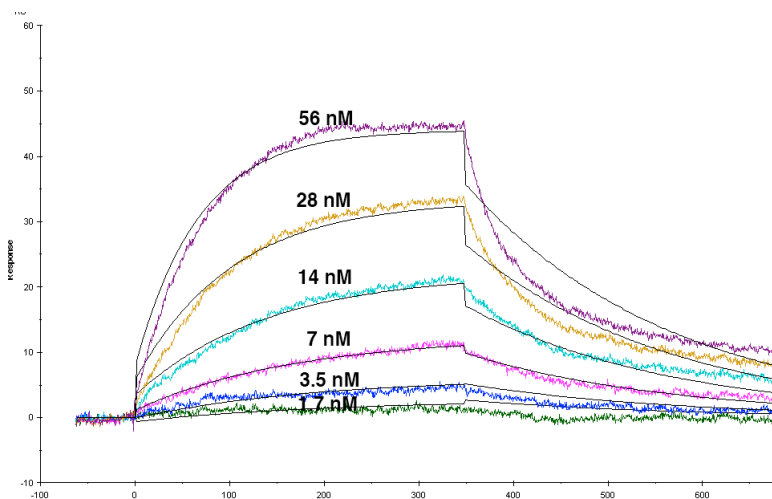


Figure S6: N-terminal triligand selection on the basis of epitope binding characteristics. Triligand candidates from the C terminal screen are tested for binding to the target epitope. The ligand **C-t1**, with L-Az4-hdGGf as the third peptide arm, shows good binding and is chosen as the C-terminal triligand **C-tL**.



k_a (1/Ms)	k_d (1/s)	K_D (M)
1.8e5	0.0046	2.5e-8

Figure S7: Sensograms from Surface Plasmon Resonance (SPR) experiment, immobilizing **N-tL** on SA chip and using Akt2 protein as the analyte. A. The data were fit to a simple 1:1 binding model using global analysis in Biacore T100 Evaluation software. The equilibrium dissociation constant K_D is 25 nM, which is in close agreement with the ELISA EC_{50} value of 19 nM.

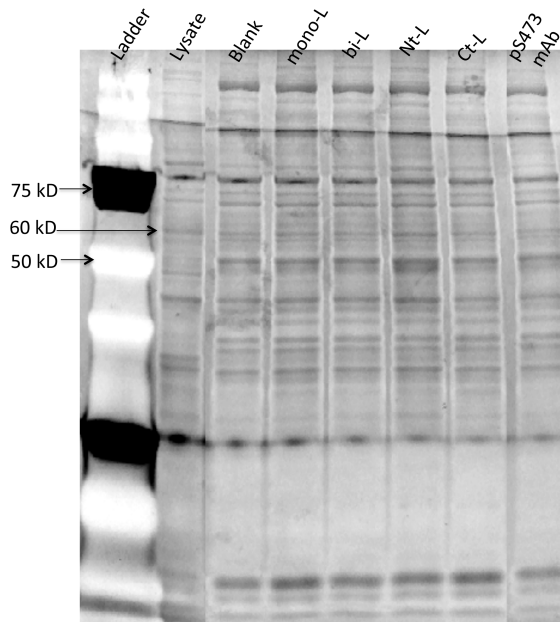


Figure S8: Coomassie staining of gel from immunoprecipitation by different ligands/ antibody. The biotinylated ligands (blank, **mono-L**, **bi-L**, **N-tL**, **C-tL**) or biotinylated antibody (pS473 mAb) are immobilized on streptavidin-agarose beads. The cell lysate (lysate) is directly diluted in the sample buffer and ran as a control. The band at 60 kD is for the Akt protein. All the eluted samples (blank to pS473 mAb) have similar non-specific protein pulldown.

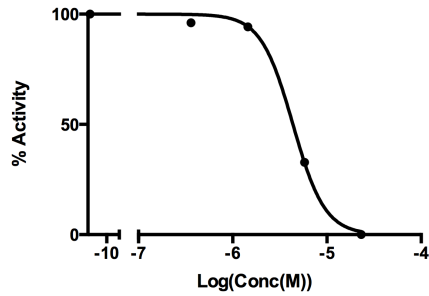
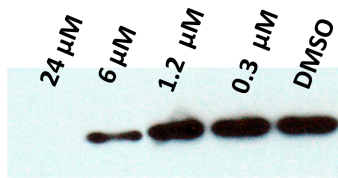


Figure S9: Inhibition of kinase activity of the Akt2 protein when treated with C-tL. The in vitro phosphorylation of GSK3 α/β substrate by active Akt2 is inhibited when treated with increasing concentrations of the C-tL ligand. The solutions are ran on a gel and visualized by treatment with pGSK3 α/β Ser 21/9 antibody. The western blot image was scanned and the signal corresponding to different concentrations of C-tL was quantitated using ImageJ. The density was normalized to the signal for no inhibitor (DMSO control) to generate an activity percentage value curve in GraphPad Prism 6.0. An EC₅₀ value of 4 μM is obtained from the curve using non-linear regression.

Table S1: OBOC peptide libraries used in screens.

Formula	Components	Unique sequences	Fraction Screened
Library A: D-Pra-XXXXX-10%M-TG	X = 18 D amino acids except D-Met and D-Cys	1,889,568	40% for 1 ^o ligand screen 80% for 3 ^o ligand screen for N-tL
Library B: XXXXX -D-Pra-10%M-TG	X = 18 D amino acids except D-Met and D-Cys	1,889,568	40% for 2 ^o ligands
Library C: L-Az4- XXXXX-10%M-TG	X = 18 D amino acids except D-Met and D-Cys	1,889,568	80% for 3 ^o ligand screen for C-tL

Table S2: Hit sequences from biligand (**bi-L**) screen with 25 nM target peptide. Consensus motifs are highlighted.

X1	X2	X3	X4	X5	D-Pra
h	n	G	i	i	D-Pra
h	n	G	r	e	D-Pra
h	r	y	y	G	D-Pra
v	n	r	r	f	D-Pra
h	n	G	G	d	D-Pra
a	y	p	h	f	D-Pra
G	f	r	r	f	D-Pra
r	G	f	f	l	D-Pra
h	n	G	y	G	D-Pra

Table S3: Hit sequences from biligand screen with 10 nM target peptide. Consensus motifs are highlighted.

X1	X2	X3	X4	X5	D-Pra
v	y	y	r	h	D-Pra
h	n	G	a	i	D-Pra
f	h	y	y	y	D-Pra
f	y	h	k	h	D-Pra
p	f	q	h	f	D-Pra
s	h	f	y	t	D-Pra
v	h	G	a	a	D-Pra
y	h	q	y	G	D-Pra

Table S4: Hit sequences from N terminal triligand (N-tL) screen.

D-Pra	X1	X2	X3	X4	X5
D-Pra	k/l*	f	q	f	r
D-Pra	r	d/n*	r	f	r
D-Pra	y	v	y	r	f
D-Pra	s	s	G	r	y
D-Pra	y	y	r	f	g
D-Pra	s	f	r	r	f
D-Pra	s	v	r	f	r
D-Pra	i	k/l*	r	r	a
D-Pra	r	q/t*	k/l*	w	r
D-Pra	r	q/t*	s	r	r
D-Pra	r	r	i	y	y
D-Pra	r	f	G	r	q/t*

*Alternative amino acid signals from poor resolution of the amino acid standards of the Edman sequencing machine.

Table S5: Elimination of peptide binders to antibody from N terminal triligand (**N-tL**) screen

D-Pra	X1	X2	X3	X4	X5	Color	Probability of being right sequence
D-Pra	k	f	q	f	r	light	0.25
D-Pra	l	f	q	f	r	light	0.25
D-Pra	k	f	t	f	r	light	0.25
D-Pra	l	f	t	f	r	light	0.25
D-Pra	r	d	r	f	r	No color	0.5
D-Pra	r	n	r	f	r	medium	0.5
D-Pra	y	v	y	r	f	light	1
D-Pra	s	s	G	r	y	No color	1
D-Pra	r	r	i	y	y	dark	1
D-Pra	y	y	r	f	G	No color	1
D-Pra	s	f	r	r	f	light	1

Table S6: Hit sequences from C terminal triligand (C-tL) screen. Consensus motifs are highlighted.

L-Az4	X1	X2	X3	X4	X5
L-Az4	h	d	G	s	q
L-Az4	h	d	G	w	w
L-AZ4	h	d	G	i	v
L-Az4	h	d	G	d	w
L-Az4	h	d	G	G	- *
L-Az4	h	d	G	d	r
L-Az4	h	d	G	G	f
L-Az4	h	d	G	G	e
L-Az4	h	d	G	s	f
L-Az4	h	d	G	q	k
L-Az4	h	d	G	s	a
L-Az4	h	d	G	k	f
L-Az4	r	l	e	a	v

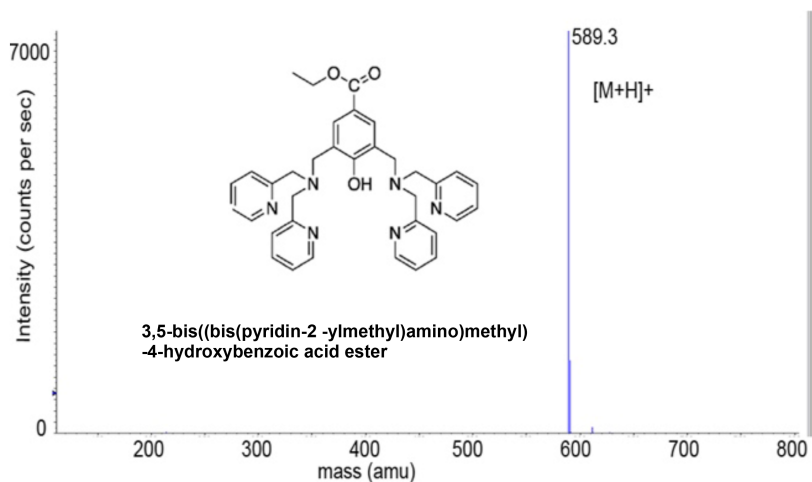
* no signal

Supplementary Information Materials and Methods:

