Developing peptide based capture agents for diagnostics and therapeutics

Thesis by
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In Partial Fulfillment of the Requirements for the degree
of
Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY
Pasadena, California
2013
(Defended May 16, 2013)

DEDICATIONS

I dedicate this dissertation to my father, friend, philosopher and guide, Mr. Dilip Kumar Nag

ACKNOWLEDGEMENTS

I want to thank my advisor, Professor J. Heath for giving me an opportunity to work with him. I have always been inspired by Jim's vision of science and his ability to define scientific challenges in new and unconventional ways. Throughout my graduate research, Jim has been very supportive, allowing me to define and evolve my own projects. I would also like to thank the members of my thesis committee, Professor David Tirrell, Professor Robert H. Grubbs, and Professor Long Cai for their support and advice.

I immensely enjoyed interacting with Professor Barry Sharpless, Professor Valery Fokin, Dr. Suresh Pitram and Dr. Jason Hein of the Scripp's Research Institute, San Diego, when we were developing the capture agent technology and would like to thank them for their valuable advice. I have also enjoyed long and successful collaboration with Dr. Su Seong Lee, Dr. Jaehong Lim, Dr. Junhoe Cha, Sylvia Tan, Shi Yun Yeo, and Yiran Zheng from the Institute of Bioengineering and Nanotechnology, Singapore. I want to specially thank Dr. Su Seong Lee for his support and advice.

I would like to thank the current members of my subgroup, Kaycie M. Butler, Jessica Pfeilsticker, Ryan Henning, Joeseph Varghese, Blake Farrow, Dr. Aiko Umeda, Baoqing Zhou and Jingxin Liang for their feedback on my research. I started my graduate life working with Dr. Heather Agnew and Dr. Rosemary Rohde. Heather and Rose have been good mentors, answering my every question and training me. Dr. Steven Millward helped me with details of biochemical experiments. I would like to thank Heather, Rose and Steve for the guidance they provided. All the other graduate students, postdoctoral scholars, and staff in the Heath lab have also befriended and guided me.

I have been lucky to work with several exceptional undergraduate students, Abdul Ahad Tariq, Mary Beth Yu and Belen Alvarez during my years at Caltech. Their work has been instrumental in finishing my dissertation.

During my research I have used resources from other research laboratories as well as Caltech central facilities. I thank Dr. Jost Vielmetter of the Protein Expression Center and Professors David A. Tirrell for the use of the SPR. I thank Professor David A. Baltimore for ready access to the ELISA plate reader. I thank Dr. Mona Shahgholi for assistance with MALDI-MS measurements. I thank Professor Carl S. Parker for the unconditional lab access he has provided and thank the Caltech Protein/Peptide Micro Analytical Laboratory for acquiring ESI-TOF spectra.

I would like to thank the Institute for Collaborative Biotechnologies, the Army Research Office, the National Cancer Institute and the Bill and Melinda Gates Foundation for funding my research.

Finally, I would like to acknowledge my family for the love and support they have provided. Though my father is no longer with us, he continues to be a source of inspiration to me. Ever since my childhood, I have been inspired by my mother's determination and self-reliance. My brother has always nurtured me and pushed me to attain my life's ambition. My husband, Dr. Samir Das, is also my lab mate and co-author of a major portion of my dissertation work. Samir and I make a great team, and I hope we continue to collaborate in the future.

ABSTRACT

Iterative in situ click chemistry (IISCC) is a robust general technology for development of high throughput, inexpensive protein detection agents. In IISCC, the target protein acts as a template and catalyst, and assembles its own ligand from modular blocks of peptides. This process of ligand discovery is iterated to add peptide arms to develop a multivalent ligand with increased affinity and selectivity. The peptide based protein capture agents (PCC) should ideally have the same degree of selectivity and specificity as a monoclonal antibody, along with improved chemical stability. We had previously reported developing a PCC agent against bovine carbonic anhydrase II (bCAII) that could replace a polyclonal antibody. To further enhance the affinity or specificity of the PCC agent, I explore branching the peptide arms to develop branched PCC agents against bCAII. The developed branched capture agents have two to three fold higher affinities for the target protein.

In the second part of my thesis, I describe the epitope targeting strategy, a strategy for directing the development of a peptide ligand against specific region or fragment of the protein. The strategy is successfully demonstrated by developing PCC agents with low nanomolar binding affinities that target the C-terminal hydrophobic motif of Akt2 kinase. One of the developed triligands inhibits the kinase activity of Akt. This suggests that, if targeted against the right epitope, the PCC agents can also influence the functional properties of the protein. The exquisite control of the epitope targeting strategy is further demonstrated by developing a cyclic ligand against Akt2. The cyclic ligand acts as an inhibitor by itself, without any iteration of the ligand discovery process. The epitope targeting strategy is a cornerstone of the IISCC technology and opens up new opportunities, leading to the development of protein detection agents and of modulators of protein functions.

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Chapter 1: Introduction

1.1 Introduction

As scientists develop increasing insight in the sciences of genomics and proteomics, a molecular picture of disease development and progression is gradually evolving[1]. It is essential to translate this vast resource of scientific knowledge into clinical platforms, so that one can envisage a future, each individual has his/her genetic and proteomic 'fingerprints' or biomarker patterns recorded. Monitoring this fingerprint will help not only in treatment, but also in disease prevention. While this picture of personalized medicine is a lofty goal, it is not an unattainable one if broken down into smaller, more attainable, goals. One of the challenges that must be addressed in realizing the goal is the development of a rapid, inexpensive and minimally invasive way of measuring biomarkers[2]. In my thesis I focus on a small but vital part of this challenge, developing a rapid, inexpensive way of measuring protein biomarkers.

A biomarker is defined as an indicator of a specific biological state, which can be used to evaluate the risk of contraction or monitor the presence of a disease. Biomarkers can also provide guidance to targeted therapy and assess the response of therapy. While all the three different types of biomolecules, DNA, RNA and proteins, can act as biomarkers, protein biomarkers deserve a special mention[3]. Changes in the genetic level, such as DNA mutation, are manifested in changes in the protein levels. Additionally, proteins can undergo post-translational modifications, like phosphorylation, ubiquitination or glycolysation, that play key roles in modulating various biological processes like cell cycle, signal transduction[4], which are almost always perturbed in disease development and progression. Current traditional diagnostics platforms use a limited number of biomarkers, typically 1-2, for prognosis e.g. measuring LDL for cholesterol. These assays typically use monoclonal antibodies in a Enzyme-Linked ImmunoSorbent Assay (ELISA) format. While monoclonal antibodies have levels of affinity and selectivity, they are expensive, especially if instead of one or two biomarkers one aims to measure a large panel of protein

biomarkers. The lack of stability of monoclonal antibodies with variation of temperature and humidity also adds significant additional costs to their shipping, handling, and storage. The third limiting aspect of monoclonal antibodies is their batch-to-batch variation in composition, which can alter the quantitative character of protein assays. Despite all these drawbacks, antibodies still remain the gold standard protein detection in different clinical and diagnostics platforms due to their high affinity and selectivity.

