# Chapter 3: Epitope targeting strategy: developing capture agents targeted against C terminal hydrophobic motif of the Akt2 protein

## 3.1 Introduction

The term epitope targeting is widely referred to in antibody development literature. An epitope is a part of an antigen that is recognized by the immune system, specifically by antibodies, T cells and B cells. An epitope can be a continuous or discontinuous stretch of amino acids within the antigen[1]. The ability of the antibodies to specifically target one vital region (generally ~10 amino acids) within a protein is its major advantage, as in most of todays biological analytic and diagnostic platforms, it is not only sufficient to recognize a protein, but it is vital to identify certain states or mutations of the protein (e.g. phosphorylation of a certain residue[2] or the point mutation of an amino acid[3]). In this chapter, we describe the development of a peptide based capture agent to specifically target a certain region of the protein. This strategy will henceforth be referred to as the epitope targeting strategy, the epitope being, in this case, a continuous region of the protein. In this strategy, the target protein region is chemically synthesized as a peptide so that it can be modified to incorporate an azide/alkyne handle. The peptide not only acts as the catalyst, but also as reaction component when screened against a peptide library that contains the complementary click handle. For the peptide that binds in proper region and in proper orientation, the weak peptide peptide interaction is strengthened by the covalent bond formation between the azide and alkyne functionalities. Once the peptide binder to the target peptide is identified, the screening process is repeated to develop a biligand and finally a triligand. In the multiligand screens, the entire protein is used as the target.

The peptide target is a 32mer peptide fragment from the C terminal of Akt2. There were several reasons for choosing this target. This peptide region is flexible and unstructured, so that its tertiary structure is not resolved in solved crystal structures of Akt2. It is likely that this peptide fragment is on the surface of the protein and not buried inside the protein. Since the target

is only a portion of the protein rather than the entire protein, to make the strategy successful, the target peptide should obey two criteria: firstly, it should be surface exposed on the entire protein and secondly, the properties of the flexible modified target peptide should not be significantly different from the peptide fragment in the entire protein. This target peptide satisfied all the structural criteria.

The selected target fragment was also selected on the basis of its functional significance, as it is the site of a serine phosphorylation and contains a hydrophobic motif that acts as an allosteric activator of the Akt2 protein. Akt collectively refers to three isoforms (Akt1, Akt2, and Akt3), and is a member of the serine/threonine AGC protein kinase family[4,5]. Akt plays a central regulatory role in growth factor signaling, and serves as a key node in the phosphatidylinositol 3-kinase (PI3k) signaling pathway. Over expression and/or hyperactivation of Akt is associated with breast[6], ovarian[7], colon[8], pancreatic[9], and bile duct[10] cancers, making Akt an attractive drug and diagnostic target[11]. Ser474 is located in the hydrophobic motif (HM) of the C-terminal tail, and is phosphorylated by mTOR Rictor[12]. The phosphorylated HM acts as an allosteric activator of Akt2 by binding to a hydrophobic groove in the N-lobe of Akt2 and enhancing the kinase activity 10-fold[13,14]. In the context of current studies showing that ATP competitive inhibitors of Akt2 can cause hyperphosphorylation of the protein[15,16], it can be worthwhile to find binders to the C terminal kinase regulatory domain, as such binders would have a high potential to regulate kinase activity.

Initially we explored two alternative methods of modifying the target peptide with a click handle. The azide group provides an anchor site for an in situ click reaction[17,18] while the biotin label is used as a screening and assay handle. In the first method, the 32-mer C-terminal polypeptide fragment of Akt2 (amino acids 450-481) with the phosphorylated Serine 474 (p-S474) is modified with a dinuclear Zn (II) DPA type complex, which has an appended biotin label and an azide handle. The dinuclear Zn(II)DPA selectively binds to the phosphate anion[19]

and provides an initial in situ click reaction site adjacent to the phosphorylated residue azide functionality close to the hydrophobic motif. In the second method, the azide handle is incorporated inside the 32mer C terminal polypeptide fragment with pSer474, by substituting an amino acid within the peptide. The hydrophobic motif FPQF(pS)YS is left unperturbed and I479, separated by two amino acids from the hydrophobic motif, is replaced by L-azidolysine. However, as screens in both methods yield very similar ligands, we pursue only the first method. The monoligand obtained from the first method is expanded through further screens against the peptide and protein to develop several biligands and ultimately two triligands. In this chapter, I describe the process of epitope targeting and the development of triligand peptides. In the next chapter, the developed multiligands are characterized and their effects on the kinase activity of the Akt2 protein are explored.

## 3.2 Materials and methods

### 3.2.1 Materials

Fmoc amino acids were purchased from Anaspec (San Jose, CA) and AAPPTec (Louisville, KY) and used as received. TentaGel S-$NH_2$ resin (diameter 90 μm, capacity 0.28 mmol/g) was obtained from Anaspec (San Jose, CA) 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. (Gibbstown, NJ) and used for synthesis of biotinylated peptides. Amide Sieber resin (capacity 0.3-0.6 mmol/g) purchased from Anaspec (San Jose, CA) 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. (Gibbstown, NJ), ChemPep (Miami, FL), and Sigma-Aldrich (St. Louis, MO) respectively. DMF (N, N′-dimethylformamide), piperidine, TFA (trifluoroacetic acid, 98% min. titration), and TES (triethylsilane) were purchased from Sigma-Aldrich (St. Louis, MO). 5-Azido-pentanoic acid was

purchased from Bachem Americas, Inc. (Torrance, CA). BCIP (5-Bromo-4-chloro-3-indolyl phosphate) was purchased from Promega. Active Akt2 (with N terminal His6 tag) was purchased from Abcam (Cambridge, MA). Mouse anti biotin antibody-Alkaline Phosphatase conjugate was purchased from Sigma Aldrich.

## 3.2.2 Methods

### 3.2.2.1 Synthesis of phosphate binding dinuclear metal ligand complex

**3,5-bis((bis(pyridin-2 -ylmethyl)amino)methyl)-4-hydroxybenzoic acid ester** (Compound **1**)**:** N, N–di(2-picolyl) 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[20,21]. Then dicholromethane (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 dicholoromethane /methanol /ammonium hydroxide afforded light yellow semi solid. Calculated mass: [M+H] 588.6 Observed mass: [M+H] 589.29

**3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoic acid** (Compound **2**)**:** The purified semisolid compound **2** 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]+ 561.25, [M-H]- 559.25 Observed mass: [M+H]+ 561.25, [M-H]- 559.4

**$Zn_2$L-Az4-$PEG_2$-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 $N_\alpha$-Fmoc protecting group was removed by treating with 20% piperidine in NMP. 1.5 equivalents of compound **2** 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 compound **2** 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.

#### 3.2.2.2 Verification of binding of $Zn_2$L-Az4-PEG$_2$-Biotin to phospho-amino acids and a phosphate containing peptide

422 μM solution of $Zn_2$L-Az4-PEG$_2$-Biotin was made dissolving the HPLC purified solid in 10 mM tris borate buffer (TBS) (pH 8). Saturated solutions of pure phosphoserine, phosphotyrosine and pSrc substrate Ac-I-pY-GEF (Novabiochem) was made in the buffer. The *$Zn_2$L-Az4-PEG$_2$-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 mixed in a 1:1 ratio with the matrix, and subjected to Maldi TOF in a positive mode, show the peaks corresponding to (*$Zn_2$L-Az4-PEG$_2$-Biotin* – pSer), (*$Zn_2$L-Az4-PEG$_2$-Biotin* – pTyr) and (*$Zn_2$L-Az4-PEG$_2$-Biotin* – Ac-I-pY-GEF)[22]. The pure compound *$Zn_2$L-Az4-PEG$_2$-Biotin* mixed with the THAP matrix yields the major peak corresponding to its mass.