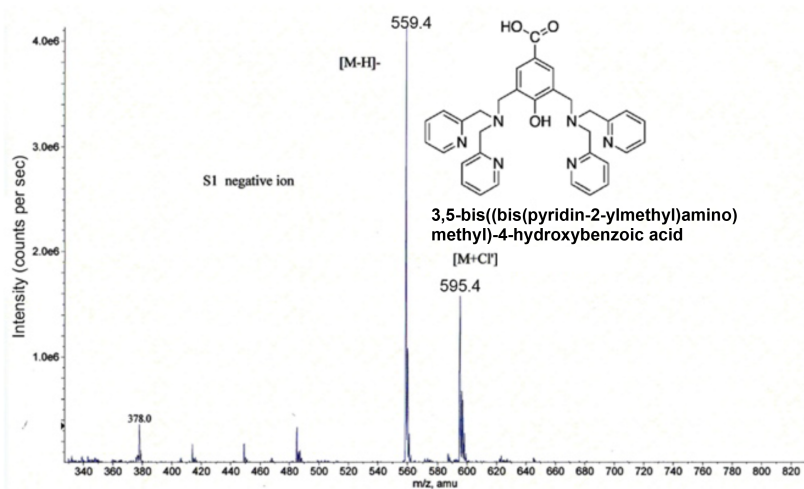
Materials: Fmoc amino acids were purchased from Anaspec and AAPPTec and used as received. TentaGel S-NH₂ resin (diameter 90 μm, capacity 0.28 mmol/g) was obtained from Anaspec and utilized for OBOC library construction. Biotin NovaTag™ resin, Biotin – PEG NovaTag™ resin, Fmoc – NH - (PEG)₂ - OH (13 atoms) were obtained from EMD Chemicals, Inc. and used for synthesis of biotinylated peptides. Amide Sieber resin (capacity 0.3-0.6 mmol/g) purchased from Anaspec was used for synthesis of protected peptides. NMP (1-methyl-2-pyrrolidinone), HATU ((2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate) and DIEA (N,N'-diisopropylethylamine) used in peptide synthesis were bought from EMD Chemicals, Inc., ChemPep and Sigma-Aldrich respectively. DMF (N,N'-dimethylformamide), piperidine, TFA (trifluoroacetic acid, 98% min. titration), and TES (triethylsilane) were purchased from Sigma-Aldrich. 5-Azido-pentanoic acid was purchased from Bachem Americas, Inc. BCIP (5-Bromo-4-chloro-3-indolyl phosphate) was purchased from Promega.

Active Akt2 (with N terminal His₆ tag) was purchased from Abcam. Inactive Akt2 (with N terminal His₆ tag) was purchased from BPS Bioscience. Active Akt1 and Akt3 (with N terminal His₆ tag) used in ELISA assays were purchased from Sigma Aldrich. Mouse anti biotin antibody-Alkaline Phosphatase conjugate used in screens was purchased from Sigma Aldrich. Anti His₆ mouse antibody, goat anti mouse IgG - Alkaline Phosphatase conjugate used in screens were purchased from Abcam. Mouse anti biotin monoclonal antibody- Horse Radish Peroxide conjugate was purchased from Cell Signaling. Anti His₆ mouse monoclonal antibody and goat anti mouse IgG-Horse Radish Peroxide conjugate were bought from Abcam. Anti Akt (pan) rabbit antibody (11E7), and mouse anti-rabbit antibody- Horse Radish Peroxide conjugate, used in Western blot and dot blot, were purchased from Cell Signalling. Biotinylated mouse PhosphoS473 (pS473) Akt antibody used in immunoprecipitation and PhosphoS473 Akt antibody used in western blot was purchased from Cell Signaling. Non-radioactive Akt kinase assay kit was purchased from Cell Signaling.

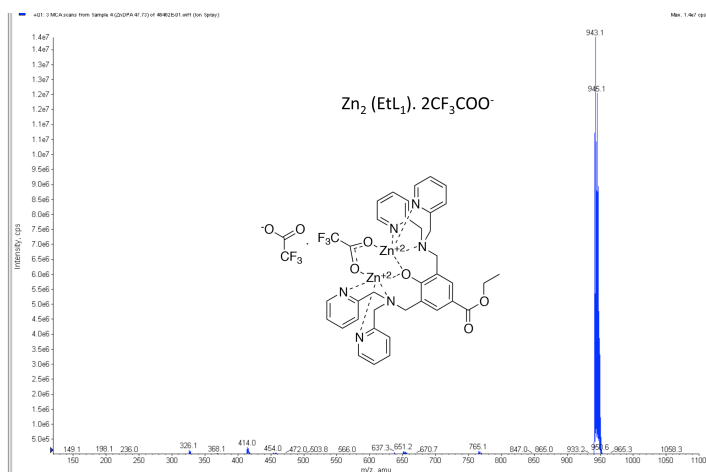
Synthesis of ethyl 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoate [H(EtL₁)]: N,N-di(2-picoly) amine (2.50 g, 12.5 mmol) in ethanol/water/HCl (30 mL/90 mL/0.6 mL of 2M) was added to paraben (830 mg, 5 mmol) and paraformaldehyde (475 mg, 15.67 mmol). The mixture was heated under reflux for 3 days and then allowed to cool to room temperature^[1]. Then dichloromethane (300 mL) and water (100 mL) was added to the reaction mixture and a liquid phase extraction was done. The organic phase, containing the compound, was washed once with 300 mL of water and dried over anhydrous sodium sulphate. A yellowish gummy semisolid was obtained after evaporation of solvent. Column chromatography on silica gel with eluents dichloromethane /methanol /ammonium hydroxide afforded light yellow semi solid. Calculated mass: [M+H] 588.6 Observed mass: [M+H] 589.29



Synthesis of 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoic acid [H(L₁)]: The purified semisolid was dissolved in 2 M NaOH in 1:1 ethanol/water solution and stirred at 60 °C for 2 days. Then the solution was neutralized by concentrated hydrochloric acid. The compound was extracted with methanol and used in further synthesis. Calculated mass: [M-H] 559.25; Observed mass: [M-H] 559.4.



Synthesis of 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoxo dizinc complex [Zn₂(EtL₁)]: 2 equivalents of zinc acetate was dissolved in methanol and added to 1 equivalent of ethyl 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoate and stirred overnight at room temperature. The solvent was removed under reduced pressure and the solid was purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector) using a C18 reversed phase semi-preparative column (Phenomenex Luna 10 μm, 250 × 10 mm). Calculated mass: 944.48; Observed mass: 945.10.



Synthesis of 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoxo dizinc complex [Zn₂(L₁)-Az₄-PEG₂-biotin]: D,L-Fmoc-azidolysine was coupled to Biotin – PEG - NovaTag resin (coupling efficiency 0.48 mmole/g) following standard Fmoc solid phase synthesis protocol. The N_ε-Fmoc protecting group was removed by treating with 20% piperidine in NMP. Then 1.5 equivalents of 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoic acid were coupled overnight to the resin. The molecule was cleaved off the resin using a cocktail of TFA, TES and double distilled water (95:2.5:2.5), precipitated in ice cold ether and lyophilized. The crude solid was used in further synthesis. 2 equivalents of zinc acetate was dissolved in methanol and added to 1 equivalent of 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoic acid and stirred overnight at room temperature. The solvent was removed under reduced pressure and the solid was purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector) using a C18 reversed phase semi-preparative column (Phenomenex Luna 10 μm, 250 × 10 mm). Calculated mass: [M].2H₂O 1369.45 Observed mass: [M].2H₂O 1369.4 (Figure S1D).

Verification of binding of dizinc complex to phosphoamino acids and phosphopeptide: 500 μM solution of Zn₂(EtL₁) or Zn₂L₁-Az₄-PEG₂-Biotin was made dissolving the HPLC purified solid in 10 mM tris borate buffer (TBS) (pH 8). Saturated solutions of pure phosphoserine, phosphotyrosine, Akt peptide C-terminal motif with 13 amino acids (**phospho-Akt2-13mer**) and pSrc substrate Ac-I-pY-GEF was made in the buffer. The Zn₂(EtL₁) or Zn₂L₁-Az₄-PEG₂-Biotin solution was added to either of the saturated solutions in a 1:1 ratio. A fresh matrix was prepared by dissolving 2,4,6-trihydroxyacetophenone (THAP) in 10 mM

tris borate buffer (pH 8) with 50% acetonitrile (20 mg/ml). Each solution was mixed in a 1:1 ratio with the matrix, and subjected to Maldi TOF in a positive mode. Two controls containing only the Zn₂ EtL₁ or Zn₂L₁-Az4-PEG₂-Biotin solution were also subjected to the Maldi TOF. In the mixed solutions, the peaks corresponding to the adduct of the dinuclear Zinc complex to the phosphoamino acid or phosphopeptides were observed (Figure S1).

Solid phase peptide synthesis:

General protocol: Peptides were synthesized on Rink Amide MBHA, Biotin Novatag, Biotin PEG Novatag and Sieber Amide resin either manually or on the Titan 357 Automatic Peptide Synthesizer (AAPPTec, Louisville, KY). Amino acid solutions in NMP (2 equivalents), with 2 equivalents of HATU and 6 equivalents of DIEA were used for the amino acid coupling reaction. For removal of N_α-Fmoc protecting groups, a solution of 20% piperidine in DMF was used.

Acylation: The resin was treated with a solution of anhydrous acetic anhydride and 2,6-lutidine (Sigma) in DMF (acetic anhydride: 2,6-lutidine: DMF; 5:6:100), twice for ten minutes at room temperature. The excess reagents were removed by five washes with DMF.

Cleavage of side chain protected peptides: The peptides were synthesized on Sieber Amide resin. The resin was treated three times for one minute with 1% TFA/DCM and then washed with DCM. The peptide solution was neutralized by adding 2 equivalent DIEA and rotavaped. The semisolid was dissolved in filtered DMSO, HPLC grade acetonitrile and double distilled water and purified on the HPLC.

Cleavage of side chain deprotected peptide: The peptides were synthesized on the Rink Amide MBHA, Biotin Novatag or Biotin PEG Novatag resin. The resin was treated with a TFA cleavage solution (TFA: TES: ddH₂O; 95:2.5:2.5) for two hours at room temperature. The cleavage solution was filtered through a Gooch filter crucible and added dropwise to an ice cooled solution of diethyl ether.

HPLC purification of peptides: All the peptides were purified using a gradient of double distilled water and HPLC grade acetonitrile and 0.1% TFA on the RP-HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector) using a C18 reversed phase semi-preparative column (Phenomenex Luna 10 μm, 250 × 10 mm).

Protocol for on bead copper (Cu) catalyzed azide alkyne cycloaddition (CuAAC) click reaction: On bead Cu catalyzed click reactions were performed with the azide on bead and the alkyne in solution. The resin was treated with 2 equivalents of the relevant alkyne, 1.5 equivalents of CuI (Sigma) and 2.5 equivalents of ascorbic acid (Sigma), in a solution of 20% piperidine in DMF. The reaction was performed overnight at room temperature. The excess copper was removed from the resin by washing extensively with a Cu chelating solution (5% (w/v) sodium diethyl dithiocarbamate, 5% (v/v) DIEA in DMF).