To address the high demand for protein detection agents with antibody like sensitivity in diagnostics platforms while overcoming the inherent disadvantages of antibodies, scientists have tried to develop artificial alternatives to antibodies. There have been two approaches for this development. One is a protein engineering approach using an alternative small and robust protein scaffold with favorable folding and stability, and easy bacterial expression. Notable examples are affibody[5] that is based on Z domain of protein A and DARPins that use ankyrin repeat motifs[6]. The second approach is chemical development of artificial protein capture agentsusing a bottom up approach. Artificial protein capture agents have been synthesized using a variety of using biological building blocks like nucleic acids[7], peptides[8], and carbohydrates[9]. Chemical approaches such as phage display[10,11], nucleic acid aptamers[12], mRNA display[13], OBOC library[14], have been used in to develop artificial antibody like molecules that have been used in detection, imaging, therapeutics etc. Phage display library technology has been extensively used to isolate both linear[15] and cyclic peptide[16] based protein capture agents. However phage display libraries are comprised of L-amino acids, so that peptides isolated from the library are protease sensitive.
An alternative to the phage display library is the One-bead one-compound (OBOC)[17] peptide or peptide mimetic libraries. While in OBOC libraries one can easily modulate chemical stability by incorporating artificial amino acids, OBOC libraries have smaller chemical diversities than phage libraries. Peptide ligand discovery is made typically with pentameric to octameric peptide

libraries, with lower diversity than phage display libraries. This is because in OBOC libraries the on bead peptides have to be highly pure, and coupling of each amino acid decreases the purity.

To exploit the chemical flexibility of comprehensive, OBOC libraries of oligopeptides to develop high affinity, high selectivity protein capture agents, we married the OBOC peptide ligand discovery process with "in situ click chemistry". "Click chemistry" describes a class of chemical reactions that quickly generate substances by joining small modular units[18]. Copper catalyzed Azide Alkyne Cycloaddition (CuAAC) is the quintessential 'click' reaction, where Cu acts as a catalyst in the reaction of azide and alkyne and create a 1,2,3 triazole linkage. A variant of click chemistry, 'in situ click chemistry'[19-21], is particularly relevant to our discussion. In 'in situ click chemistry' the biological target, rather than Cu, acts as the reaction catalyst. The target acts as a scaffold, bringing together complementary building block reagents to create the triazole linkage. In the demonstration of "in situ click" strategy, libraries of small molecules, based upon components of a known inhibitor, were used as building blocks and the assembled compound was this known inhibitor, targeting the active site of the target enzyme. It was observed that the reaction had high selectivity, selecting not only the right building blocks, but also the right orientation between the building blocks, to create a specific regioisomer.

Iterative In Situ Click Chemistry (IISCC)[22,23] is a further development of this approach that we have developed for developing peptide based protein capture agents. Unlike the original approach that expanded upon a known inhibitor, in this approach we use large comprehensive libraries. This allows us to sample a much larger chemical space. In this process, the target is screened against OBOC peptide libraries to produce a peptide binder, typically having low micromolar affinity for the target protein. This peptide is referred to as the 1° ligand or monoligand. The 1° ligand is modified with an alkyne or azide to form a monoligand anchor. The protein is treated with the monoligand anchor and then screened against an OBOC library containing the complementary click handle. The protein acting as a catalyst, selects a second

binder (2° ligand), and clicks it with the anchor peptide to form a biligand. The biligand, modified with a new click handle (biligand anchor), is used, along with the protein, in the next round of ligand discovery. The process is then iterated to form a triligand, etc., until we achieve the desired levels of selectivity and affinity. The iterative nature of ligand discovery allows us to overcome the length limitation of OBOC peptide libraries. In the first demonstration of the IISCC technology, Agnew et al developed a triligand PCC Agent against bovine carbonic anhydrase II (bCAII) demonstrated that the developed PCC Agents could replace the antibody for carbonic anhydrase in protein assays like dot blots and ELISA. The target Carbonic anhydrase II was chosen as it has earlier been extensively characterized to study ligand-protein interactions. The protein has two distinct binding pockets and hence bind bivalent ligands[21,24]

Chapter 1 of my thesis explores the IISCC technology to develop branched PCC agents against bovine carbonic anhydrase II. The reported PCC agents for bCAII had not been allowed to branch on the peptide backbone but was grown off at the C terminal or the N terminal. Hence the process did not exploit the architectural flexibility that arises from the modular nature of the PCC agents. If a PCC agent is allowed to evolve via branching on the 1° or 2° ligand, the architectural space scanned vastly increases. Peptide dendrimers containing branching amino acid cores and surface peptide chains have been reported to have higher binding affinities[25]. Constraining a peptide by branching or cyclizing it increases the geometric constraints and reduces the conformational entropy of the molecule[26] which results in higher affinity. In the current work, starting from the same 1° ligand for bovine carbonic anhydrase, we allow development of branched PCC agents where one peptide arm is built off the backbone of another peptide arm. We discovered that branched ligands developed this way have affinities 2-3 times better than the original linear triligand reported. In ‘In situ click chemistry’ with protein as a catalyst, the protein may yield either anti (1,4), or syn (1,5) regioisomers a mixture of the two as the product. The multiligands that are produced in bulk and characterized, are synthesized

through CuAAC reaction, which means that in the characterized ligands the peptide arms are anti to each other and linked through a 1,4-triazole linker. 1,5 regioisomers are difficult to synthesize in bulk. We overcome this technical difficulty by developing a mimic of the 1,5 triazole linker. This allows us to characterize the PCC agents where the individual peptide arms are syn to each other. In the case of bCAII, we observe that the PCC agent with the 1,5 triazole linker mimic has higher affinity than the 1,4 triazole linked PCC agent.

While one of the major advantages of the PCC capture agent is the generality of the approach, the original process of development does not allow one to target a specific region of the protein, unless one can start with a literature molecule that is known to bind to the region of interest such as the active site. Without this knowledge it is almost impossible to develop PCC agents that can distinguish between subtle variations of the protein structure. However, in diagnostics platforms it is often observed that the salient protein marker parameter is not the quantity of the protein but changes in the protein structure. Disease prognosis may require detection of phosphorylation, glycosylation and other post-translational modifications. Certain non-antibody protein capture agents have been developed to recognize specific post-translational modifications like acylation[27]. Nevertheless, there is no general strategy that can be used to develop these reagents. In chapter 3, we outline a general strategy to chemically develop a peptide based capture agent specifically targeting a certain region of the protein. We describe the epitope targeting strategy and the development of peptide based PCC ligands that target the phosphorylated C-terminal hydrophobic motif of the protein Akt2. Akt, an AGC kinase, plays a central regulatory role in growth factor signaling, and serves as a key node in the phosphatidylinositol 3-kinase (PI3k) signaling pathway[28,29]. In the fully active Akt2 protein, two residues T309 and S474 are phosphorylated. T309 is located near the ATP and substrate binding pocket. It is the primary phosphorylation site required for the activity of the kinase. S474 is located in the C terminal of Akt2, and is part of unstructured region of the protein. However,

S474 is known to be important for the kinase activity of the protein and phosphorylation at S474 enhances the kinase activity 10-fold[30]. In the context of current studies showing that ATP competitive inhibitors of Akt can cause hyperphosphorylation of the protein[31], we thought it was worthwhile to find binders to the C terminal kinase regulatory domain, as such binders would have a high potential to regulate kinase activity. In this epitope targeting strategy, the target protein region is chemically modified to incorporate an azide/alkyne handle. The target, the 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 and provides an initial in situ click reaction site adjacent to the phosphorylated residue azide functionality close to the hydrophobic motif. This phosphopeptide/metal complex is then subjected to an in situ click screen against a large one-bead-one-compound (OBOC) library of acetylene-containing 6-mer peptides to identify an initial (1^o) peptide ligand. For an in situ click reaction, the binding target itself (here the 32-mer peptide) provides a highly selective catalytic scaffold for promoting the click reaction between an azide and an alkyne to produce a triazole linkage. The process of sequential in situ click chemistry is then utilized to expand the 1^o ligand into a biligand, and then the biligand into two triligands.