**Figure 3.1: Synthesis of dinuclear Zn chelator $Zn_2$L-Az4-$PEG_2$-Biotin.**

Starting from paraben, 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoic acid is synthesized (compound **2**). Compound **2** is coupled via amide coupling to PEG containing biotin novatag resin. The compound cleaved off bead and treated with Zn acetate yields *$Zn_2$L-Az4-$PEG_2$-Biotin*.

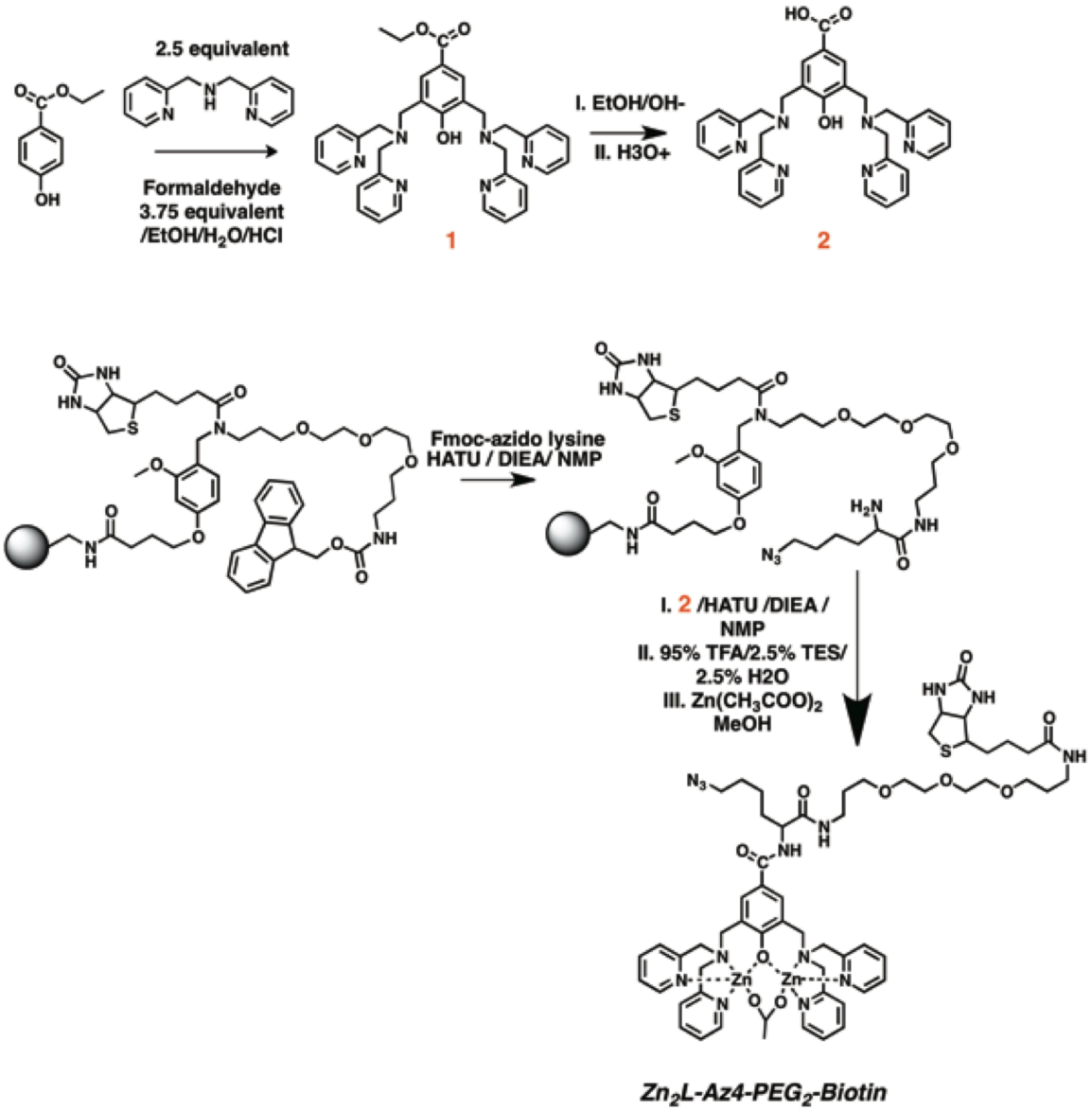
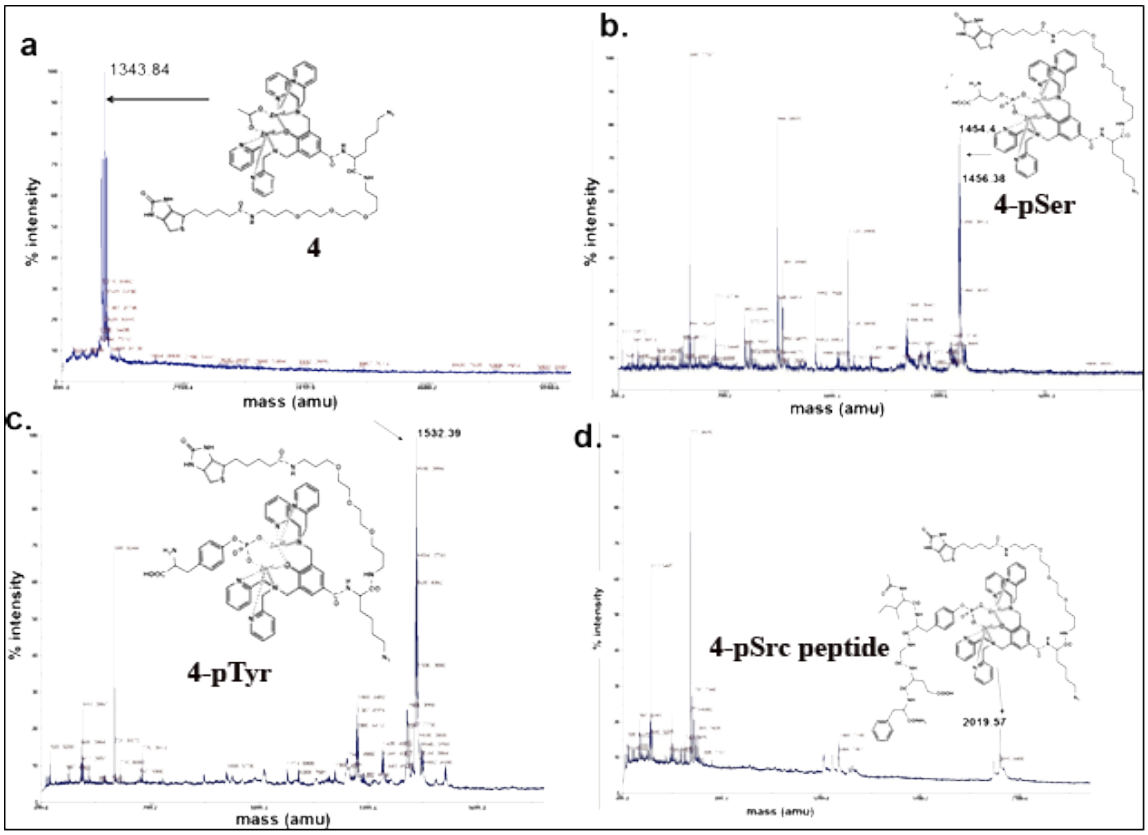