Peptide library Synthesis:

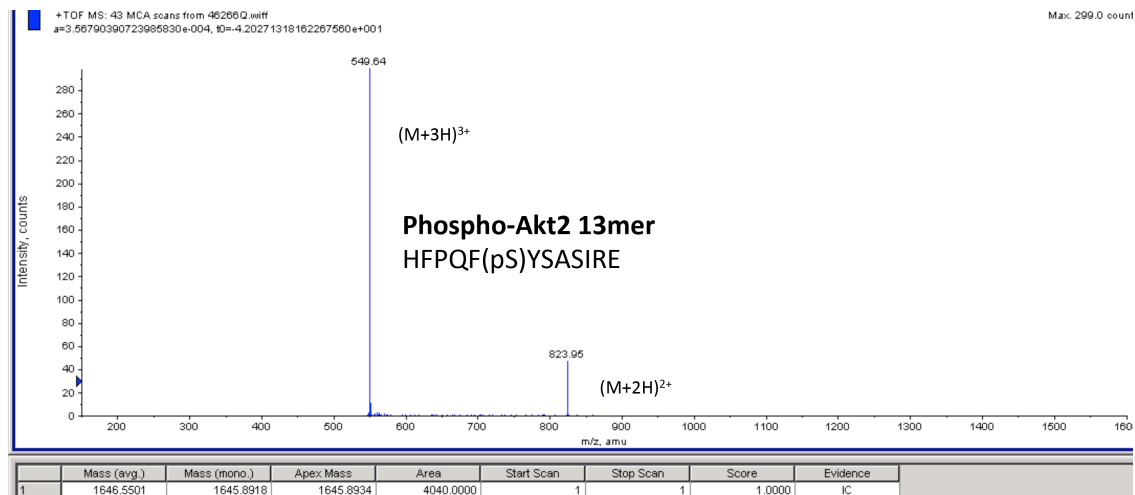
Randomized OBOC^[2] libraries of hexapeptides were synthesized using the Titan 357 Automated Peptide Synthesizer (AAPPTec) on 90 μm polyethylene glycol-grafted polystyrene beads (TentaGel S-NH₂, 0.28 mmol/g, 2.86 × 10⁶ beads/g). All the libraries used unnatural D amino acids including Fmoc-D-

propargylglycine. In library C, for azide incorporation, Fmoc-L-azido lysine (Anaspec) was coupled to the N termini of the on bead peptides. All the libraries contained 10% D-Methionine at the C terminal, for compatibility with MalDI-TOF/ TOF sequencing. The 10% Methionine was incorporated following literature protocol^[3].

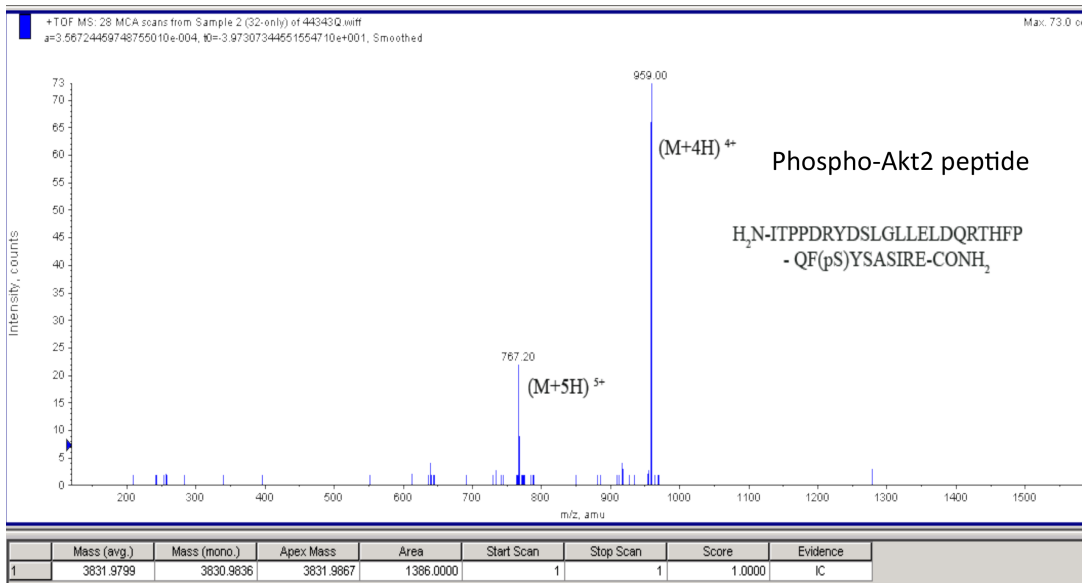
Synthesis and characterization of peptide ligands and intermediates:

Synthesis and characterization of target peptide sequence (phospho-Akt2 peptide) and C terminal 13 amino acid long phospho-peptide (phospho-Akt2-13mer): The 32mer target peptide sequence containing amino acids 450-481 of Akt2, ITPPDRYDSLGLLELDQRTHFPQF(pS)YASASIRE (**phospho-Akt2 peptide**), and the 13mer peptide sequence containing amino acids 469-481 of Akt2, HFPQF(pS)YASASIRE (**phospho-Akt2-13mer**), was synthesized on Rink Amide MBHA resin, using the Titan 357 peptide synthesizer. Fmoc-Ser(OPO₃Bzl)-OH (AaPPTec) was used for the incorporation of phosphoserine in the peptide. It was cleaved by TFA/TES/ddH₂O, precipitated in cold ether and purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Calculated mass: 1645.67; observed mass: 1646.55.

Mass spectrometric analysis of phospho-Akt2-13mer: ESI -TOF MS: m/z; 823.95 (M+2H)²⁺; 549.6 (M+3H)³⁺

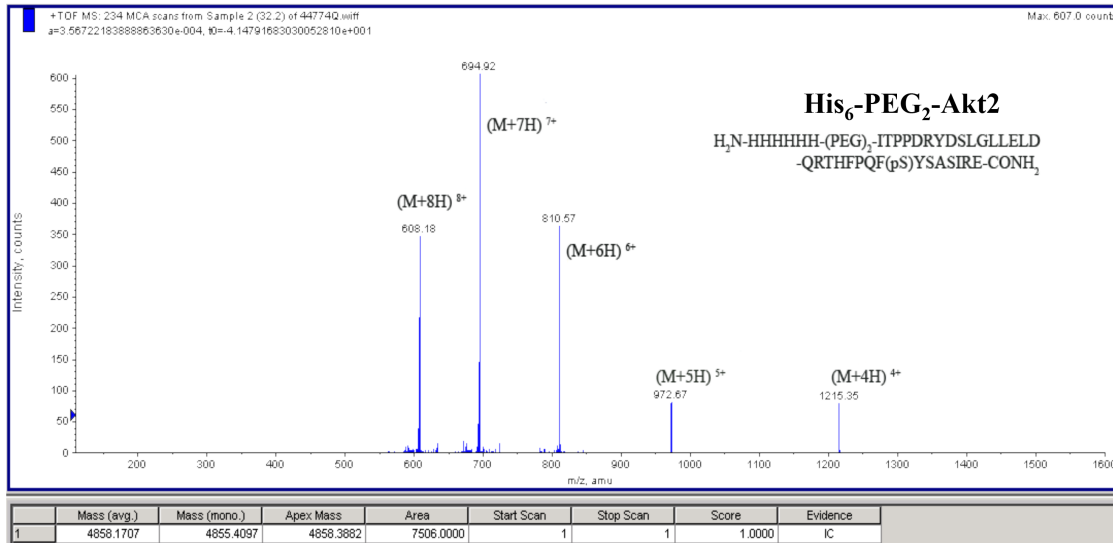


Mass spectrometric analysis of phospho-Akt2 peptide: Calculated mass: 3832.0; Observed mass: 3831.98; ESI –TOF MS: m/z; 767.2 (M+5H)⁵⁺, 959.0 (M+4H)⁴⁺

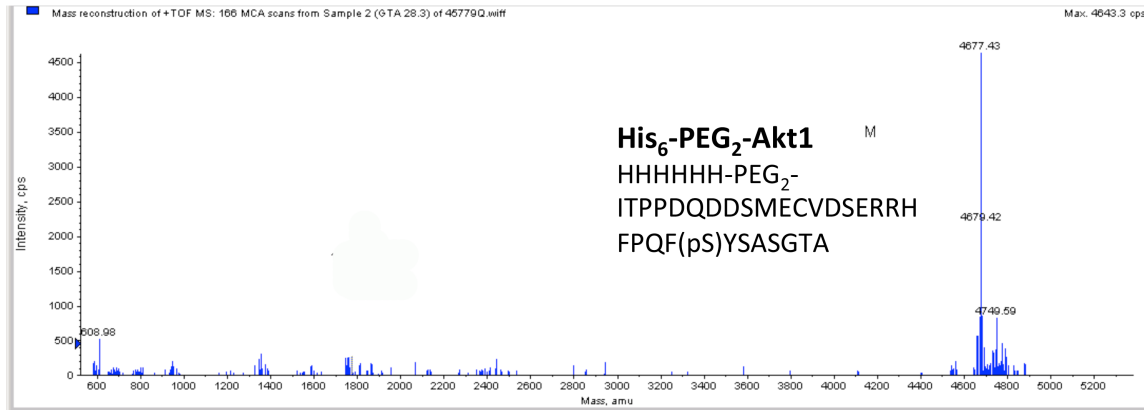


Synthesis and characterization of His₆ tagged target phospho-Akt2 peptide (His₆- PEG₂- phospho – Akt2) and corresponding C terminal peptides from Akt1 (His₆- PEG₂- phospho – Akt1) and Akt3 (His₆- PEG₂- phospho – Akt3): The target sequence, amino acids 450-481 of Akt2, with pS474, and corresponding C terminal sequences of Akt1 (449-480) and Akt3 (448-479) were synthesized on Rink Amide MBHA resin, using above mentioned procedure. Fmoc-NH-(PEG)₂-OH was then coupled with each peptide. Then six successive couplings were done with Fmoc-L-His(Trt)-OH. The peptides were cleaved by TFA/TES/ddH₂O, precipitated in cold ether and purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC.