In chapter 4, we describe the assays used to choose the peptide arms, and characterize the final developed ligands in additional assays. The three triligand candidates have low nanomolar binding affinities. One triligand is selective for the Akt2 and over its homologous isoforms Akt1 and Akt3. We also explore their effect on the kinase activity of Akt. Remarkably, the two triligands, sharing the same biligand fragment, were found to have opposite effects on the kinase activity of the Akt2 protein. While one of the ligands enhances the kinase activity of the protein, the other ligand inhibits the kinase activity.

In the epitope targeting strategy, we are trying to detect peptide-peptide interaction, which is typically weaker than peptide-protein interaction and tougher to detect. If the 1° ligand chosen has a high affinity for its peptide epitope, it significantly simplifies the epitope targeted PCC agent development process. This led us to explore cyclic peptides, which have higher affinity and selectivity than their corresponding linear counterparts. For demonstrating of this process, cyclic ligands were developed against the C terminal fragment from the protein Akt2, amino acids 450-481. In chapter 5 we describe the development of OBOC CuAAC cyclized peptide libraries, which are then screened against the peptide epitope to isolate a cyclic peptide monoligand. We demonstrate that the isolated monoligand binds to the peptide epitope and the full-length protein. The cyclic ligand has a low nanomolar binding affinity, rather than a micromolar binding affinity that is characteristic of a linear 1° ligand. The ligand is also found to inhibit the kinase activity of the protein. Future studies shall be done to develop the biligand and to study the effects in live cells.

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Chapter 2: Development of branched capture agents against bovine carbonic anhydrase II

2.1 Introduction

Biologically[1,2] or chemically synthesized protein capture agents that can detect protein in diagnostics platforms and can be easily functionalized remain a very lucrative research area. Peptides discovered through phage display[3-5], antibody or protein[6] fragments[7] and nucleic acid[5,8,9] aptamers represent some classes of protein capture agents. Each class of artificial capture agents have unique advantages and disadvantages[6,10,11].In the past we have reported development of a new class of protein capture agents. An in situ protein-catalyzed multiligand capture agent was developed against bovine carbonic anhydrase II. The capture agent showed nanomolar binding affinity and could be used as an antibody substituent[12]. The PCC agent developed was linear, meaning that peptides, acting as modules, were joined either N terminal to N terminal or C terminal to N terminal. In the following chapter we extend the earlier work to develop branched capture agents against bCAII starting from the same monoligand peptide. Branching of peptides introduces geometric constraints to the PCC agent and reduces the conformational entropy of the molecule[13], which should result in higher affinity. Branching of peptide have also been reported to increase the protease resistance[14]. In the current work we explore two approaches to find the most appropriate branching point of an anchor peptide towards branched ligands via in situ click screen. In one approach a branching point is introduced in the biligand stage through screening of peptide libraries containing azido-modified artificial amino acids at random positions. In the second approach, an amino acid within a linear biligand is replaced with an artificial amino acid containing an azide or acetylene functional group. Hydrophobicity and structural similarity are used as criteria for the replacement. The branched biligands are further used in triligand screens to develop branched triligands. Some of the branched triligands are found to have ~3 fold higher affinity than the linear triligand developed earlier. We also develop a mimic of the 1,5 disubstituted 1,2,3 triazole linkage and determine if the 1,4 vs 1,5 orientation of the peptide arms in the developed multivalent ligands have any effect on the affinity for the protein.

2.2 Materials and Methods

2.2.1 Materials

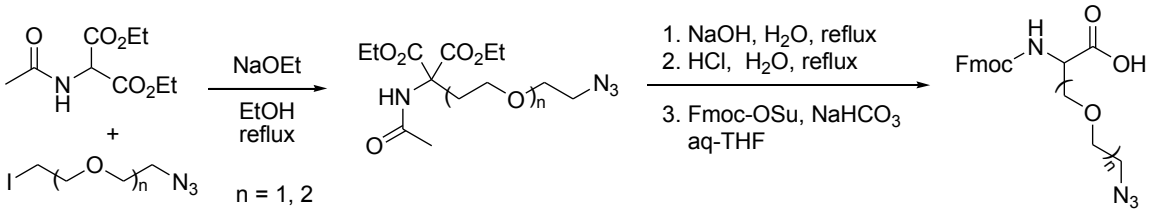
Fmoc D amino acids were purchased from Anaspec (San Jose, CA) and AAPPTec (Louisville, KY). TentaGel S-NH_2 resin (diameter 90 μm, capacity 0.28 mmol/g) used in OBOC library construction was bought from Anaspec (San Jose, CA). Bulk peptide synthesis was done either on Biotin NovaTag™ resin (EMD) or Rink Amide MBHA resin (Anaspec). Peptide synthesis reagents NMP (1-methyl-2 -pyrrolidinone), HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate) and DIEA (N,N′-diisopropylethylamine) were bought from EMD Chemicals, Inc. (Gibbstown, NJ), ChemPep (Miami, FL), and Sigma-Aldrich (St. Louis, MO) respectively. The target protein Bovine carbonic anhydrase II from bovine erythrocytes was bought from Sigma-Aldrich.

2.2.2 Methods

2.2.2.1 Synthesis of azide and alkyne containing artificial amino acids

Artificial amino acids 21- 35 (Figure 2.7) containing either the free azide functionality or alkyne functionality were synthesized. Amino acids **21**-**27**, **34**-**35** were synthesized following literature methods[15-17]. Amino acids **28**-**33** were developed by modification of literature methods[18,19]. The details of synthesis of amino acids **28**-**33** are as follows:

Figure 2.1: Synthesis of unnatural amino acids containing PEGylated azide side chains.



To a solution of pre-dried diethyl acetamidomalonate (7.35g, 3.3mmol), sodium ethoxide solution (0.82 g, 12 mmol in 40 ml of ethanol) was added at ambient temperature and stirred for 2 hours at 40°C. Azidoalkyliodide (4.0 mmol) was added and the reaction mixture was refluxed at 85 °C for 24 hours. The mixture was extracted with ethyl acetate, washed with water and brine, dried over $MgSO_4$ and then concentrated. Flash column chromatography (hexane:ethyl acetate = 2:1) was performed to give a purified product diethyl 2-acetamido-2-(3-azidopropyl) malonate (> 80%) as a clear oil. Diethyl 2-acetamido-2-(3-azidopropyl) malonate (2.6 mmol) was dissolved in 35 ml of 10% NaOH solution and the mixture was refluxed for 4 hours. The solution was then acidified to pH = 2.0 with concentrated HCl and concentrated under reduced pressure. The residue was dissolved in 70 ml of H_2O and refluxed for 4 hours. After cooling to ambient temperature, the mixture was acidified to pH 3.0 with 10% HCl solution and extracted with ethyl acetate. The organic layers were concentrated to give yellow oil, into which 54 ml of 2.0 N HCl solution was added. The mixture was refluxed for 2 hours and then concentrated in vacuum. The salt was then dissolved in a THF/H_2O (55:45) mixture and $NaHCO_3$ (10 eq.) was added with stirring at 0 °C. Fmoc-OSu (1.1 eq.) was added and the reaction mixture was stirred for 4 hours. THF was removed under reduced pressure and the resulting mixture was extracted with diethyl ether. The combined aqueous layers were acidified to pH 2.0 using concentrated HCl and extracted with ethyl acetate. The organic layers were washed with brine, dried over dried over $MgSO_4$ and then vacuum concentrated to obtain the final product which was further purified by column chromatography to yield the white product.