**Figure 3.2: Evidence of binding of dinuclear zinc chelator to phospho amino acids and phospho peptide.**

Structure and Maldi TOF spectra of the Zn chelator *$Zn_2$L-Az4-PEG$_2$*-Biotin and complexes of *$Zn_2$L-Az4-PEG$_2$-Biotin* with phosphorylated amino acids and phosphorylated peptides. Mass spectrum of a) pure *$Zn_2$L-Az4-PEG$_2$-Biotin* (observed mass M.2H2O = 1369, (M-N2).2H2O = 1343.8 ; expected mass M.2H2O = 1369.45, (M-N2).2H2O = 1343.45). b) *$Zn_2$L-Az4-PEG$_2$-Biotin* - phospho-serine (observed mass =1454.4; expected mass = 1454.43) c) *$Zn_2$L-Az4-PEG$_2$-Biotin* - phospho-tyrosine (observed mass = 1532.39 ; expected mass = 1532.46). d. Mass spectrum and structure of *$Zn_2$L-Az4-PEG$_2$-Biotin* - Ac-I-pY-GEF (observed mass =2019.57; expected mass = 2019.86).



### 3.2.2.3 Peptide library Synthesis

Randomized OBOC[23] libraries of hexapeptides were synthesized using the Titan 357 Automated Peptide Synthesizer (AAPPTec) on 90 μm polyethylene glycol-grafted polystyrene beads (TentaGel S-$NH_2$, 0.28 mmol/g, 2.86 x 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[24].

**Table 3.1: Summary of libraries used in screens against the Akt2 protein.**

| Formula | Components | Number of unique sequences |
|---|---|---|
| Library A: D-Pra-XXXXX-10% M-TG | X = 18 D amino acids except D-Met and D-Cys | 1,889,568 |
| Library B: XXXXX -D-Pra-10%M-TG | X = 18 D amino acids except D-Met and D-Cys | 1,889,568 |
| Library C: Az4- XXXXX -10%M-TG | X = 18 D amino acids except D-Met and D-Cys | 1,889,568 |

### 3.2.2.4 Screening with One Bead One Compound (OBOC) peptide library

*Screen for mono-L:*

50 nM solution of target phospho-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 metal chelated anchor *$Zn_2$L-Az4-$PEG_2$-Biotin* was added to the 50 nM solution of the phosphor-peptide-1 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. The screen was conducted using library A, of the form D-Pra-XXXXX-10% M-TG. 250 mg of beads were used in the screen. The beads were equilibrated in binding buffer (25 mM tris hydrochloride pH 7.5, 150 mM NaCl, 0.1% BSA, 0.05% Tween 20) by shaking for 8 hours. The complex formed by incubating the target phospho-peptide with *$Zn_2$L-Az4-$PEG_2$-Biotin*, denoted as *Complex-1*, 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 three times with binding buffer, thrice with TBST (0.05% tween 20/TBS) and three times 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 $MgCl_2$) (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 solution. The hit beads were picked with a pipette tip and transferred to a spinnex tube. The turquoise color of the hit beads was removed by washing with DMF. The proteins on the beads were stripped buffer. The exact screen protocol was repeated, 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 7.5 M guanidium hydrochloride (pH 2) and water, and sequenced using the Edman Sequencer.

**Table 3.2: Sequences of hits from the monoligand (*mono-L*) screen.**

| **D-Pra** | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ |
|---|---|---|---|---|---|
| D-Pra | w | k | v | k | l |
| D-Pra | w | k | v | k | l |

*Screen for bi-L:*

*Prescreen:*

2 batches of 135 mg of library B of form XXXXX-D-Pra- 10% M- TG were washed in water, and swelled overnight in binding buffer. 20 μM and 50 μM solutions of compound 4 was added to the beads and shaken for 10 hours at room temperature. The beads were washed thrice, for fifteen minutes, 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, wash buffer 2, TBS and then washed once with AP buffer. 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 compound **4** or the detection antibody, were picked up manually. The clear beads were stringently washed with DMF, guanidium hydrochloride and water as described in the previous paragraph.

*Product screen:*

The washed beads from each prescreen were dried, then swelled overnight in binding in 8 ml fritted polypropylene solid-phase synthesis tubes. In two separate eppendorf tubes, 20 μM and 50 μM solution of compound **4** was incubated overnight at room temperature with 10 nM and 25 nM target phospho-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 Table 3.3 and Table 3.4.

**Table 3.3: Hit sequences from the biligand (*bi-L*) screen with 25 nM target peptide.**

| $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | 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 3.4 Hit sequences for biligand screen with 10 nM target peptide.**

| $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | 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 |

*Screen for N-term-tri-L:*

*Prescreen:*

500 mg of library A of the form D-Pra-XXXXX-10%M- TG were swelled in binding buffer 2 overnight. The beads were incubated with 25 μM solution of *Anchor-3N* (Figure 3.10) 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 reequilibrated 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 $MgCl_2$, 0 . 0 5 % (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 $MgCl_2$). 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 preclear screen were swelled overnight in binding buffer 2. 25 μM solution of biligand anchor was incubated for 30 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 reequilibrated 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 2 (50 nM Akt2 protein, preincubated with 73.5 μM biligand anchor 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 binding buffer 2. A 1:1000 dilution of mouse anti $His_6$ 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 3.5. 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-$NH_2$ 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 $His_6$ 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 was recorded in Table 3.6, 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.

**Table 3.5: Hit sequences for N terminal triligand (*N-term-tri-L*) screen.**

| D-Pra | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ |
|---|---|---|---|---|---|
| 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 3.6: Elimination of peptide binders to the detection antibody for the N terminal triligand (*N-term-tri-L*) screen.**

| D-Pra | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | 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 |

Screen for *C-term-tri-L*:

*Prescreen:*

500 mgs of library C of form $H_2N$-Az4-XXXXX-10%M-TG was swelled in binding buffer 2 overnight. The beads were incubated with 100 μM solution of *Anchor-3C* in binding buffer for two hours at 4°C. The same screening protocol as for the prescreen for *N-term-Tri-L* screen is 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 2. 100 uM solution of *Anchor-3C* was incubated for thirty minutes at 4°C with 50 nM Akt2 in binding buffer. The same screening protocol as for the *product screen* for *N-term-Tri-L* is followed. The sequences are recorded in Table 3.7.

**Table 3.7: Hit sequences from the C terminal triligand (*C-term-tri-L*) screen.**

| L-Az4 | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ |
|---|---|---|---|---|---|
| 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 |

### 3.2.2.5 Bulk peptide synthesis

Fully deprotected peptides were synthesized on Rink Amide MBHA resin. Biotinylated peptides were synthesized on Biotin Novatag resin. Polyethylene glycol linker between the biotin tag and the amino acids were achieved by coupling Fmoc-$PEG_2$-COOH (13 atoms) directly to the Biotin Novatag resin before coupling other amino acids. Side chain protected peptides used in certain steps of synthesis were synthesized on Sieber Amide resin. Fmoc-Ser($OPO_3Bzl$)-OH (AAPPTec) was used for the incorporation of phosphoserine in the phosphorylated peptides.