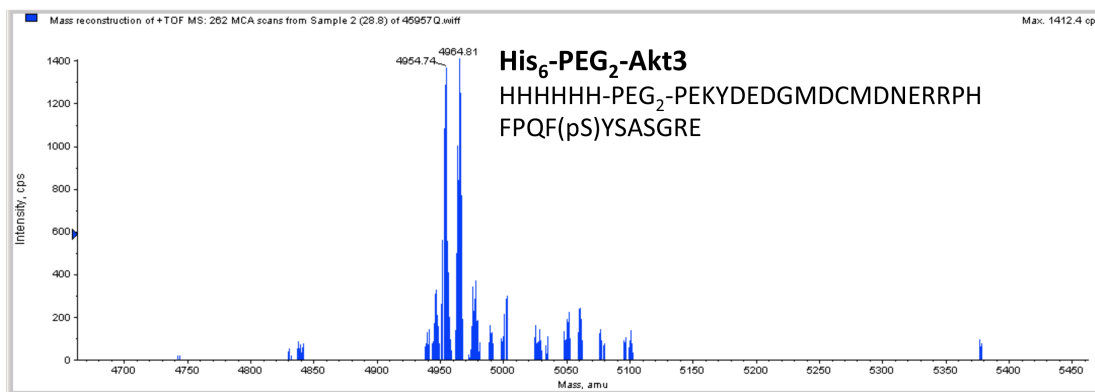
His₆- PEG₂ - phospho - Akt2: Calculated mass: 4858.1; Observed mass: 4858.1; ESI -TOF MS: m/z; 608.18 (M + 8H)⁸⁺, 694.92 (M + 7H)⁷⁺, 810.57 (M+6H)⁶⁺, 972.67 (M+5H)⁵⁺, 1215.35 (M+4H)⁴⁺



His₆- PEG₂ - phospho - Akt1: Calculated mass: Q-TOF: (M+H) 4676.8, (M.4H₂O) 4748.8; Observed mass: (M+H) 4677.43, (M.4H₂O) 4749.59



His₆- PEG₂ - phospho - Akt3 peptide: Q-TOF: Calculated mass (M+K+H₂O) 4953.1, (M+3K) 4965.10;
Observed mass: (M+K+H₂O) 4954.74, (M+3K) 4964.81.



Synthesis and characterization of mono-L: Fmoc-NH-PEG₂-OH was coupled using standard Fmoc protocol on Biotin Novatag resin. 1.5 equivalents of D, L-Fmoc-azidolysine were coupled on the resin followed by acylation. On bead Cu catalyzed click reaction was carried out following described protocol using 2 equivalents of Fmoc-D-Pra-O^tBu. After washes with the copper chelating solution the peptide was acylated. The resultant molecule **S1** (figure S10) was cleaved off the resin using TFA cleavage solution. The crude solid was used in further synthesis.

The peptide wkvkl was made on Rink Amide MBHA resin (Anaspec) following standard Fmoc SPPS synthesis protocol. 1.5 equivalents of **S1** were then coupled to the peptide. After TFA cleavage the ligand **mono-L** was purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Calculated mass: 1494.8. Observed mass: 1494.6

Mass spectrometric analysis of mono-L:

ESI –TOF MS: m/z ; 498.95 ($M + 3H$)³⁺, 747.92 ($M + 2H$)²⁺

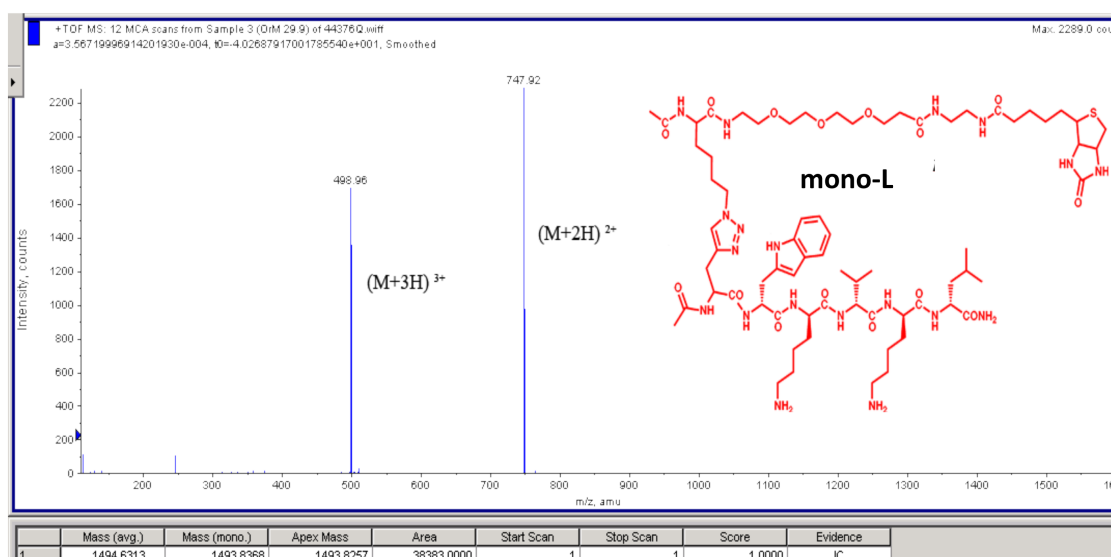
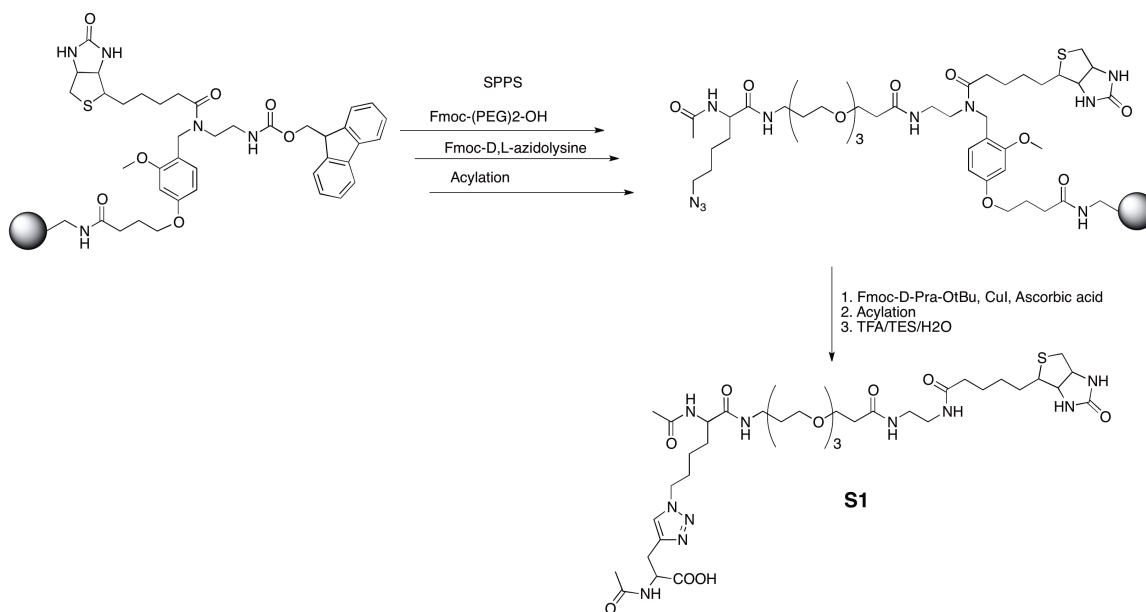


Figure S10: Synthesis of **mono-L**



Synthesis and characterization of 2: Fmoc-NH-PEG₂-OH was coupled using standard Fmoc protocol on Biotin Novatag resin. 1.5 equivalent of D, L-Fmoc-azidolysine was coupled on the resin followed by coupling of 1.5 equivalent of 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoic acid. The resin was then subjected to on bead Cu catalyzed click reaction with Fmoc - D-Pra-O^tBu (Fmoc - D-propargylglycine tertiary butyl ester). The excess copper was removed by washing with the copper

chelating solution. 5-Azido-pentanoic acid was then coupled. The resulting peptide **S2** (Figure S11) was TFA cleaved and lyophilized. The crude was used in further synthesis. The peptide wkvkl was made on Rink Amide MBHA resin following standard Fmoc SPPS synthesis protocol. 1 equivalent of peptide **S2** was then coupled to the peptide. The peptide was cleaved off using TFA cleavage solution. 2 equivalents of zinc acetate were dissolved in methanol and added to 1 equivalent of crude peptide and stirred overnight at room temperature. The solvent was removed under reduced pressure and the solid was purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Mass calculated: $[M+Na]$ 2291, $[M+Na.TFA]$ 2404 Mass observed: $[M+Na]$ 2289.98, $[M+Na.TFA]$ 2403.95

Mass spectrometric analysis of **2**:

Mass calculated: $[M+Na]^+$ 2291, $[M+Na.TFA]^+$ 2404 Mass observed: $[M+Na]^+$ 2289.98, $[M+Na.TFA]^+$ 2403.95

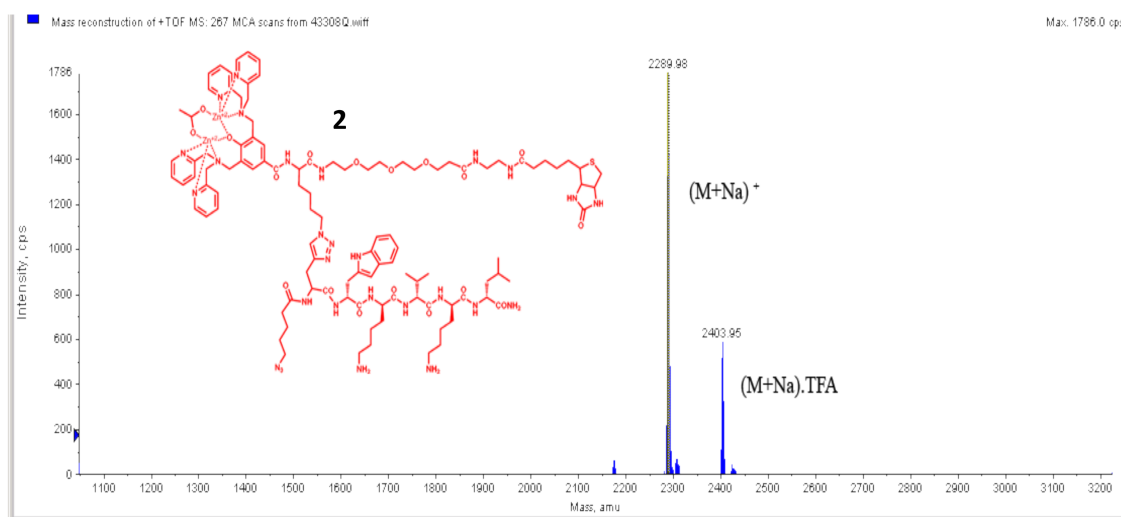
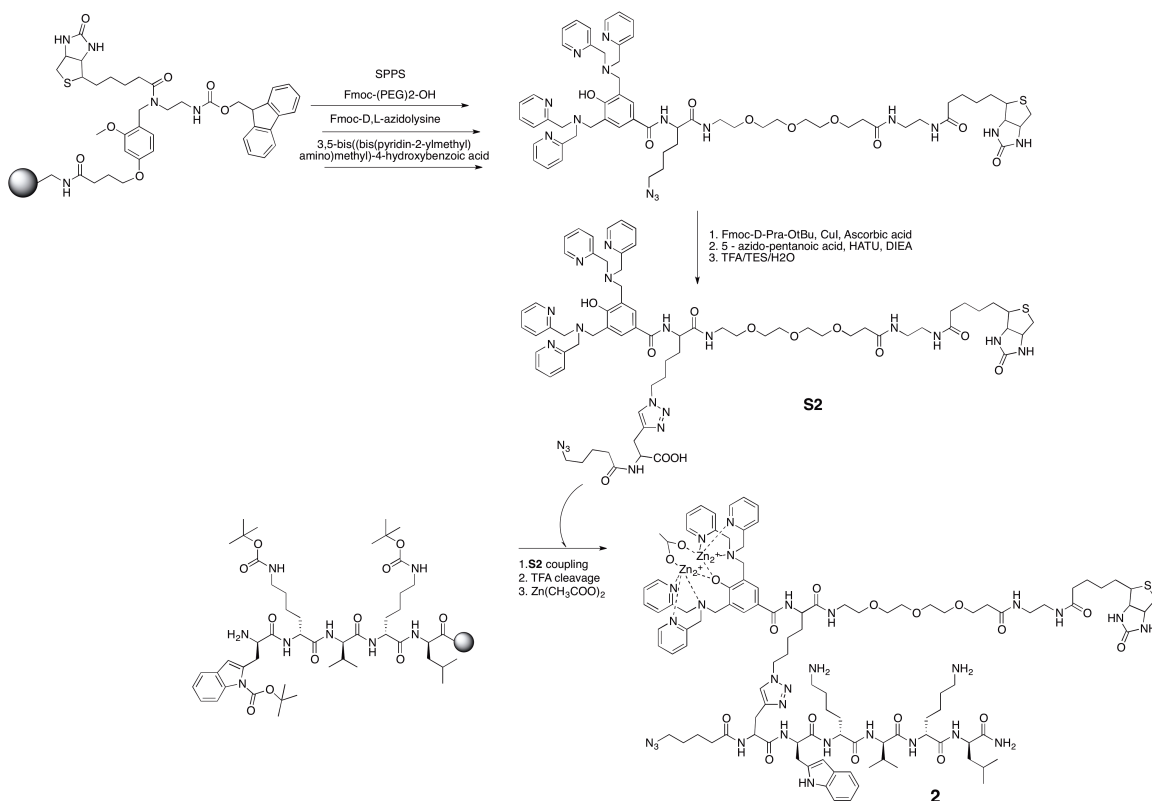