28 (n = 1):

^{1}H-NMR (400MHz, DMSO-d_6): δ 1.77-1.85 (m, 2H), 1.94-2.02 (m, 2H), 3.30-3.60 (m, 6H), 4.04-4.10 (m, 1H), 4.18-4.30 (m, 3H), 7.33 (t, 2H, *J* = 7.4 Hz), 7.41 (t, 2H, *J* = 7.4 Hz), 7.63 (d, 1H, *J* = 8.0 Hz), 7.72 (d, 2H, *J* = 7.4 Hz), 7.89 (d, 2H, *J* = 7.4 Hz), 12.85 (s, 1H).

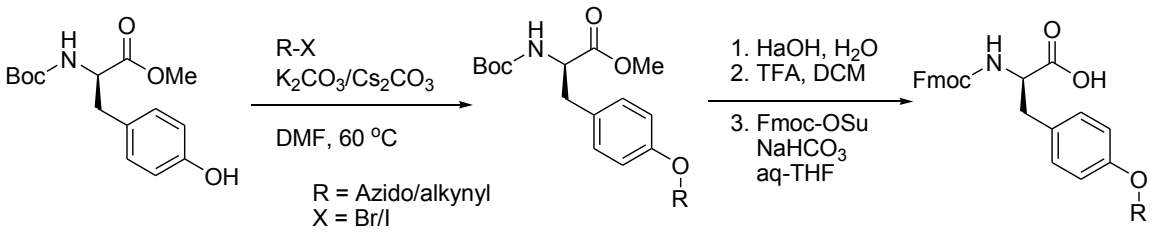
^{13}C-NMR (100MHz, DMSO-d_6): δ 31.4, 47.1, 50.4, 51.2, 66.0, 67.1, 69.5, 120.6, 125.7, 127.5, 128.1, 141.2, 144.3, 156.6, 174.4.

29 (n = 1):

^{1}H-NMR (400MHz, $CDCl_3$): δ 2.15-2.20 (m, 2H), 3.36-3.40 (m, 2H), 3.60-3.70 (m, 8H), 4.24 (t, 2H, *J* = 6.4 Hz), 4.40-4.44 (m, 2H), 4.45-4.52 (m, 2H), 6.04 (bd, 1H, *J* = 7.2 Hz), 7.32 (t, 2H, *J* = 7.4 Hz), 7.41 (t, 2H, *J* = 7.4 Hz), 7.61 (t, 2H, *J* = 7.4 Hz), 7.77 (d, 2H, *J* = 7.4 Hz).

^{13}C-NMR (100MHz, $CDCl_3$): δ 31.0, 31.4, 47.2, 50.7, 52.5, 67.0, 67.9, 69.8, 70.3, 120.0, 125.1, 127.1, 127.7, 141.3, 143.8, 156.2, 175.0.

Figure 2.2: Modification of D-phenyl alanine with an azido/acetylene group to synthesize amino acids 30 -33.



A bromide or iodide was added to Boc-protected D-phenyl alanine methyl ester in anhydrous DMF. Anhydrous K_2CO_3 (3 eq.) and Cs_2CO_3 (0.2 eq.) were added to the solution and heated at 60^{o}C overnight. Water was added to the mixture and the product was extracted with ethyl acetate. The ethyl acetate solution was washed with water three times. A crude mixture was obtained by removing the solvent under reduced pressure. The mixture was purified by column chromatography using hexane: ethyl acetate (1:5). After the product was dissolved in H_2O: MeOH: THF (1:1:3), NaOH (2 eq.) in water was added to the solution. After concentrating the mixture under reduced pressure, 2 M HCl solution was added to adjust the acidity to pH 3. The product was extracted with ethyl acetate two times. After removing the solvent, 50% TFA in DCM was added to the product and stirred overnight. After evaporating, the product was

dissolved in 40% aqueous THF. $NaHCO_3$ (10 eq.) and Fmoc-OSu (1.1 eq.) were added sequentially and stirred overnight. After removal of THF, diethyl ether was added to extract unreacted Fmoc-Osu. After discarding the organic layers, the pH of the aqueous solution was adjusted to 2.0 by adding 2M HCl solution. The product was extracted with ethyl acetate and then purified through column chromatography using DCM: MeOH (50:1).

30: ^{1}H-NMR (400MHz, $CDCl_3$): δ 2.03 (q, 2H, *J* = 6.4 Hz), 3.07 (dd, 1H, *J* = 14.4, 6.4 Hz), 3.16 (dd, 1H, *J* = 14.4, 5.2 Hz), 3.50 (t, 2H, *J* = 6.4 Hz), 3.98 (t, 2H, *J* = 6.0 Hz), 4.21 (t, 1H, *J* = 7.2 Hz), 3.36 (dd, 1H, *J* = 10.8, 6.8 Hz), 3.16 (dd, 1H, *J* = 10.8, 6.8 Hz), 4.65-4.70 (m, 1H), 5.23 (bd, 1H, *J* = 8.0 Hz), 6.82 (d, 2H, *J* = 8.4 Hz) , 7.17 (d, 2H, *J* = 8.4 Hz), 7.31 (t, 2H, *J* = 7.4 Hz), 7.41 (t, 2H, *J* = 7.4 Hz), 7.55 (t, 2H, *J* = 7.4 Hz), 7.78 (d, 2H, *J* = 7.4 Hz).

^{13}C-NMR (100MHz, $CDCl_3$): δ 28.8, 36.9, 47.1, 48.2, 54.7, 64.4, 67.1, 114.6, 120.0, 125.1, 127.1, 127.7, 127.8, 130.4, 141.3, 143.6, 143.7, 155.8, 157.9.

31: ^{1}H-NMR (400MHz, $CDCl_3$): δ 1.40-1.52 (m, 4H), 1.60-1.68 (m, 2H), 1.76-1.82 (m, 2H), 3.04-3.20 (m, 2H), 3.28 (t, 2H, *J* = 6.8 Hz), 3.92 (t, 2H, *J* = 6.4 Hz), 4.21 (t, 1H, *J* = 7.2 Hz), 4.34-4.40 (m, 1H), 4.43-4.49 (m, 1H), 4.65-4.71 (m, 1H), 5.21 (bd, 1H, *J* = 8.0 Hz), 6.82 (d, 2H, *J* = 8.4 Hz) , 7.17 (d, 2H, *J* = 8.4 Hz), 7.31 (t, 2H, *J* = 7.4 Hz), 7.41 (t, 2H, *J* = 7.4 Hz), 7.55 (t, 2H, *J* = 7.4 Hz), 7.78 (d, 2H, *J* = 7.4 Hz).

^{13}C-NMR (100MHz, $CDCl_3$): δ 23.0, 23.7, 25.7, 26.5, 28.9, 30.3, 47.1, 51.4, 67.1, 67.7, 114.6, 120.0, 125.1, 127.1, 127.8, 130.4, 141.3, 143.7, 158.3.

32:^{1}H-NMR (400MHz, $CDCl_3$): δ 3.04-3.18 (m, 2H), 3.41 (t, 2H, *J* = 4.8 Hz), 3.74 (t, 2H, *J* = 4.8 Hz), 3.85 (t, 2H, *J* = 4.8 Hz), 4.10 (t, 2H, *J* = 4.8 Hz), 4.37 (dd, 1H, *J* = 10.8, 6.8 Hz), 4.46 (dd, 1H, *J* = 10.8, 7.2 Hz), 4.62-4.70 (m, 1H), 5.27 (bd, 1H, *J* = 8.0 Hz), 6.85 (d, 2H, *J* = 8.8 Hz) , 7.06 (d, 2H, *J* = 8.8 Hz), 7.31 (t, 2H, *J* = 7.4 Hz), 7.41 (t, 2H, *J* = 7.4 Hz), 7.55 (t, 2H, *J* = 7.4 Hz), 7.79 (d, 2H, *J* = 7.4 Hz).