Peptides generally were synthesized on the Titan 357 Automatic Peptide Synthesizer (AAPPTec, Louisville, KY). Overnight coupling steps were performed manually in 8 ml fritted polysterene tubes. The protocols for the peptide general synthesis, cleavage, Cu catalyzed on bead click reaction, and HPLC protocols followed are described in chapter 2. The synthesis and characterization of the individual peptides follow:

*Target phospho-peptide:*

The 32mer target peptide sequence, amino acids 450-481 of Akt2, *ITPPDRYDSLGLLELDQRTH-FPQF(pS)YSASIRE* was synthesized on Rink Amide MBHA resin, using the Titan 357 peptide synthesizer. Fmoc-Ser($OPO_3Bzl$)-OH (AaPPTec) was used for the incorporation of phosphoserine in the peptide. Calculated mass: $[M+H]^+$ 3832. Observed mass: $[M+H]^+$ 3831.97

**Figure 3.3: Sequence of the phospho peptide used as target epitope.**
The target phospho-peptide, amino acids 450-481 of Akt2, is phosphorylated at Ser474. The hydrophobic motif is highlighted in red.

***phospho-peptide 1***

ITPPDRYDSLGLLELDQRTH-FPQF**(pS)**YS-ASIRE

*Synthesis of mono-L:*

Fmoc-NH-$PEG_2$-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-OtBu[S2]. After washes with a copper chelating solution, the peptide was acylated. The resultant molecule S1 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 ***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

**Figure 3.4: Synthesis of the intermediate S1 for the bulk synthesis of the monoligand peptide *mono-L*.**

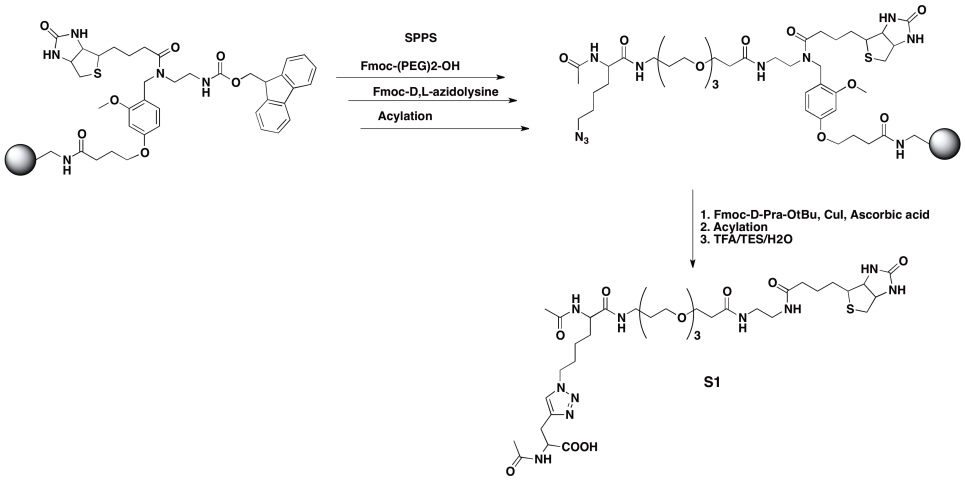
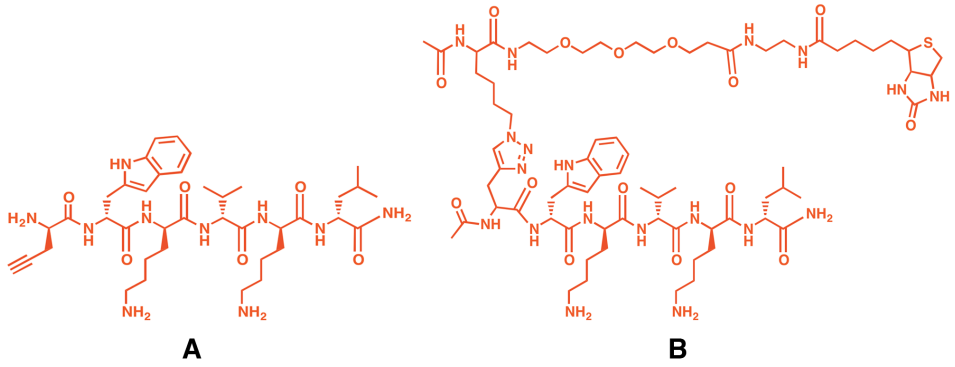



**Figure 3.5: Structure of the monoligand peptide *mono-L* developed against the target peptide.**

S1 is coupled to peptide D-Pra-wkvkl to synthesize ***mono-L.***

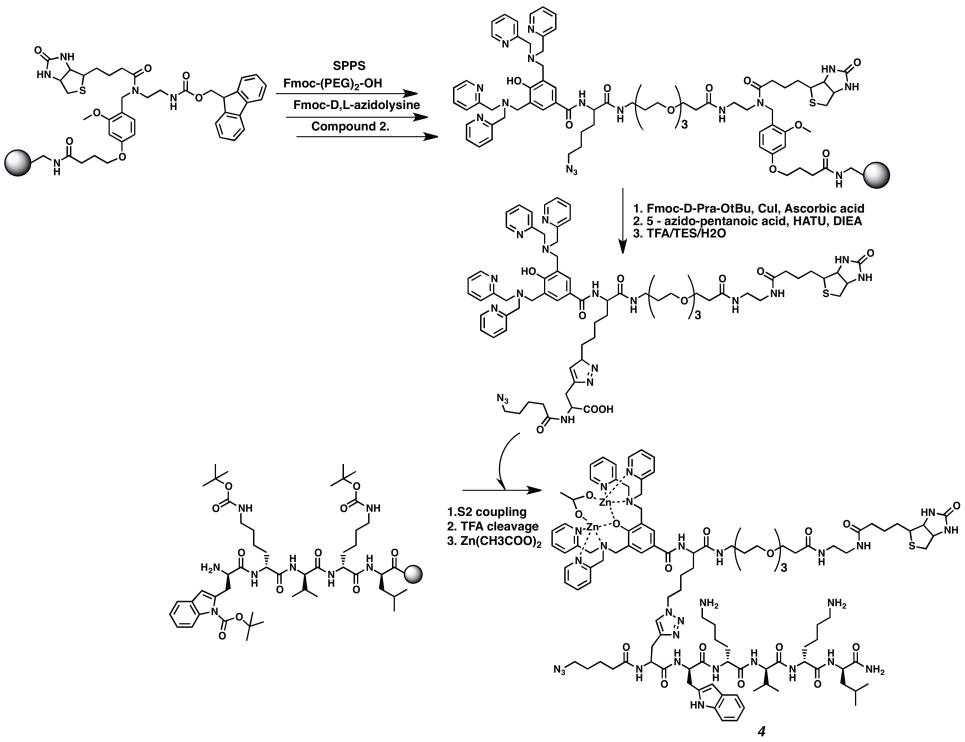


**Synthesis of compound 4:**

Fmoc-NH-$PEG_2$-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 compound **2**. The resin was then subjected to on bead Cu catalyzed click reaction with Fmoc - D-Pra-O$^t$Bu. The excess copper was removed by washing with the copper chelating solution. 5-Azido-pentanoic acid was then coupled. The resulting peptide S2 was TFA cleaved and lyophilized. The crude 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 **S2** was then coupled to the peptide. The peptide was cleaved off using TFA cleavage solution. 2 equivalents of zinc acetate was 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, compound **4**, 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

**Figure 3.6: Synthesis of the zinc chelator - monoligand complex *compound 4* used in biligand screen of Akt2.**




*Synthesis of bi-L:*

Fmoc-NH-$PEG_2$-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-OtBu. After washes with copper removing solution, 5-azido-pentanoic acid was coupled. After TFA cleavage the resultant molecule **S3** (Figure 3.7) 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$^t$Bu 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