Figure S11: Synthesis of intermediate **S2** and **2**



Synthesis and characterization of bi-L: Fmoc-NH-PEG₂-OH was coupled using standard Fmoc protocol on Biotin Novatag resin. 1.5 equivalent of D,L-Fmoc-azidolysine was coupled on the resin followed by acylation of the amine terminal. On bead click reaction was carried out with 2 equivalents of Fmoc-D-Pra-O^tBu. After washes with copper removing solution, 5-azido-pentanoic acid was coupled. After TFA cleavage the resultant molecule **S3** (figure S12) was lyophilized and the crude solid was used in further synthesis. The peptide wkvkl was made on Rink Amide MBHA resin (Anaspec) following standard Fmoc SPPS synthesis protocol. 1 equivalent of **S3** was then coupled to the peptide on bead. Fmoc-D-Pra-O^tBu was then clicked to the azido functionality on bead. After washes with the copper chelating solution, the peptide was further extended to hngyf on the N terminal using standard Fmoc SPPS synthesis. After TFA cleavage the biligand was purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Mass calculated: 2309.7 Mass observed: 2309.4

Mass spectrometric analysis of bi-L: ESI –TOF MS: m/z ; 770.44 ($M + 3H$)³⁺, 578.33 ($M + 4H$)⁴⁺

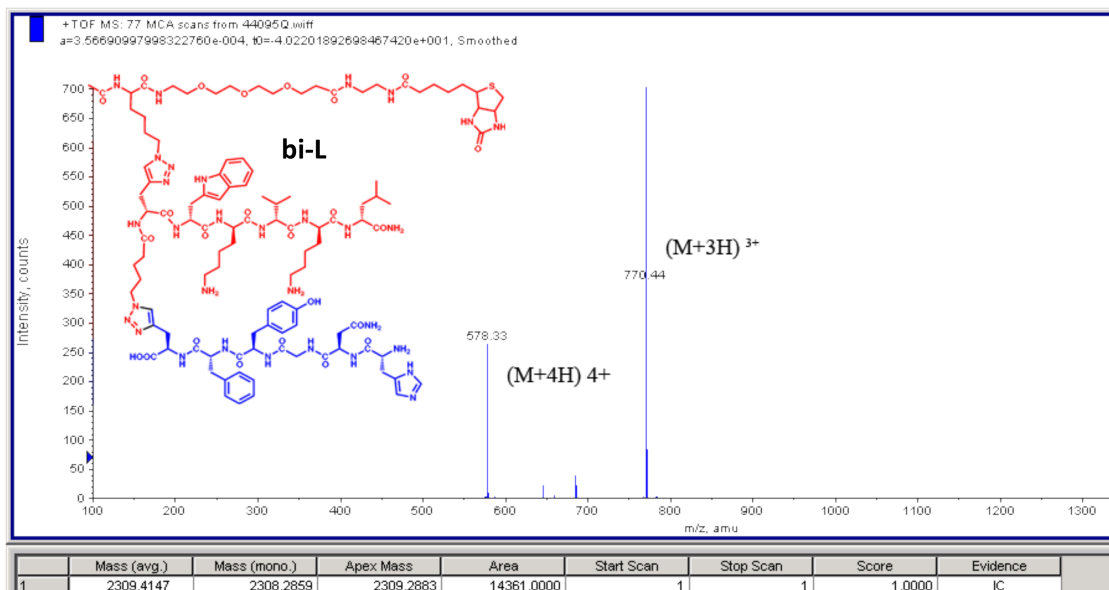
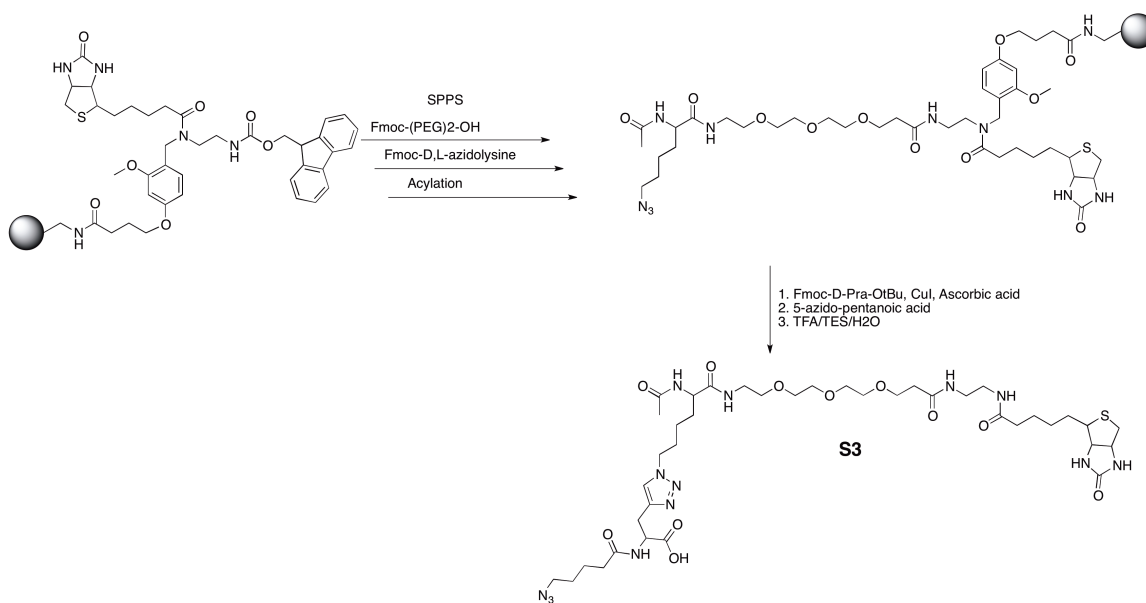


Figure S12: Synthesis of compound S3. S3 is used in the synthesis of the bi-L, Anchor-3C and C-tL



Synthesis and characterization of Anchor-3N: Fmoc-NH-PEG₂-OH was coupled using standard Fmoc protocol on Biotin Novatag resin. 1.5 equivalent of D, L-Fmoc-azidolysine was coupled on the resin followed by acylation using acetic anhydride and 2,6-lutidine in DMF. On bead click reaction with Fmoc-D-Pra-O^tBu was carried out. After washes with copper chelating solution Fmoc-L-azidolysine was coupled. Following removal of the Fmoc protecting group, the amine terminal was acylated. After TFA cleavage the

resultant molecule **S4** (figure S13) was lyophilized and the crude solid was used in further synthesis. The peptide wkvkl was made on Rink Amide MBHA resin. 1.5 equivalents of **S4** were then coupled to the peptide. On bead click reaction was carried out with two equivalents of Fmoc-D-Pra-O^tBu. After washes with copper removing solution, the peptide was further extended to hngyf on the N terminal using standard Fmoc SPPS synthesis. Fmoc-L-azidolysine was then coupled, followed by acylation of the amine terminal. After TFA cleavage **Anchor-3N** was purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Mass calculated: 2534.9 Mass observed: 2534.6