^{13}C-NMR (100MHz, $CDCl_3$): δ 36.9, 47.1, 50.7, 54.7, 67.1, 67.3, 69.8, 70.2, 114.8, 120.0, 125.1, 127.1, 127.8, 130.4, 141.3, 143.7, 155.8, 157.9, 175.8

33: ^{1}H-NMR (400MHz, $CDCl_3$): δ 3.03-3.18 (m, 2H), 3.38 (t, 2H, *J* = 4.8 Hz), 3.65-3.70 (m, 4H), 3.71-3.76 (m, 2H), 3.83-3.88 (m, 2H), 4.07-4.11 (m, 2H), 4.21 (t, 1H, *J* = 6.8 Hz), 4.36 (dd, 1H, *J* = 10.8, 6.8 Hz), 4.45 (dd, 1H, *J* = 10.8, 7.2 Hz), 5.33 (bd, 1H, *J* = 8.0 Hz), 6.84 (d, 2H, *J* = 8.8 Hz) , 7.05 (d, 2H, *J* = 8.8 Hz), 7.31 (t, 2H, *J* = 7.4 Hz), 7.41 (t, 2H, *J* = 7.4 Hz), 7.55 (t, 2H, *J* = 7.4 Hz), 7.79 (d, 2H, *J* = 7.4 Hz).

^{13}C-NMR (100MHz, $CDCl_3$): δ 36.9, 47.1, 50.6, 54.7, 67.0, 67.3, 69.8, 70.1, 70.7, 70.8, 114.7, 120.0, 125.1, 127.1, 127.8, 130.4, 141.3, 143.8, 155.8, 157.9, 175.4, 176.8.

2.2.2.2 Procedure for peptide synthesis

Peptides were synthesized using standard Solid Phase Fmoc chemistry (SPPS)[20] either manually or automatically. Peptides were synthesized on Rink Amide AM resins (Anaspec, loading = 0.31 mmol/g), Biotin Novatag Resin (EMD Biosciences, loading 0.5 from mmol/g), and Rink Sieber Amide resin (Anaspec, loading = 0.4 mmol/g) on a 200 mg scale. Peptides were manually synthesized in fritted polypropylene tubes using a 180° shaker (St. John Associates; Beltsville, MD). A 24-port SPE vacuum manifold system (Grace; Deerfield, IL) was used for draining the coupling and deprotection solutions. Automated synthesis was done using the Titan 357 (AAPPTec) automated peptide synthesizer. Before the peptide synthesis, the resin was swelled in NMP for 2 hours. For coupling amino acids to the resin, it was treated for 30 minutes with 2 equivalents of an Fmoc protected amino acid, (0.2 M in NMP), 2 equivalents of TBTU (0.2 M solution in NMP), and 5 equivalents of DIEA (0.2 M solution in NMP). For removal of the Fmoc group from the N terminal, the resin was treated with 20% pyridine in NMP for 15 minutes twice. In between the coupling and deprotection steps, the resin was washed five times for 1 minute with NMP. For N- terminal acylation, the peptide containing resin was swelled in DMF and treated with 5:6:10 solution of acetic acid, 2,6 lutidine and DMF for 10 minutes twice.

Following the treatment the excess reagent was removed by multiple washes with DMF. For peptide cleavage off the resin, the resin was treated with a trifluoroacetic acid cleavage cocktail (TFA/water/triisopropylsilane (TIS) (95/2.5/2.5, v/v/v)) mixture at room temperature for 2 hours. The cleavage solution was concentrated and then added drop-wise into ice-cold diethyl ether to precipitate the peptide. The resulting crude peptides were lyophilized. 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 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).

Synthesis of biotin tagged peptides:

Biotin was incorporated at the C terminal for all the peptides, with a polyethylene glycol between the biotin tag and the rest of the peptide sequence. The peptides were synthesized either by directly using biotin novatag resin (which incorporates a biotin on the C terminal of the peptide) or by appending a biotin on the side chain of a lysine on Rink Amide MBHA resin. For the later method, Fmoc-lysine(Mtt)-OH (Mtt = 4-methyltrityl) was coupled to Rink Amide resin using standard SPPS chemistry. The Mtt group was selectively removed by three consecutive washes with trifluoroacetic acid (3%), triisopropylsilane (3%) and DCM (94%). 1.5 equivalents of Biotin-NHS and 5 equivalents of DIEA were added to the resin Fmoc-$(PEG)_2$-CO_2H (20

atoms, EMD Biosciences) was coupled to the resin to introduce a polyethylene glycol (PEG) spacer. The synthesized peptides were purified to > 98% purity.

2.2.2.3 Measurement of the Relative Hydrophobicity Coefficients of amino acids

To compare the relative hydrophobicities of the synthesized artificial amino acids with natural amino acids, a literature method was adapted[21]. Briefly, a series of Ac-X-GLF-OH peptides were synthesized (X is the natural or unnatural amino acid) and their retention times were measured using an autosampler equipped Waters Acquity UPLC system (Waters Acquity UPLC® BEH C18 column, flow-rate of 0.3 ml/minutes, temperature 25°C, eluent of 10% acetonitrile and 0.1% TFA in water). The hydrophobicity coefficients of the amino acid X in the synthesized peptides were calculated as the retention time of the X-substituted peptide minus the retention time of Ac-GGLF-OH peptide. The relative hydrophobicity coefficients are presented in Figure 2.8.

2.2.2.4 Construction of Peptide Libraries

Randomized OBOC libraries of penta or heptapeptides were synthesized using an automatic synthesizer Titan 357 (AAPPTec) via standard split-and-mix methods, on TentaGel S-NH2 beads. Unless otherwise specified, non-natural D-stereoisomers were used at every possible position in the peptide sequence. For all the coupling steps, standard SPPS method with Fmoc chemistry was used. The resin was swelled in NMP for 2 hours in a big Collective Vessel (CV) and divided into multiple equal-mass aliquots for the cycles of coupling and deprotection with different amino acids in the smaller Reaction Vessels (RV). For libraries used at IBN, Fmoc-methionine was coupled to the entire resin in the first coupling step to make the library MALDI-TOF compatible. The amino acid side-chain protective groups were then removed by treatment with TFA cleavage cocktail mixture for 2 hours. The on bead peptide library was washed with DCM, methanol and water, and stored. The peptide libraries used for various screens are summarized in Table 2.1.

Table 2.1: One Bead One Compound (OBOC) peptide libraries used in screens.