**Figure 3.7: Synthesis of intermediate S3 for bulk synthesis of the biligand *bi-L*, biligand anchor *anchor-3C* and the triligand *C– term–tri-L*.**

**S3** is used in the synthesis of the ***bi-L***, ***Anchor-3C*** and ***C– term–tri-L*.**

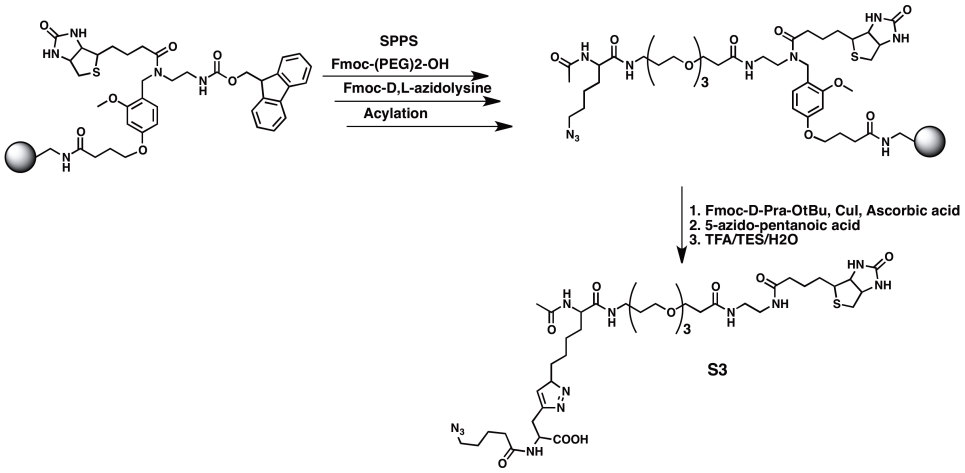
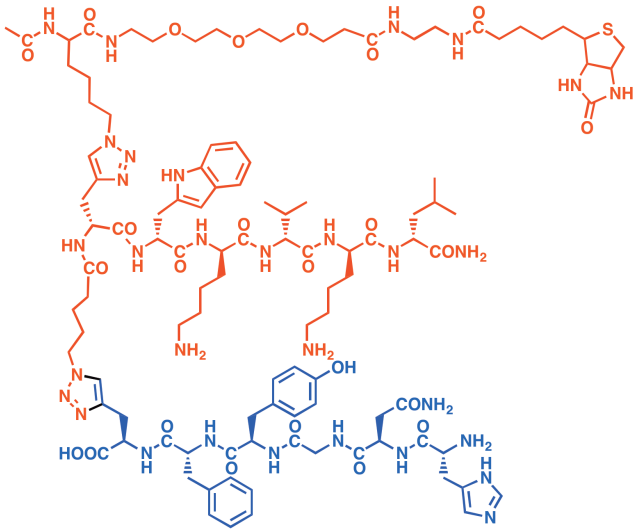



**Figure 3.8: Structure of the biligand *bi-L* isolated in the screen against the target peptide.**



*Synthesis of Anchor-3C:*

The peptide $NH_2$-wkvkk(Alloc) was made on Rink Amide MBHA resin following standard Fmoc SPPS synthesis protocol. 1 equivalent of **S3** (Figure 3.7) was then coupled to the resin. On bead click reaction was carried out overnight using 1.5 equivalents of Fmoc-D-Pra-O$^t$Bu. 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[S3]. 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

*Synthesis of Anchor-3N:*

Fmoc-NH-$PEG_2$-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 solution in DMF. On bead click reaction with Fmoc-D-Pra-O$^t$Bu 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 3.9) 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-OtBu. 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

**Figure 3.9: Synthesis of intermediate S4 for the bulk synthesis of the biligand anchor *anchor-3N* and the triligand *N-term-tri-L.***

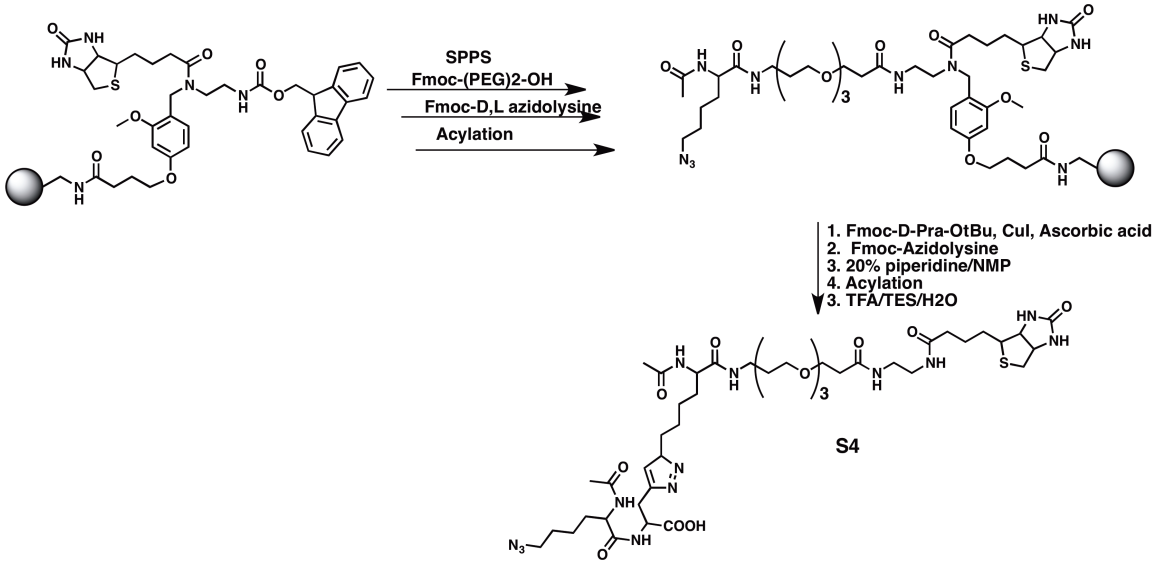
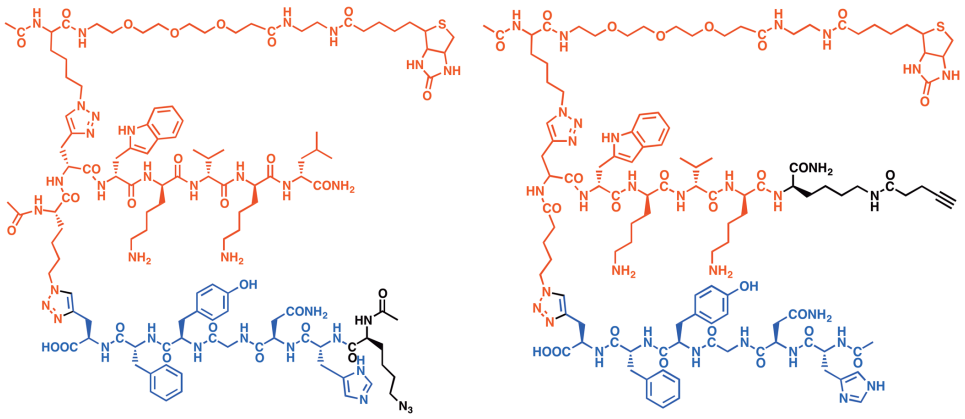