Mass spectrometric analysis of Anchor-3N: ESI –TOF MS: m/z; 634.60 (M + 4H)⁴⁺

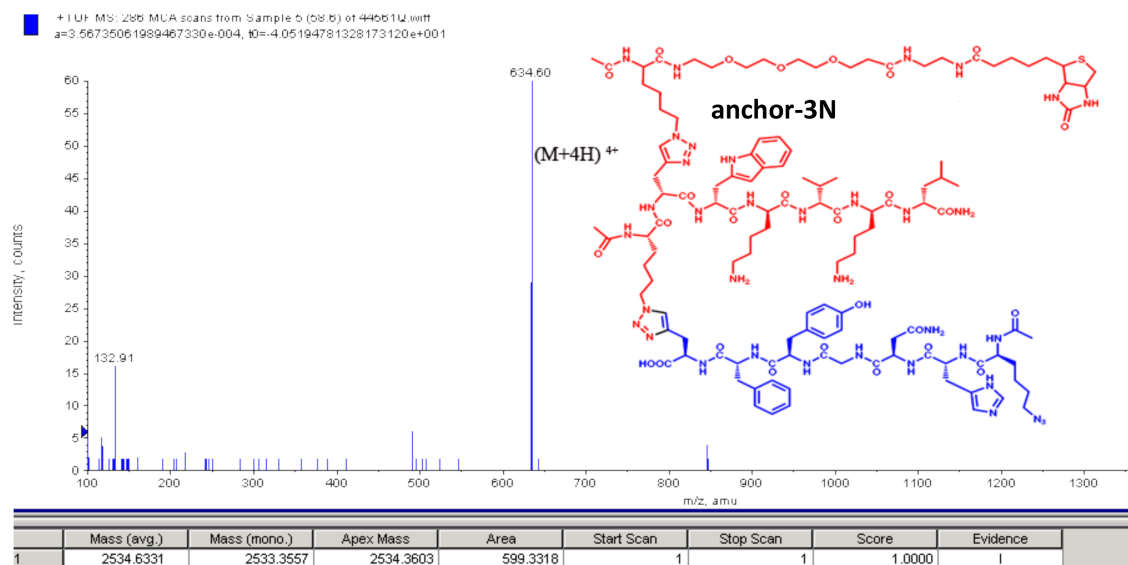
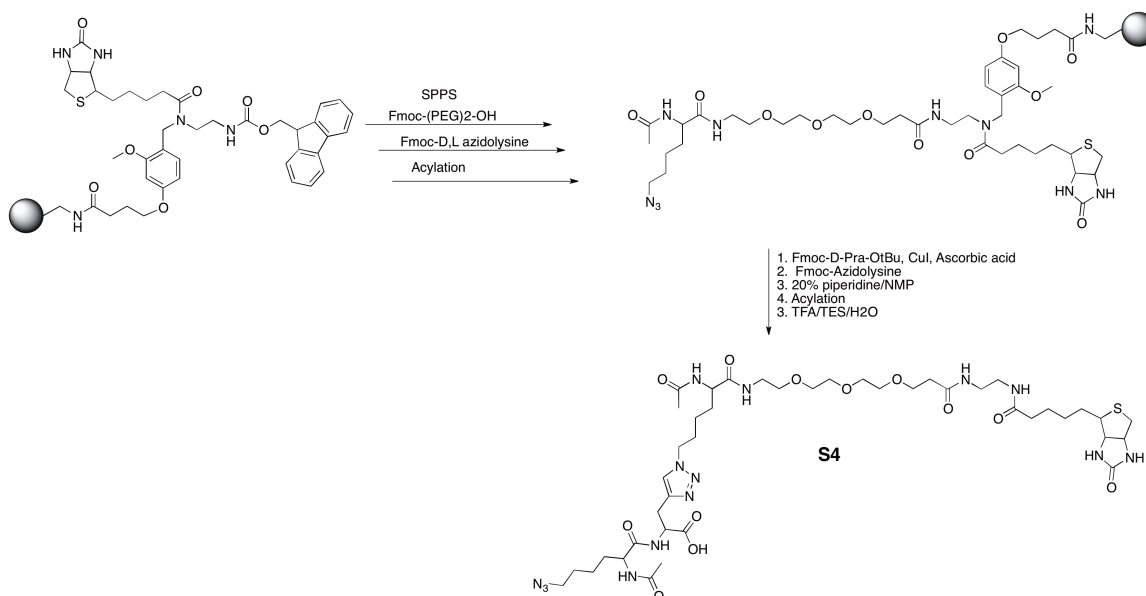
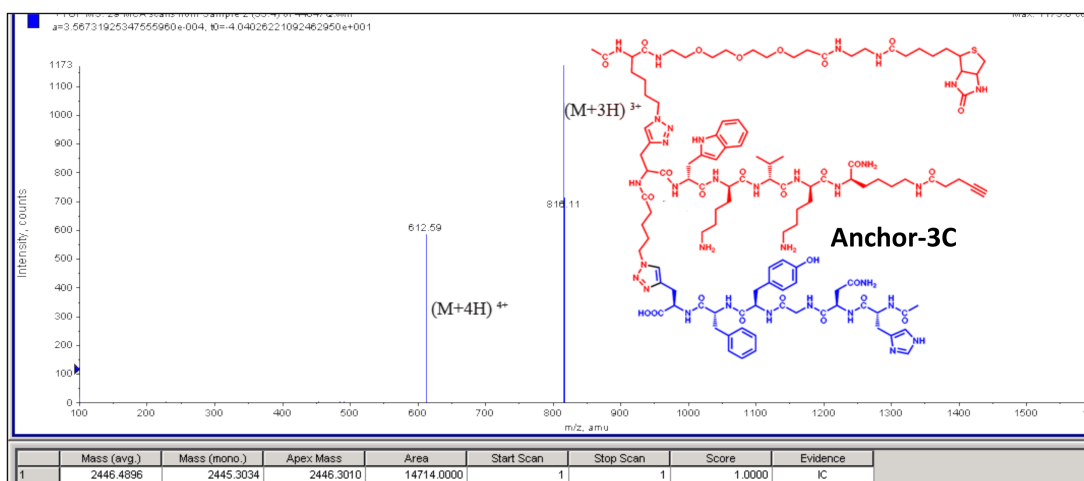


Figure S13: Synthesis of intermediate compound **S4** for **anchor-3N** and **N-tL**



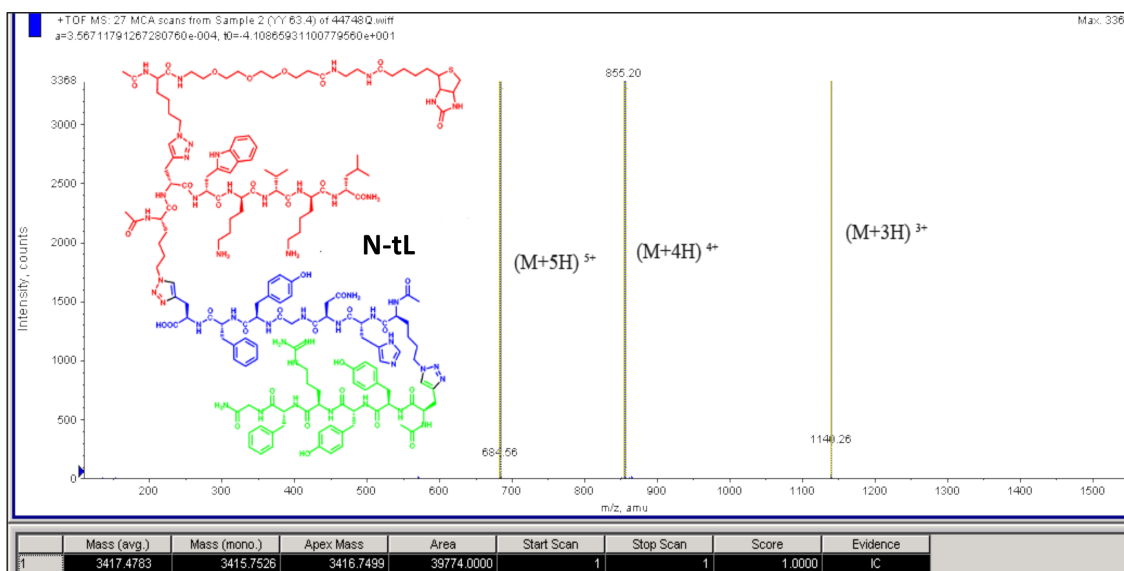
Synthesis and characterization of Anchor-3C: The peptide NH₂-wkvkk(Alloc) was made on Rink Amide MBHA resin following standard Fmoc SPPS synthesis protocol. 1 equivalent of **S3** (figure S12) was then coupled to the resin. On bead click reaction was carried out overnight using 1.5 equivalents of Fmoc-D-Pra-O^tBu. After washes with the copper chelating solution, the peptide was further extended to hngyf on the N terminal using standard Fmoc SPPS synthesis. Then it was acylated again. The alloc side chain of Fmoc-D-lysine(Alloc)-OH was deprotected using standard alloc deprotection technique^[4]. Then 4-pentynoic acid was coupled. After TFA cleavage, **Anchor-3C** was purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Mass calculated: 2446.8; Mass observed: 2446.5

Mass spectrometric analysis of **Anchor-3C**: ESI –TOF MS: m/z; 816.11 (M + 3H)³⁺, 612.59 (M + 4H)⁴⁺



Synthesis and characterization of N-tL: Side chain protected version of Ac-D-Pra-yyrfG-CONH₂ was made on Amide Sieber resin. The protected peptide was cleaved off using 1% TFA in DCM and purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. **Anchor-3N** was made on resin as described and then acylated using standard acylation method. On bead click reaction was carried out for the bead bound **Anchor-3N** with 2 equivalents of side chain protected purified peptide Ac-yyrfG-CONH₂. The resin was washed with copper chelating solution. The peptide was cleaved off the resin with the TFA cleavage solution and purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Mass calculated: 3417.9 Mass observed: 3417.5

Mass spectrometric analysis of N-tL: ESI –TOF MS: m/z; 684.56 (M+5H)⁵⁺, 855.20 (M + 4H)⁴⁺, 1140.26 (M + 3H)³⁺

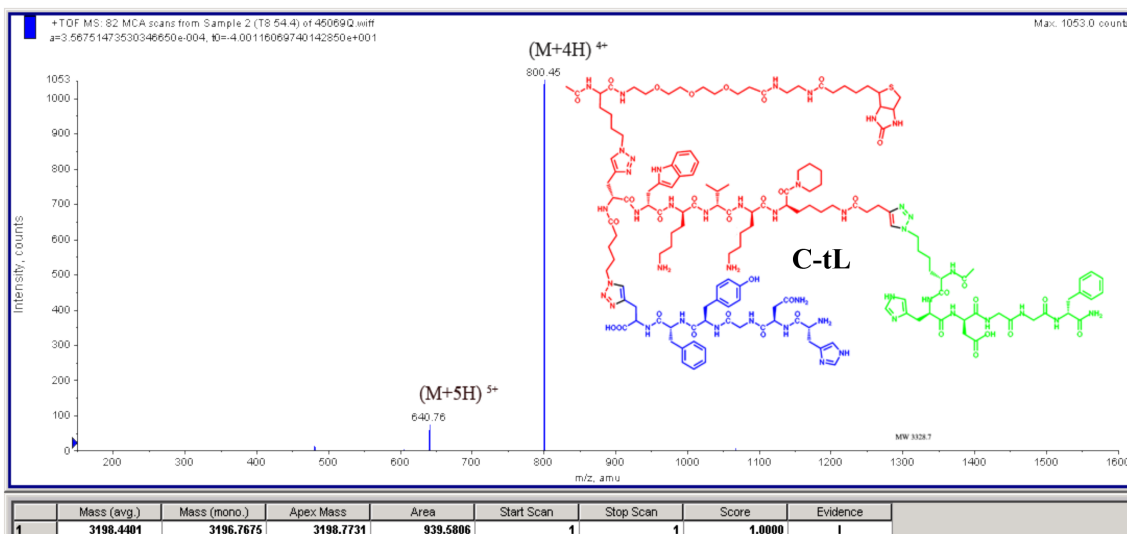


Synthesis of D-Lys (pentyne) amide: Boc-D-Lys(Fmoc)-OH was coupled with rink amide resin. Then 4-pentynoic acid was coupled with it, after standard piperidine deprotection. The dried resin was cleaved with TFA cocktail and purified using a gradient of water and acetonitrile and 0.1% TFA on the prep-HPLC. Mass calculated: (M+H) 225 Mass observed: 226

Synthesis and characterization of C-tL: Ac-L-Az4-hdGGf (Az4 = azidolysine) was made on Rink Amide MBHA resin. On bead click reaction of the peptide on resin with D-Lys(pentyne) amide was carried out overnight at room temperature with 2 equivalents of D-Lys(pentyne) amide. The resin was washed with the copper chelating solution and further extended to wkvk on the N terminal using standard Fmoc SPPS synthesis. Then, 1.5 equivalent of **S3** was coupled to the peptide. An on bead click reaction was carried out with Fmoc-D-Pra -O^tBu. After washes with copper chelating solution, the peptide was further extended to hngyf on the N terminal using standard Fmoc SPPS synthesis. The dried resin was cleaved with TFA

cleavage solution and purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Mass calculated: 3199.65; Mass observed: 3198.44.