Library	Sequence	Amino acids used	Number of unique sequences
A	$X_1X_2X_3X_4X_5X_6$-TG	X_i= D-Trp, D-Lys, D-Ile, Gly, D-Leu, D-Val, D/L- azidolysine	117649
B	$X_1X_2X_3$-D/L-Az4-X_5X_6-D-Met-TG	X_i = D-Trp, D-Lys, D-Leu, D-Val, D-Ile, Gly	15552
C	$X_1X_2kX_4$-D/L-Az4-X_6w-D-Met-TG	X_1= all 18 D amino acids except D Met and D-Cys; X_2= D-Lys(Alloc)-OH, D-Trp, D-Val; X_4= D-Arg, D-Asn, D-Gln, D-Asp, D-Lys, D-Ser, D-Thr, D-His, D-Ala, Gly, D-Val, D-Trp; X_6 = D-Arg, D-Asn, D-Gln, D-Asp, D-Lys, D-Ser, D-Thr, D-His, D-Ala, Gly, D-Leu, D-Phe	15552
D	Acetyl-D-Pra-$X_1X_2X_3X_4X_5X_6$-L-Met-TG	D-Pra = D-Propargylglycine,; X_i = D-Ala, Gly, D-Leu, D-Ile, D-Val, D-Phe, D-Trp, D-Arg, D-His, D-Lys, D-Asp, D-Glu, D-Asn, D-Gln, D-Ser, D-Thr, D-Tyr, D-Pro	34012224
E	$X_1X_2X_3X_4X_5X_6$-L-Met-TG	X_1= D-Lys, D-Arg, D-Val, D-Leu, D-Phe, D-Ile, D-Tyr, D-Trp; X_2 = D-Ile, D-Arg, D-Phe, D-Val, D-Leu, D-Lys, D-Trp, D-Tyr; X_3= D-Phe, D-Arg, D-Ile, D-Val, D-Leu, D-Lys, D-Thr; X_4 = D-Tyr, D-Phe, D-Arg, D-Trp, D-Val, D-Lys, D-Ile, D-Leu X_5 = D-Arg, D-Ile, D-Lys, D-Phe, D-Val, D-Tyr, D - His, D-Asn; X_6 = D-Arg, D-Lys, D-Phe, D-Tyr, D-Val, D-Trp, D-His, D-Ile	262144
F	D/L-Az4-$X_1X_2X_3X_4X_5$-TG	Xi = all 18 D amino acids except D-Met and D-Cys	3,779,136

2.2.2.5 Screening procedures

For screens with the florescent labeled protein, bCAII was labeled with Alexa Fluor 647 (Microscale Protein Labeling Kit, Invitrogen) following the manufacturer's protocol. The different screens for biligand and triligands are summarized in Table 2.2. Details of each type of screen i.e., target, or product, and each type of sorting, i.e., automatic or manual is provide below.

Table 2.2: Summary of screens for the development of branched PCC agents for bovine carbonic anhydrase II.

Screen	Library	bCAII concentration	Peptide anchor	Peptide concentration	Type of Screen	Type of sorting	Ligand/ motif Isolated
BB1	A	10 nM	**37**	20 μM	target	manual	xxx-Az4-xx
BB2	B	10 nM, 1 nM and 500 pM	**37**	20 μM, 2 μM, 1 μM	target	manual	x-k-x-Az4-x-w
BB3	C	10 nM	**37**	40 μM	target	manual	***38, 39***
TRI1	D	10 nM	**42**	20 μM	target	automated	N/A
TRI2	E	10 nM	**42**	20 μM	target	automated	***45, 46***
TRI3	F	10 nM	**40**	20 μM	product	manual	***48***, ***49, 50, 51***

In situ click target screen:

Nanomolar solutions of Alexa Fluor 647-labeled bCAII (PBS, pH 7.4, 0.1% Tween-20, 0.1% bovine serum albumin (BSA), 0.05% NaN_3) was pre-incubated for 2 hours with the micromolar solutions of the corresponding anchor peptide (table 2.2) in binding buffer for 2 hours at 25^{o}C in a polypropelene vessel. 200 mg of the relevant OBOC peptide library was swelled in the binding buffer in a separate vessel for 1 hour at 25^{o}C. The pre-incubated solution of Alexa Fluor® 647-labeled bCAII and the biligand peptide anchor 40 were added to the equilibrated on bead peptide library. The library was incubated for ~16 hours at 25^{o}C. Non-specifically bound

proteins were eliminated by multiple washes, first with the binding buffer and then with 0.1%Tween 20 in PBS. The details of each individual screen are tabulated in table 2.2.

Automated sorting of fluorescent protein:

The assayed library was transferred into the sample vessel of a COPAS Plus (Union Biometrica) automated bead sorter, and diluted with 200 ml of PBS buffer (pH=7.4). A two-step sorting was applied. The first sorting was performed at high flow rate (> 80 beads per seconds) to sort out 1~2% of beads according to fluorescence intensity. The sorted bright beads were washed with DI water several times, transferred into the sample vessel, and diluted with 200 ml of DI water. The second sorting was carried out at low flow rate (5 to 10 beads per second). In the second sorting, positive beads were directly sorted into a 96 titer well plate with conical-shaped wells. Sequencing of these beads was performed with MALDI-TOF/TOF and the semi-automated algorithm.

Manual sorting of fluorescent protein:

Following the screen and washes, the library was transferred to glass microscope slides and imaged using the GenePix 4200 array scanner (λ_{ex} = 635 nm). The beads with the highest fluorescence intensities were considered hit beads and picked up manually using a glass micropipette. The picked beads were washed with 7.5 M guanidium hydrochloride (pH= 2.0) for 1 hour followed by ten washes with double distilled water. Each bead was loaded on a cartridge on the Edman Sequencer (494 Procise cLC Sequencer, Applied Biosciences).

In situ click product screen:

Bovine carbonic anhydrase II was incubated with ~2000x concentrated biotinylated anchor peptide overnight at 25 ^{o}C. The OBOC peptide library is swelled in the binding buffer overnight and then added to the preincubated protein solution. The screen was done for 7.5 hours at room temperature, followed by washes with the binding buffer, wash buffer 1 (PBS + 0.05% NaN3 + 0.1% Tween 20) and PBS. The beads were treated with 7.5 M guanidium hydrochloride

(pH = 2) for an hour and washed ten times with Millipore water. The beads were swelled back in binding buffer and incubated for an hour with a 1:10,000 diluted solution of anti-biotin mAb-alkaline phosphatase (Sigma Aldrich) in the binding buffer. The beads were washed multiple times with the wash buffer and PBS. The beads in AP buffer (100mM Tris-HCl [pH 9.0], 150mM NaCl, 1mM MgCl2) were transferred to plastic petri dishes. 10 ml of BCIP solution (33 μl BCIP/ 10 ml AP buffer) was added to each petri dish. After 30 minutes, beads that have anti-biotin mAb-alkaline phosphatase binding to them turn turquoise due to the reaction of BCIP with the alkaline phosphatase. These beads were picked up manually using a micropipette and decolorized by washing with dimethyl formamide. Following treatment with 7.5 M guanidium hydrochloride (pH= 2.0) for 1 hour and stringent water washes, the beads were equilibrated in binding buffer. The library was then treated with biotin saturated anti-biotin –alkaline peroxide antibody. After an hour, the beads were washed as in the previous screen and treated with BCIP solution. Due to ligand competition, the beads that bind to the biotin-binding site of the antibody were colorless in this screen while the nonspecific binders to the antibody turned turquoise. The colorless beads were picked up manually, washed with 7.5 M guanidium hydrochloride (pH= 2.0) for 1 hour followed by ten washes with Millipore water. Each bead was separately loaded on an Edman Sequencer cartridge and sequenced on the Procise sequencer.

Table 2.3: Hit sequences from the first biligand screen BB1.

The library A of form $X_1X_2X_3X_4X_5X_6$-TG (where X_i= D-Trp, D-Lys, D-Ile, Gly, D-Leu, D-Val, D/L- azidolysine TG = tentagel S NH2 resin, diversity 117649) is screened with anchor peptide **37** with the Alexa-647 labeled bCAII protein.

X_1	X_5	X_3	X_4	X_5	X_6
I	f	k	Az4	v	w
i	k	v	Az4	i	w
f	k	w	Az4	i	w
v	k	v	Az4	i	w
w	k	v	Az4	i	w
w	k	l	Az4	i	w
f	k	f	Az4	f	f
Az4	k	f	Az4	i	w
Az4	k	w	G	G	l

Table 2.4: Hit sequences from the second generation biligand screen BB2.

The library B of form $X_1X_2X_3$-D/L-Az4-X_5X_6-D-Met-TG (where X_i = D-Trp, D-Lys, D-Leu, D-Val, D-Ile, Gly, Met=L-Methionine, TG = tentagel S NH2 resin, 15552 unique sequences) is screened with anchor peptide **37** with the Alexa-647 labeled bCAII protein in the biligand screen.