**Figure 3.10: Structure of biligand anchor peptides *Anchor-3N* and *Anchor-3C*.**

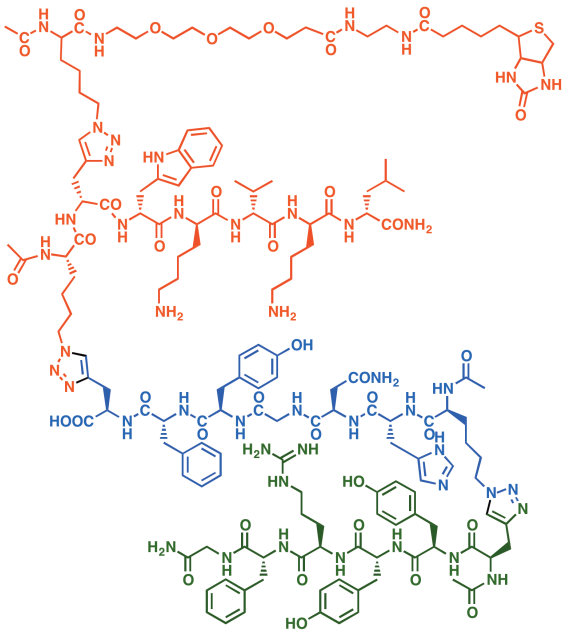
A. *Anchor-3N* is used to the Akt2 protein before screening against a alkyne containing OBOC library A. *Anchor-3C* is used to the Akt2 protein before screening against a alkyne containing OBOC library.




*Synthesis of N-term-tri-L:*

Side chain protected version of Ac-D-Pra-yyrfG-$CONH_2$ 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-$CONH_2$. 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

**Figure 3.11: Molecular structure of the triligand *N-term-tri-L*.**



*Synthesis of N-term-tri-dimer:*

Fmoc-NH-$(PEG)_2$-OH was coupled using standard Fmoc protocol on Biotin Novatag resin. Fmoc-Dap (Fmoc)-OH was then coupled, providing two amine terminals for further amino acid couplings, which happened in parallel. Following the coupling of Fmoc-NH-$(PEG)_2$-OH, wkvkl was synthesized on both of the amine termini. The intermediate compound **S5** (Figure 3.12) was then added to provide two azide click handles. After click reaction with Fmoc-D-Pra-O$^t$Bu, the peptide was extended to hnGyf on the N terminal and then acylated. On bead Cu catalyzed click reaction was carried out with the resin bound peptide and 2 equivalents of side chain protected HPLC purified Ac-D-Pra-yyrfG. After treatment with the Cu chelating solution, the peptide was dried, cleaved off resin with TFA cleavage solution and HPLC purified. Mass calculated: 7245.2 Mass observed: 7244.97

**Figure 3.12: Synthesis of intermediate compound S5 for bulk synthesis of the triligand *N-term-tri-L-dimer.***

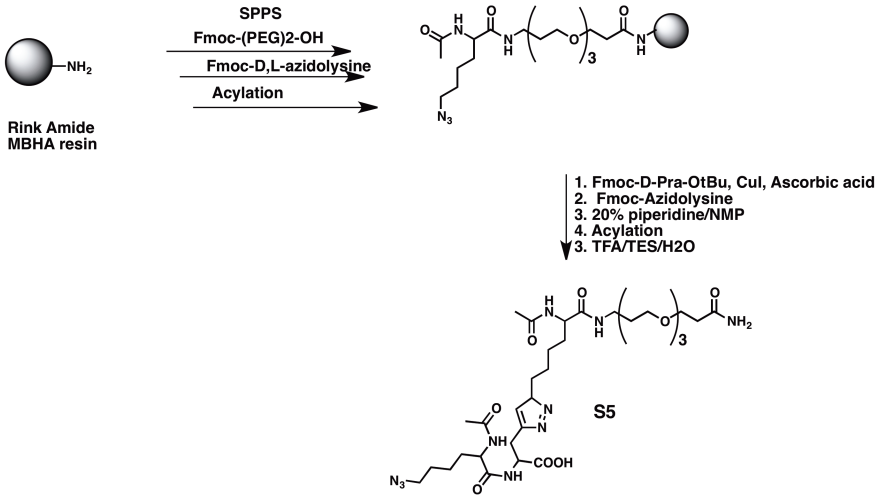
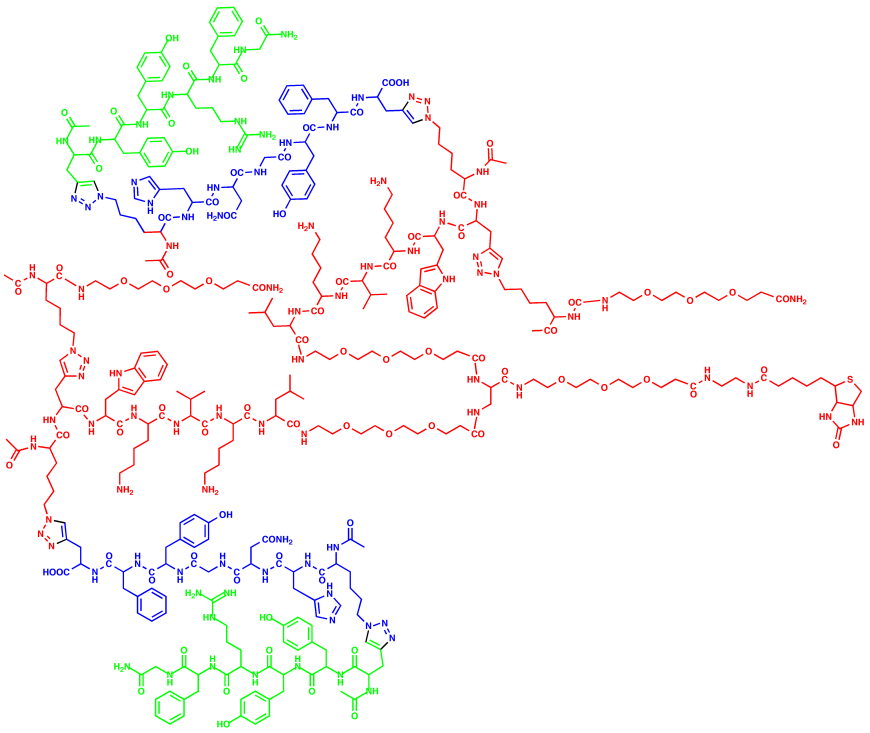


**Figure 3.13: Molecular structure of the triligand *N-term-tri-dimer.***



*Synthesis of D-Lys (pentyne) amide:*

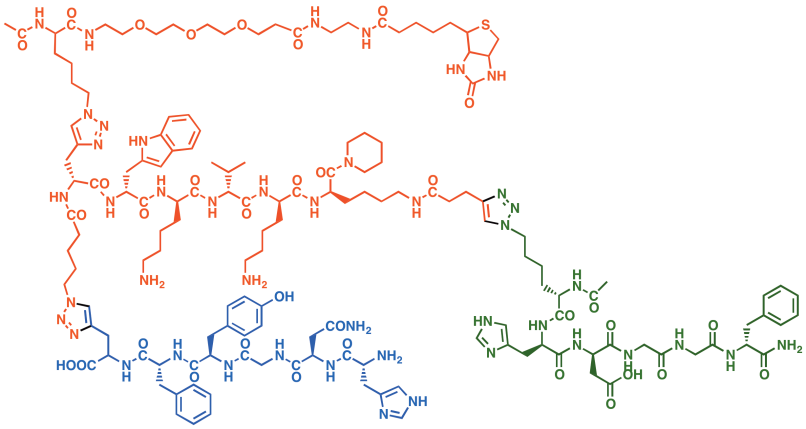
Boc-D-Lys(Fmoc)-OH was coupled with rink amide resin. Then 4-pentynoic acid was coupled with it, after standard piperidine deprotection. The dried resin was cleaved with TFA cocktail and purified using a gradient of water and acetonitrile and 0.1% TFA on the prep-HPLC. Mass calculated: (M+H) 225 Mass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

*Synthesis of C-term-tri-L*:

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$^{t}$Bu. 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: 3199.6 Mass observed: 3198.4.