Mass spectrometric analysis of C-tL: ESI –TOF MS: m/z; 640.76 (M+5H)⁵⁺, 800.45 (M+4H)⁴⁺



Screening with One Bead One Compound (OBOC) peptide library:

Screen for mono-L:

50 nM solution of the Akt2 peptide was made by diluting 0.5mg/ml DMSO stock in 25 mM tris chloride, 150 mM NaCl, 2 mM KCl, pH 8) (TBS). 100 μM solution of the Zn₂L-Az4-PEG₂-Biotin was added to the 50 nM solution of the **phospho-Akt2 peptide** and shaken overnight at room temperature. Before the addition to the OBOC library, Bovine Serum Albumin (BSA) and Tween 20 was added to the solution to make the final concentrations 0.1 % BSA and 0.05% tween 20 in the buffer. 250 mgs of library A (D-Pra-XXXXX-10%M-TG, supplementary table S1) were used in the screen. The beads were equilibrated in 0.1% BSA, 0.05% Tween 20/TBS (binding buffer) by shaking for 8 hours. The Zn₂L-Az4-PEG₂-Biotin –Akt2 peptide solution was added to the swelled beads and shaken overnight at room temperature. The beads were washed three times with the binding buffer. A 1:10,000 dilution of mouse anti biotin monoclonal antibody-Alkaline Phosphatase conjugate (Sigma) in binding buffer was added to the beads. The beads were then washed thrice with binding buffer, thrice with TBST (0.05% tween 20/TBS) and thrice with TBS.

A BCIP solution was freshly prepared by adding 33 ul of BCIP (50 mg/ml) stock solution in 10 ml of Alkaline Phosphatase buffer (100 mM Tris-HCl, pH 9.0, 150 mM NaCl, 1 mM MgCl₂) (Promega). The beads were washed once with the Alkaline Phosphatase buffer, and then treated with the fresh BCIP solution. The hit beads turned turquoise blue due to a colorimetric reaction of Alkaline Phosphatase with

BCIP. The reaction was quenched after one hour with 0.1 N HCl. The hit beads were picked with a pipette tip and transferred to an eppendorf tube. The turquoise color of the hit beads was removed by washing with DMF. The proteins on the beads were stripped by washes with 7.5 M guanidium hydrochloride pH 2.0 solution. Then beads were then equilibrated in buffer. The screen was repeated on this small set of beads, this time using a preincubated mixture of 2.5 mM biotin and 1:10,000 dilution of a mouse anti biotin monoclonal-Alkaline Phosphatase conjugate (Sigma) as the secondary antibody. On addition of the BCIP, the true hits, due to competition with biotin, remain clear. The clear beads were manually picked, washed with guanidium hydrochloride and water, and sequenced using the Edman Peptide Sequencer.

Screen for bi-L:

Prescreen: 2 batches of 135 mg of library B (XXXXXX-D-Pra- 10% M- TG, Supplementary table S1) were washed in water, and swelled overnight in binding buffer (25 mM Tris-Cl (pH = 7.7), 150 mM NaCl, 2 mM KCl, 0.1% (v/v) Tween-20, and 0.1 % BSA). 20 μ M and 50 μ M solutions of **2** (figure S2B) was added to the beads and shaken for 10 hours at room temperature. The beads were washed thrice, for fifteen minutes each, with the binding buffer. A 1:10,000 dilution of mouse anti biotin antibody-Alkaline Phosphatase conjugate (Sigma) in binding buffer was added to the beads. The beads were washed three times, for fifteen minutes each, with wash buffer 1 (25 mM Tris-Cl (pH = 7.7), 150 mM NaCl, 2 mM KCl, 0.1% (v/v) Tween-20), followed by three fifteen minute washes with wash buffer 2 (25 mM Tris-Cl (pH = 7.7), 150 mM NaCl, 2 mM KCl). The beads were then developed in BCIP solution for 35 minutes and quenched with 0.1 N HCl. The blue hit beads, which were background binders to the mono-L-Zn chelator or the detection antibody were picked up manually. The clear beads were stringently washed with DMF, 7.5 mM guanidium hydrochloride, pH 2.0, and double distilled water.

Product screen: The washed beads from each prescreen were dried and swelled overnight in binding in 8 ml fritted polypropylene solid-phase synthesis tubes. In two separate eppendorf tubes, 20 μ M and 50 μ M solution of **2** was incubated overnight at room temperature with 10 nM and 25 nM **phospho-Akt2 peptide** solution respectively, in binding buffer. 4 mL of each of the two solutions were added to a precleared swelled bead batch and the tubes were shaken at room temperature for ten hours. The beads were washed three times, for fifteen minutes each, with wash buffer 1 followed by three fifteen minute washes with wash buffer 2. The beads are then developed in BCIP solution for thirty-five minutes and quenched with 0.1 N HCl. The blue hit beads were picked up manually, stringently washed with DMF, guanidium hydrochloride and water, and sequenced on the Edman Sequencer. The sequences from the biligand screen are given in tables S2 and S3.

Selection of final biligand bi-L: Several biligand candidates were synthesized by Cu catalyzed click reaction which contained either a direct hit sequence (hnGyG—D-Pra, hnGre-D-Pra, hnGai - D-Pra, hnGii - D-Pra, hnGGd - D-Pra) or the consensus sequence from the amino acid histograms (hnGyf - D-Pra from figure S3) as the 2^o peptide. Biligands with the second peptide arm as hnGyG - D-Pra (**Bi1**), hnGyf - D-

Pra (**Bi2**), hnGre -D-Pra (**Bi3**), hnGai - D-Pra (**Bi4**), hnGii – D-Pra (**Bi5**), hnGGd – D-Pra (**Bi6**) were tested for immunoprecipitation of Akt from OVCAR3 cells. Eluents from the immunoprecipitation from **Bi1**, **Bi2**, **Bi3** and **Bi4** were run on a western blot and eluents from the immunoprecipitation from **Bi5** and **Bi6** are blotted directly on nitrocellulose and treated with anti-Akt pan antibody. The western blot was given in figure S4 and the dot blot did not show any Akt pulldown. On the basis of the pulldown efficiency **Bi2**, which has hnGyf - D-Pra as the 2° peptide arm, was chosen as the final biligand candidate.

Screen for N-tL:

Prescreen: 500 mg of library A (D-Pra-XXXXXX-10%M- TG, Supplementary table S1) were swelled in binding buffer (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂, 0.1% BSA, 0.05% Tween-20) overnight. The beads were incubated with 25 µM solution of **anchor-3N** in binding buffer for two hours at 4°C. The beads were washed three times for five minutes each, with binding buffer. The beads were treated for two hours with 7.5 M Guanidium chloride (pH = 2) and washed ten times with double distilled water. The beads were equilibrated in binding buffer. A 1:10,000 dilution of mouse anti biotin antibody-Alkaline phosphatase conjugate (Sigma) in binding buffer was added to the beads. The beads were washed three times, for five minutes each, with wash buffer 3 (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂, 0.05% (v/v) Tween-20), followed by three five minute washes with wash buffer 4 (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂). The beads were then developed in BCIP solution for thirty minutes and quenched with 0.1 N HCl. The blue hit beads, which were background binders to the **anchor-3N** or the antibody were separated from the rest of the beads. The clear beads were stringently washed with DMF, guanidium hydrochloride and water and used in the product screen.

Product screen: The clear beads from the prescreen were swelled overnight in binding buffer (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂, 0.05% (v/v) Tween-20, and 0.1% BSA). 25 µM solution of **anchor-3N** was incubated for thirty minutes at 4°C with 50 nM Akt2 in binding buffer. The solution was added to the beads and shaken at 4°C for two hours. The beads were washed three times, for five minutes each, with the binding buffer. The beads were treated for two hours with 7.5 mM guanidium chloride (pH = 2) and washed ten times with double distilled water. The beads were re-equilibrated in binding buffer. A 1:10,000 dilution of mouse anti biotin antibody-alkaline phosphatase conjugate (Sigma) in binding buffer was added to the beads. The beads were washed three times, for five minutes each, with wash buffer 3 followed by three five minutes washes with wash buffer 4. The beads were then developed in BCIP solution for thirty minutes and quenched with 0.1 N HCl. The blue hit beads were picked up manually, stringently washed with DMF, guanidium hydrochloride and water, and used in the target screen.

Target screen: The washed hit beads from the product screen were swelled overnight in binding buffer (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂, 0.05% (v/v) Tween-20, and 0.1% BSA). 50 nM Akt2 protein, preincubated with 73.5 µM **anchor-3N** for thirty minutes at 4°C, was added to the swelled beads and the beads were shaken for ninety minutes at 4°C. The beads were washed three times, for five

minutes each, with the binding buffer. A 1:1000 dilution of mouse anti His₆ antibody (Abcam) in binding buffer was added to the beads and incubated for an hour with shaking at 4°C. Following three five minute washes with the binding buffer, a 1:10,000 diluted solution of anti mouse –alkaline phosphatase (Sigma) was added and the shaken for one hour at 4°C. The beads were washed three times for five minutes each with wash buffer 3, followed by three five minute washes with wash buffer 4. The beads were then developed in BCIP solution for thirty minutes and quenched with 0.1 N HCl. The blue hit beads were picked up manually, stringently washed with DMF, guanidium hydrochloride and water, and sequenced on the Edman Sequencer. The sequences obtained are listed in Table S4. Because of poor resolution of amino acid standards in the Edman Peptide Sequencer during that time, some peptides were assigned to have either one of two amino acids in some positions.