Protein concentration	X_1	X_2	X_3	Az4	X_5	X_6
10 nM	w	k	v	Az4	l	w
10 nM	v	k	v	Az4	i	w
10 nM	w	k	l	Az4	i	w
10 nM	i	k	w	Az4	f	w
10 nM	w	k	f	Az4	i	w
10 nM	v	k	w	Az4	i	w
10 nM	w	k	i	Az4	v	w
10 nM	v	k	w	Az4	i	w
10 nM	i	k	f	Az4	i	w
10 nM	f	k	f	Az4	f	w
10 nM	v	k	w	Az4	f	w
1 nM	v	k	w	Az4	f	w
500 pM	w	k	v	Az4	l	w

Table 2.5: Hit sequences from the third generation biligand screen BB3.

The library C of form $X_1X_2kX_4$-Az4X_6w-D-Met-TG (where X_1= all 18 D amino acids except D Met and D-Cys; X_2= D-Lys(Alloc)-OH, D-Trp, D-Val; X_4= D-Arg, D-Asn, D-Gln, D-Asp, D-Lys, D-Ser, D-Thr, D-His, D-Ala, Gly, D-Val, D-Trp; X_6 = D-Arg, D-Asn, D-Gln, D-Asp, D-Lys, D-Ser, D-Thr, D-His, D-Ala, Gly, D-Leu, D-Phe) is screened with anchor peptide **37** and Alexa-647 labeled bCAII protein .

X_1	**X_2**	**k**	**X_4**	**Az4**	**X_6**	**w**
k	w	k	w	Az4	l	w
r	v	k	w	Az4	i	w
v	w	k	v	Az4	i	w
k	w	k	v	Az4	l	w
a	v	k	v	Az4	l	w
i	v	k	w	Az4	l	w
i	w	k	v	Az4	f	w

Table 2.6: Hit sequences from the first generation triligand screen TRI1

Library D of form Acetyl-D-Pra-$X_1X_2X_3X_4X_5X_6$-L-Met-TG (where D-Pra = D-Propargylglycine,; X_i = D-Ala, Gly, D-Leu, D-Ile, D-Val, D-Phe, D-Trp, D-Arg, D-His, D-Lys, D-Asp, D-Glu, D-Asn, D-Gln, D-Ser, D-Thr, D-Tyr, D-Pro) is screened against bCAII- biligand **42** complex.

1	vgvwfk	38	nyfkrr	77	hrfyrr
2	viifkr	39	fttlvk	78	rGfykr
3	wklwfr	40	lrfyli	79	yryrlr
4	hiifnk	41	aiflrr	80	klkyrr
5	alfiir	42	kyrylr	81	srryry
5	hdtfvr	43	rfrrfa	82	rlfyth
6	afffrl	44	ldnvvk	83	Gwyvrr
7	hiryrk	45	ylfflr	84	krwwnr
7	vwtygh	46	lvallk	85	frkrhr
8	iwvryd	47	lrlllr	86	tiiyrh
9	vflrnv	48	kwrirl	87	kvyfnr
10	nvarhp	49	ffrtlv	88	tllykk
11	lrlyfr	50	iviwkr	89	rrfrlr
12	rfrlwl	51	kwrhwr	90	yyrfrk
13	vkiyvr	52	iaiwlr	91	khyrrw
14	kiyvrf	53	llvvyr	92	kiifsr
15	vvtsvr	54	nwkryr	93	rvvwfr
16	vrlylk	55	llhvfr	94	vklflk
17	rilfhw	56	rfwklk	95	frvwfg
18	kwyfyr	57	sryrhr	96	ryffkw

19	yifflr	58	kfhrrk	97	ftvllr
20	lraylr	59	yafflr	98	riyvrv
21	wrrfrr	60	kfyyrv	99	lrkwlw
22	ytGlfk	61	kiryfr	100	rfvkvf
23	pypyl	62	nwkwrk	101	wpherd
24	nrGnhr	63	khwrrr	102	pfdlw
25	Iiiyrs	64	rkawlr	103	fyyrk
26	yylvkr	65	fitrkf	104	pwfwG
27	llhltk	66	kwvver	105	rfvkvf
28	wpvpvf	67	ywlvkr	106	fkrkir
29	frvysf	68	lffrwv	107	wriyir
30	nfyyri	69	fafyvr	108	lfirly
31	rwklrr	70	wirirk	109	vfvkkl
32	kwtrei	71	hifirk	110	llrlay
33	wirGfy	72	rifvfr	111	rlrfhk
34	vyrkyk	73	llfyrk	112	prfyky
35	iyifrk	74	hyrkkw	113	rvkwkk
36	yrwrkf	75	ywflkk		
37	rkywkr	76	fkkyyr		

Table 2.7: Hit sequences from the second generation triligand screen TRI2

Library E of form $X_1X_2X_3X_4X_5X_6$-L-Met-TG (X_1= D-Lys, D-Arg, D-Val, D-Leu, D-Phe, D-Ile, D-Tyr, D-Trp; X_2 = D-Ile, D-Arg, D-Phe, D-Val, D-Leu, D-Lys, D-Trp, D-Tyr; X_3= D-Phe, D-Arg, D-Ile, D-Val, D-Leu, D-Lys, D-Thr; X_4 = D-Tyr, D-Phe, D-Arg, D-Trp, D-Val, D-Lys, D-Ile, D- Leu X_5 = D-Arg, D-Ile, D-Lys, D-Phe, D-Val, D-Tyr, D - His, D-Asn; X_6 = D-Arg, D-Lys, D-Phe, D-Tyr, D-Val, D-Trp, D-His, D-Ile) is screened against Alexa-647 labeled bCAII in presence of biligand **42**.

1	**yrlfvr**	18	**lilfnr**	35	**yklffr**
2	kyfakf	19	**lklwfk**	36	**vklwfk**
3	**vrlffk**	20	**kyyfrf**	37	**kyffrf**
4	**vklwlk**	21	**lklwlk**	38	**kyffrf**
5	rfwfkw	22	**vklwfk**	39	yvtfvk
6	yirnrr	23	**ykifvr**	40	**kyffrf**
7	**kyffrf**	24	**kyffrf**	41	yrrfri
8	**kyffkf**	25	kfffrv	42	**vkifvr**
9	iitfnr	26	ifyrh	43	**lilfnr**
10	**vrlffk**	27	**vklfvr**	44	rfffrv
11	**rifvri**	28	kiyffr	45	ryflrf
12	kiyffr	29	kfffr	46	**irlflk**
13	kfffri	30	**iklwlr**	47	**vivfnr**
14	**ivlfnr**	31	**irlwlk**	48	**ivkfvr**
15	**lrlwlk**	32	**fkifvr**	49	kwyffr
16	**kffyrf**	33	**lrfflk**	50	**kyffrf**
17	rkrfrh	34	lrkfri		

Table 2.8: Hit sequences from the first generation screen TRI3 using the product screening method.

The library F of form D/L-Az4- $X_1X_2X_3X_4X_5$-TG (Xi = all 18 D amino acids except D-Met and D-Cys) is screened with biligand anchor peptide **40** with the Alexa-647 labeled bCAII protein in the triligand screen.