**Figure 3.14: Molecular structure of the triligand *C-term-tri-L*.**



## 3.3 Results and discussion

### 3.3.1 Synthesis of the dinuclear zinc chelator $Zn_2$L-Az4-PEG$_2$-Biotin and verification of its binding to phospho amino acids and phosphopeptide

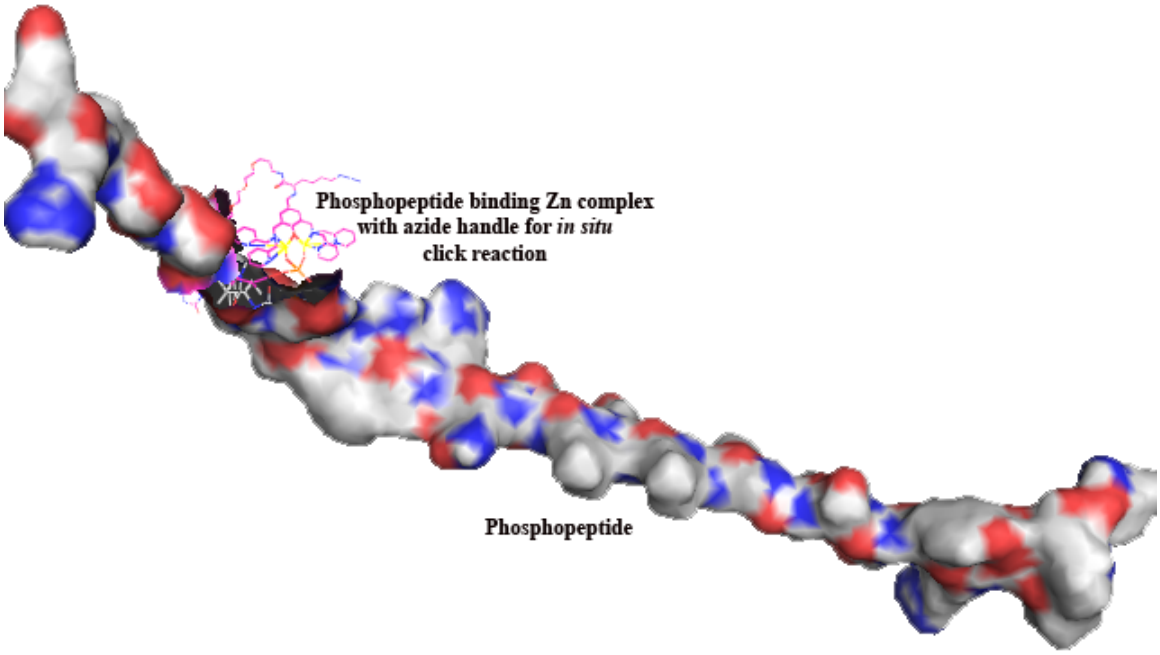
Dinuclear Zn(II)-dipyridylamine (DPA) complexes, due to their avidity for phosphate anions over other anions such as carbonate, sulphate, or chloride, have been used, for example, in the development of fluorescence-based sensors of kinase activity [21], for the detection of bacterial infection sites [22]. In this work, the property of dinuclear Zn(II)DPA to selectively bind to the phosphate anion is combined with the technique of in situ protein catalyzed click chemistry to develop a new strategy of chemical epitope targeting. Ethyl 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoate (compound **1**,Figure 3.1) was synthesized by the

aromatic Mannich reaction of 2.5 equivalents of N,N –di(2-picolyl)amine and 3.75 equivalents of formaldehyde with ethyl 4-hydroxobenzoate under reflux conditions. Alkaline hydrolysis of the ester yielded 3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzoic acid (compound **3**, Figure 3.1), a heptadentate ligand. The carboxylic acid group on **2** is compatible with solid phase Fmoc based peptide synthesis. To add an azido tag and a biotin tag to **2**, Fmoc -azidolysine was coupled using standard SPPS protocols to Biotin PEG Novatag resin, followed by the coupling of ***3*** to the resin. After cleaving the compound off the resin with trifluoroacetic acid and subsequent HPLC purification, a treatment in solution with Zinc acetate yielded the desired ligand *$Zn_2$L-Az4-$PEG_2$-Biotin* (Figure 3.1). The property of *$Zn_2$L-Az4-$PEG_2$-Biotin* to selectively bind to phospho-serine, phospho-tyrosine and a phospho-peptide (pSrc substrate) was demonstrated by incubating *$Zn_2$L-Az4-$PEG_2$-Biotin* with the respective amino acids or peptide in borate buffer, followed by Maldi TOF in a positive mode, that yielded the peaks for *$Zn_2$L-Az4-$PEG_2$-Biotin* -pSer, *$Zn_2$L-Az4-$PEG_2$-Biotin* -pTyr and *$Zn_2$L-Az4-$PEG_2$-Biotin* -pSrc complexes[23] (Figure 3.2).

### 3.3.2 Modification of peptide fragment of Akt2

For the development of an anchor ligand targeted against the Akt2 C-terminal phospho-peptide, we reacted *$Zn_2$L-Az4-$PEG_2$-Biotin* with the phosphorylated, C-terminal Akt2 polypeptide (amino acids 450-481 with p-S474) to provide an initial (temporary) in situ click reaction site adjacent to the phosphorylated residue. This complex is *Complex-1* (Figure 3.15).