Elimination of peptides binding to anti-Akt antibody: Since a preclear screen was not performed against the mouse-Anti His₆ antibody and the anti-mouse-alkaline phosphatase antibody, to eliminate peptide binders to the antibodies, all the dark colored peptide hits obtained in the earlier target screen were synthesized on Tentagel – S - NH₂ resin. 10 beads of each sequence was taken in spinnex tubes, equilibrated in binding buffer, and then treated with a 1:1000 diluted solution of mouse anti His₆ antibody (Abcam) for an hour at 4°C. Following three five minute washes with the binding buffer, a 1:10,000 diluted solution of anti mouse –alkaline phosphatase was added and the shaken for one hour at 4°C. The beads were washed three times for five minutes each with wash buffer 3, followed by three five minute washes with wash buffer 4. The beads were then developed in BCIP solution for thirty minutes and quenched with 0.1 N HCl. The color intensity of the different sequences are recorded in Table S5, along with the probability that the sequence is a binder to the protein and not a background binder (clear beads). The probability arises from the poor resolution of certain amino acid standards in the Edman sequencer during sequencing the hits from the N terminal triligand target screen.

Selection of final N terminal triligand N-tL: Two sequences were observed (table S5) which were not background binders (remain clear in the antibody binder elimination screen) and which had the full probability of being the right sequence. These two 3° peptides, D-Pra-yyrfG and D-Pra-ssGry, were clicked to **anchor-3N** by CuAAC reaction and tested as triligand candidates (**N-tri1** and **N-tri2** respectively). These were used to immunoprecipitate Akt2 from OVCAR3 cells (figure S5). The first triligand candidate **N-tri1** being more efficient was chosen as the final N terminal triligand **N-tL**.

Screen for C-tL:

Prescreen: 500 mgs of library C (H₂N-Az4-XXXXX-10%M-TG, table S1) was swelled in binding buffer overnight. The beads were incubated with 100 µM solution of **anchor-3C** in binding buffer for two hours at 4°C. The protocol for the prescreen for **N-tL** screen was followed. The blue hit beads, which were background binders to the **anchor-3C** or the detection antibody were separated from the rest of the beads.

The clear beads were stringently washed with DMF, guanidium hydrochloride and water and used in the product screen that followed.

Product screen: The washed beads from the prescreen were swelled overnight in binding buffer 100 μ M solution of **anchor-3C** was incubated for thirty minutes at 4°C with 50 nM Akt2 in binding buffer. The solution was added to the beads and shaken at 4°C for two hours. The protocol for the **N-tL** product screen was followed. The blue hit beads were picked up manually, stringently washed with DMF, guanidium hydrochloride and water, and sequenced on the Edman Sequencer. The sequences are recorded in Table S6.

Selection of the final C terminal triligand C-tL: Four triligand candidates were synthesized with L-Az4-hdGGf (**C-t1**), L-Az4-hdGsf (**C-t2**), L-Az4-hdGww (**C-t3**), L-Az4-hdGkf (**C-t4**) as the 3° peptide arm. Binding of the candidates to the target peptide epitope was determined in an ELISA assay (Figure S6) and the ligand **C-t1** emerged as the best binder. C-t1 was chosen as the C terminal triligand **C-tL**.

Epitope/ Protein Selectivity Assay:

1.25 μ M biotinylated ligand was prepared by diluting the 1 mM stock in binding buffer (25 mM tris chloride, pH= 7.4, 150 mM NaCl, 0.1% BSA, 0.05% Tween 20). The prepared ligand solution or 0.125 % DMSO in buffer (buffer control) was immobilized on a High Capacity Streptavidin 96 well plate (Thermo Scientific). After washing away the excess ligand, the plate was blocked overnight with 1% BSA /25 mM tris chloride, pH= 7.4/ 150 mM NaCl, 0.05% Tween 20. 2.5 μ M solutions of the His₆ tagged target phosphopeptide epitope Akt2 amino acids 450 – 481, His₆ tagged Akt1 amino acids 449-480, His₆ tagged Akt3 amino acids 448-479 were added to each of the wells. Following three washes with the binding buffer, the plate was treated for an hour with a 1:1000 dilution of anti His₆ mouse monoclonal antibody. A 1:10,000 dilution of goat anti mouse antibody-Horse Radish Peroxide conjugate in binding buffer was added to the wells. The plates were washed four times, five minutes each, with 0.05% Tween 20/ TBS (25 mM tris chloride, pH= 7.5, 150 mM NaCl) and once with TBS (25 mM tris chloride, pH= 7.5, 150 mM NaCl). Color was developed by adding TMB substrate (KPL) to each well. The reaction was quenched with 0.5 M H₂SO₄. The A450 measured on a 96-well plate reader. The Net A450 was obtained by subtracting the absorbance value for the blank control (no immobilized ligand) from each of the triplicate values obtained for the ligand-epitope interaction.

The selectivity assay with full length His₆ tagged Akt1, Akt2 and Akt3 was performed following the same protocol, using 25 nM protein instead of 2.5 μ M His₆ tagged peptide epitope.

Measurement of binding affinity of N-tL by Surface Plasmon Resonance:

A Biacore T100 machine was used for SPR experiments. A Streptavidin Chip (Series S, G.E. Healthcare) was conditioned as per manufacturer's recommendation. A stock of 1 mM biotinylated ligand was diluted into HBSP+ Buffer (G.E. Healthcare) to a final concentration of 100 nM

and ~150 RU was immobilized on the chip. Serial dilutions of the Akt2 protein were made in HBSP+ buffer for the experiment with the immobilized N-tL. The solution was flowed over the chip at 50 μ L/min. Binding and dissociation were carried out at 10°C with a contact time of 350 sec, a dissociation time of 390 sec, and a stabilization time of 200 sec, with buffer blanks between each concentration. The response was double corrected, using an unmodified reference flow cell and the response for the 0 concentration of Akt2 on the sample flow cell. The data was fitted to the kinetic Langmuir 1:1 binding model using the Biacore Evaluation software for T100 to calculate the dissociation constant.

OVCAR3 lysate pulldown assay:

OVCAR3 cells (ATCC) were grown in RPMI-1640 media containing 20% fetal bovine serum. Passage three cells were grown to ~80% confluence. Cells were lysed with lysis buffer (10 mM Tris-Cl (pH = 7.5), 100 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS (w/v), 0.5% deoxycholate, 1 mM DTT, 1 mM EDTA, 1X PhosStop phosphatase inhibitors (Roche), 1X Complete protease inhibitors (Roche)) and the amount of protein measured using BCA Protein Assay Kit (Pierce Biotechnologies, Inc). Streptavidin-agarose resin solution (EMD) was swelled in TBST (25 mM tris chloride, pH= 7.5, 150 mM NaCl, 0.05% Tween-20). Each of the biotinylated ligands was immobilized on the Streptavidin-agarose resin by adding 7.2 μ L of 1 mM ligand stock (DMSO) to 10 μ L of the swelled streptavidin-agarose resin. For the blank control, the resin was treated with 7.2 μ L of 1 mM biotinylated acyl glycine. Biotinylated pS473 antibody (Cell Signaling) was added in a 1:200 ratio as per vendor's suggestion. After shaking overnight at 4°C, 50 μ M D-biotin was added to the resin to block any remaining sites. The resin was washed with TBST five times, for fifteen minutes each. The resin was then swelled in binding buffer for two hours. To each of the immobilized ligands, 200 μ L OVCAR3 cell lysate (1.9 mg/ml) was added. The tubes were shaken at 4°C for 18 hours. The beads were extensively washed, three times for fifteen minutes in binding buffer, three times (fifteen minutes) in TBST, and three times (fifteen minutes) in TBS to remove unbound proteins. The resin bound proteins were eluted by adding 40 μ L of 2X SDS-PAGE sample loading buffer (BioRad) and heating at 95°C for five minutes. A 1:10 diluted sample of lysate in sample loading buffer was prepared by mixing 1 μ L of the sample with 9 μ L sample loading buffer and denaturing the sample by heating at 95°C for five minutes. 10 μ L of each sample was loaded on a 12% SDS-PAGE gels (BioRad) and run for 80 minutes at 110 volts. Three identical gels were loaded and run at the same time. One gel was used for Coomassie staining with Coomassie Fluor Orange Protein Gel Stain (Molecular Probes) following the manufacturer's protocol. The two other gels were transferred to nitrocellulose membranes by the semidry transfer method, blocked for two hours at 4°C with 5% non-fat milk, and treated overnight at 4°C with a 1:1000 dilution of pan-Akt rabbit monoclonal antibody (Cell Signaling Technology) and a 1:1000 dilution of pS473 Akt rabbit monoclonal antibody (Cell Signaling Technology), respectively, in 0.5% non-fat milk. The membranes were washed and treated for an hour with a 1:10,000 dilution of monoclonal mouse anti-rabbit- HRP secondary antibody (Cell Signaling Technology) in 0.5% milk. After five washes of five minutes each with TBST and one wash of five

minutes with TBS, the blots were developed with West Dura ECL substrate (Thermo Scientific) and imaged on film.

Non-radioactive kinase assay to evaluate effect of ligands on Akt2 kinase activity:

The non-radioactive kinase assay kit for Akt2 was purchased from Cell Signaling. Ligand solutions were made in DMSO. Kinase reactions were set up in 1X kinase buffer (25 mM TrisHCl (pH 7.5), 10 mM MgCl₂, 0.01% Triton-X, 1X Complete protease inhibitor (Roche), 1X Phosstop phosphatase inhibitor (Roche)), each 25 μ L reaction mixture containing 400 ng Akt2, 400 ng GST-GSK-3 α / β fusion protein, 500 mM ATP and 0.5 μ L of peptide solutions in DMSO or DMSO only. Reactions were allowed to proceed for 30 minutes at 30°C. A reaction mixture was also prepared using a 1:2.5 dilution of anti pS473 antibody (Cell Signaling) in the kinase buffer instead of the peptide ligand or DMSO. The reactions were quenched by adding 12.5 μ L of 2X SDS sample loading buffer and heating at 95°C for five minutes. 10 μ L from each sample was loaded on an any kD SDS gel (Biorad) and run for an hour at 110 volts. Following semidry transfer of the gel to a nitrocellulose membrane, the membrane was blocked in 5% nonfat milk/TBST for an hour at 4°C, and treated overnight at 4°C with a 1:200 dilution of Phospho GSK-3 α / β Ser (21/9) rabbit antibody (Cell Signaling) in 0.5% milk/TBST. Following washes, the membrane was treated with a 1:2000 dilution of mouse anti-rabbit –HRP antibody for an hour at 4°C. After four five minute washes with TBST, and one five minute wash with TBS, the membrane was treated with West Dura ECL substrate (Thermo Scientific) and imaged on film.

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