**Figure 3.15: Complex-1 is formed by the reaction of dinuclear zinc complex with the phospho-peptide epitope.**

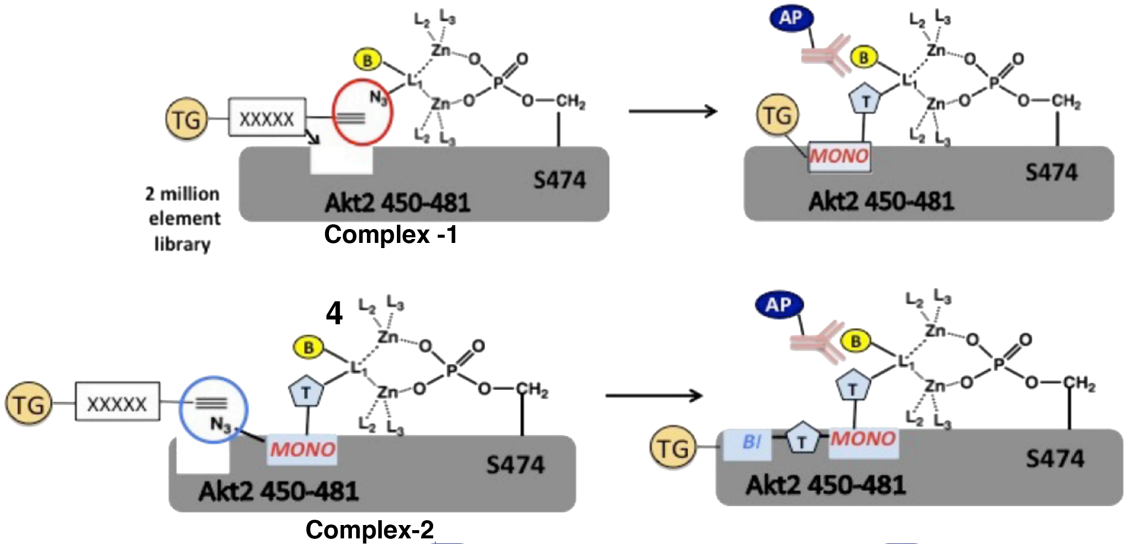


### 3.3.3 Identification of a 1$^{o}$ ligand targeted against Akt2 C terminal fragment

*Complex-1* was incubated with an acetylene-containing one-bead-one-compound (OBOC) 6-mer peptide Library A (Figure 3.16), which consisted of all possible combinations of 18 non-natural and artificial amino acids in 5 positions (Table 3.1). The OBOC library was probed with a monoclonal anti-biotin antibody conjugated with Alkaline Phosphatase (AP) enzyme (anti biotin mAb–AP). The hit beads turn turquoise colored upon treatment with the chromogenic BCIP (5-Bromo-4-chloro-3-indolyl phosphate) substrate for AP[1,2]. These hit beads (around 5000) contain both the desired in situ click hits, as well as false positives that exhibit binding to the anti biotin mAb–AP. The hit beads were stringently washed to remove all non-covalently bound biological and chemical agents, then rescreened with *Complex-1*, then probed with *biotin bound* anti biotin mAb – AP .The true hit beads remain clear when treated with BCIP. Those true hits were sequenced by Edman degradation to identify candidate 1$^{o}$ ligands. This dual screening strategy ensures that the beads identified as true hits have clicked product on-bead[3,4]. Remarkably, this strategy produced only true 2 hits (from a starting (oversampled) library size of 1.9 million sequences), which sequenced to yield the same peptide: D-Pra-wkvkl, which, when appended to azidolysine, $(PEG)_2$ and biotin label, formed *mono-L* (Figure 3.5).

**Figure 3.16: Screening strategy for developing a capture agent targeting the C terminal hydrophobic motif of the Akt2 protein.**

*$Zn_2$L-Az4-$PEG_2$-Biotin* is coupled with the Akt2 32-mer C-terminal fragment containing p-S474 through the interaction to create *Complex-1*. *Complex-1* is screened against Library A to yield 1$^o$ ligand candidates, from which a consensus 1$^o$ ligand (*mono-L*) is identified. In the next round of ligand development, compound **4** is coupled to the C-terminal fragment through the Zn chelator to create *Complex-2*. *Complex-2* is screened against Library B to identify candidate 2$^o$ ligands, from which a consensus biligand (*bi-L*) is prepared.




## 3.3.4 Identification of a biligand targeted against Akt2 C terminal fragment

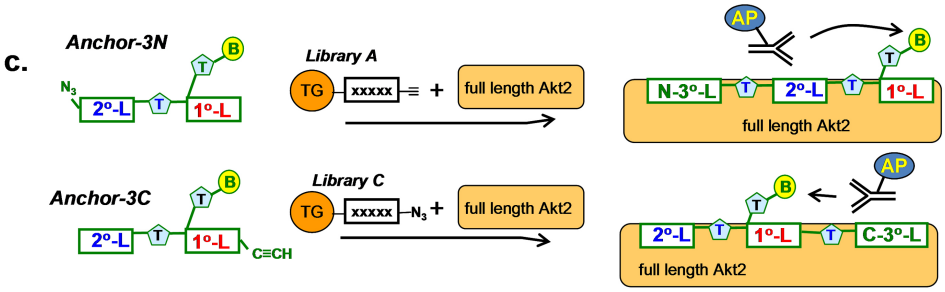
We used a similar strategy to identify candidate 2$^o$ ligands. The Zn chelator *$Zn_2$L-Az4-$PEG_2$-Biotin* was modified with peptide wkvkl and an azide on the N terminal to form compound **4**, which was then reacted with the C-terminal 32-mer polypeptide fragment of Akt2 containing p-S474 to form *Complex-2* (Figure 3.16). Library B was screened against two concentrations of *Complex-2*. To eliminate background binders to the detection antibody or to compound **4**, pre-screens were performed. The pre-screening conditions replicated actual screen conditions, except

that compound **4** was used in place of *Complex-2*. In the prescreen, Library B, equilibrated in the binding buffer, was incubated with compound **4,** and then probed with anti-biotin mAb–AP following the screening protocol. Colored beads were removed from the library, and the clear beads are subjected to a series of stringent washes and then screened against *Complex-2*. The hit beads from that screen were then treated with guanidium chloride and sequenced. The sequences exhibited a high homology, and are listed in Table 3.3 and Table 3.4. Based on the sequence homology, four peptides were chosen as potential $2^o$ ligand candidates. Simpler versions of the biligand candidates were made substituting a clicked triazole with histidine. These ligands were then compared in an immunoprecipitation assay for their ability to pulldown Akt2 from OVCAR3 cell lysate. The best performing biligand was resynthesized in bulk, this time containing no substitutions, *(bi-L)* and further characterized.

### 3.3.5 Development of Triligands against full length Akt2 protein

The biligand was separately modified at both the N- and C-termini, with acetylene- and azide-containing amino acids, respectively, to form *Anchor-3N* and *Anchor-3C* (Figure 3.10) for in situ click triligand screens. Both screens involved pre-clear steps similar to that described above. The major difference was that these screens utilized the full length active Akt2 as the target/catalyst, and employed *Libraries A* and *C* respectively. Several candidate $3^o$ ligands were identified from these screens. A subset was selected for scale-up and testing as candidate triligands (Table 3.6 and Table 3.7). The two triligands *C-term-tri-L*, *N-term-tri-L* **(**Figure 3.14**,** Figure 3.11**)** were synthesized. Since dimerizing of a peptide ligand may create a ligand with higher affinity to the protein, the ligand *N-term-tri-L,* having high affinity, was dimerized using a diaminopropionic acid and polyethylene glycol spacer. *N-term-tri-L-dimer* (Figure 3.13) were synthesized in bulk and characterized.

**Figure 3.17: Scheme describing triligand screens using *anchor-3N* and *anchor-3C*.**
*Anchor-3N* and *Anchor-3C* are synthesized by modifying *bi-L* with an azide group at the N-terminal and an alkyne group at the C-terminal of the peptide, respectively. *Anchor-3N* and *Anchor-3C* are separately screened against Library A and Library C in the presence of full length Akt2 to identify candidate 3° ligands, from which the consensus triligands are identified. The screens involve various other steps (not shown) that were designed to remove false positive hits. All hit beads are identified using alkaline-phosphatase (AP)-labeled anti-biotin antibody as a means of detecting the on-bead triazole linked product.




## 3.4 Conclusion

In this chapter I have described the development of the epitope targeting strategy. We modified the peptide fragment from C terminal of Akt2 to incorporate an azide handle. This modified complex acts as the scaffold and a reactant when exposed to a peptide library with a complementary alkyne handle. Two rounds of ligand development were done with the peptide fragment to develop a biligand. In this biligand stage we decided to eliminate the phosphate binding dinuclear zinc complex, as this type of molecule can react with several biological molecules. Then we applied the strategy of developing Iterative In situ Click capture agents by screening against the entire protein, to yield several triligands. In the next chapter we shall discuss the characteristics of the developed ligands. It is to be noted that although the peptide modification was done using a dinuclear zinc chelator, there can be several other ways of achieving this goal, the simplest of which is direct amino acid substitution in the target peptide to

insert a click handle. In fact, our preliminary data with that method yielded sequences similar to the followed method. The epitope targeting strategy is a particularly valuable strategy when the target protein has posttranslational modifications like glycosylation or phosphorylation, which are difficult to express or purify.

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