Az4	X_1	X_2	X_3	X_4	X_5
Az4	h	d	t	f	y
Az4	h	d	t	G	f
Az4	h	d	e	G	G
Az4	y	s	q	w	a

2.2.2.6 Dot blots

The affinity of the capture agents for bCAII was demonstrated through dot blot experiments in 5% nonfat dry milk in TBST [25 mM Tris, 150 mM NaCl, 2 mM KCl, 0.1% Tween 20 (pH 7.0)]. A bCAII stock solution (10 mg/mL) was prepared in PBS buffer (pH 7.4). A dilution series of bCAII solution was applied to a nitrocellulose membrane, typically ranging from 1 μg to 20 ng per spot. The membrane was blocked at room temperature for 1 hour in 5% nonfat milk/TBST, which was then washed three times with TBST for 10 minutes each. A biotinylated peptide ligand solution was prepared at 100 nM or 1 μM in 0.5% nonfat milk/TBST and incubated over the membrane for 1 hour at room temperature. After washing three times with TBST for 10 minutes, the membrane was incubated with 1:3000 streptavidin-HRP (Abcam) in 0.5% milk/ TBST for 1 hour followed by subsequent washing with TBST for 10 minutes. The

resulting membrane was detected using the chemiluminescent reagents (Amersham ECL plus Western blotting detection reagents, GE Healthcare).

2.2.2.7 ELISA with PCC agent as capture agent

Streptavidin coated 96 well plates were washed three times with 0.1% BSA/TBST. 100 μl of 200 nM solution of the biotinylated capture agent (made by dilution in 0.1% BSA/TBST of 1 mM DMSO stock) was added to each well and incubated for 2 hours with shaking at room temperature. The wells were then washed three times with 200 μl of 0.1% BSA/TBST. 100 μl of His_6 tagged recombinant carbonic anhydrase II protein solution, (from 10 μM to 1 nM in 0.1% BSA/TBST) was added to the wells. After overnight incubation of the plate at 4°C, each well was washed three times with 0.1% BSA/TBST. 100 μl of 1:1000 diluted anti-His_6 mouse monoclonal antibody (Abcam) was added to each well and incubated with shaking for an hour at 4°C. Following three washes with 0.1% BSA/TBST, 100 μl of a 1:10,000 diluted solution of anti – mouse IgG goat antibody conjugated with horseradish peroxidase enzyme (Abcam) was added and incubated for one hour at 4°C. After one hour the wells were washed five times with TBST and then 100 μl TMB (3,3',5, 5'-tetramethylbenzidine) substrate (KPL) was added per well. Upon color development, each well was quenched with 100 μl of 1 M sulfuric acid in the order the TMB substrate was added. The absorbance at wavelength 450 nm was measured using a Spectramax ELISA plate reader.

2.3 Results and discussion

The PCC agent can be visualized as a multivalent binding agent that links several non-competitive weak affinity peptide ligands to allow cooperative binding. We have developed in the past a linear triligand capture agent **44** against bovine carbonic anhydrase II (bCAII). Starting with the development of a weak peptide binder (with high micromolar dissociation constant) **36**, a specific and selective multiligand peptide binder with high nanomolar affinity had been

developed. The peptide ligands had been selected through screens such that they could be linked linearly from N terminal to C terminal. In the current work, we explore the different connectivities of the peptide ligands to create branched peptide multiligands for bCAII starting with the same monomeric peptide binder. Two different methods of developing a branched bivalent PCC agent are explored. In the first method, the reported peptide **36** is retained as the monoligand and a new 2° peptide arm is discovered thorough screening. In the second method, the earlier reported linear biligand **41** is modified in the 2° ligand arm. The newly developed branched biligands are used to screen for branched trivalent PCC agents. It is explored if the variation in connectivity of the peptide arms in the PCC agent has any positive effect on the affinity of the ligand for the bCAII protein.

2.3.1 Development of branched biligand using randomized azide library

In the previous work, the primary peptide binder **36** to the protein that had been obtained via two-generation screens, first against a comprehensive random library, and then against a smaller focused library. This ligand **36** had been modified with D-propargylglycine at the C terminal to form the anchor ligand, **37,** and used as the anchor peptide in the linear biligand screens. To develop a branched biligand containing the same primary ligand, we utilize the highest occurring amino acids from the linear library screens to develop a small focused library. To allow the protein to scan the entire conformational space the azidolysine is used as an amino acid component like all others. This results in the synthesis of an OBOC heptapeptidic library with a random number and random position of the azidolysine (library A). The library was screened against 10 nM Alexa 647-labelled bCAII pretreated 20 μM peptide **37.** After buffer washes, the beads with the highest fluorescent intensities were manually picked up. Out of the ten sequences containing azidolysine, nine contained one azidolysine, while one sequence had two azidolysines Amino acid D/L-azidolysine was conserved at X_4 (Table 2.3). This suggested that the protein actually prefers the in situ click occurring not end to end but from one end of the

anchor to the middle of the second peptide arm. X_2 and X_6 positions of the hit sequences were also found to be highly conserved. A second focused library was synthesized, where the position of the D/L-azidolysine was fixed at X_4. The library was screened against three different concentrations (10 nM, 1 nM and 500 pM) of Alexa 647-labeled bCAII in presence of peptide **37**. The beads with highest fluorescence intensity were picked. This yielded eleven beads from the 10 nM screen, and one bead each from the 1 nM and 500 pM screens (Table 2.4). The trends noticed in the first screen were even more noticeable in this screen, yielding two motifs, wkX-Az4-Xw and vkX-Az4-Xw. Biligands **38** and **39** were synthesized in which vkw-Az4-lw and wkv-Az4-lw were clicked to the peptide **37**.

To find the preference of the protein for further branching, the protein was screened with a third heptameric focused library. The aim of this library was to increase the length of the second peptide arm while simultaneously looking at the selectivity of the protein for branching at X_5 position by incorporation of D-Lys(Alloc)-OH. In situ click screens of two copies of the OBOC library with 10 nM bCAII pretreated with 40 μM anchor, after buffer washes, was imaged and the highest fluorescent intensity beads manually picked. The sequences of the beads are presented in Table 2.5. The amino acid at X_5 was always D-Trp or D-Val, indicating a strong preference of these two amino acids at this position. A hit sequence k(alloc)-wkv(Az4)lw obtained twice within the hit sequences was chosen as the candidate to further extend the second peptide arm. The alkyne functionality was incorporated on the side chain of the N terminal D-Lys by coupling Fmoc-D-Pra to the lysine side chain.

Figure 2.3: Molecular structures of the reported monoligand 36 and the monoligand anchor 37 used in the branched biligand screen.

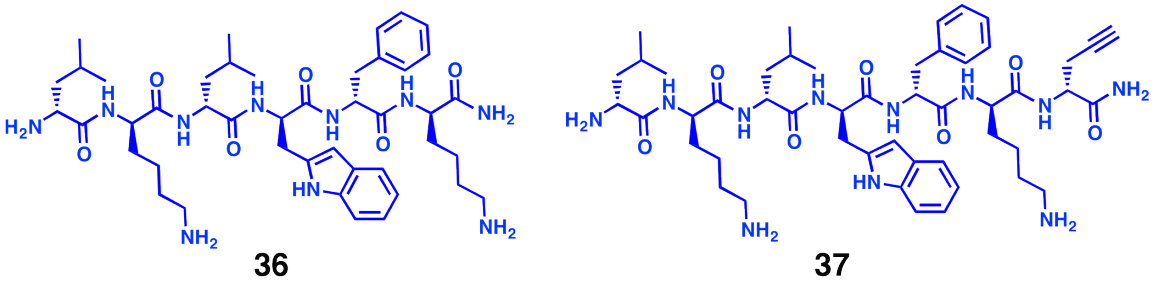


Figure 2.4: Schematic representation of target screen against bovine carbonic anhydrase II using OBOC library with multiple azides for developing a branched biligand.

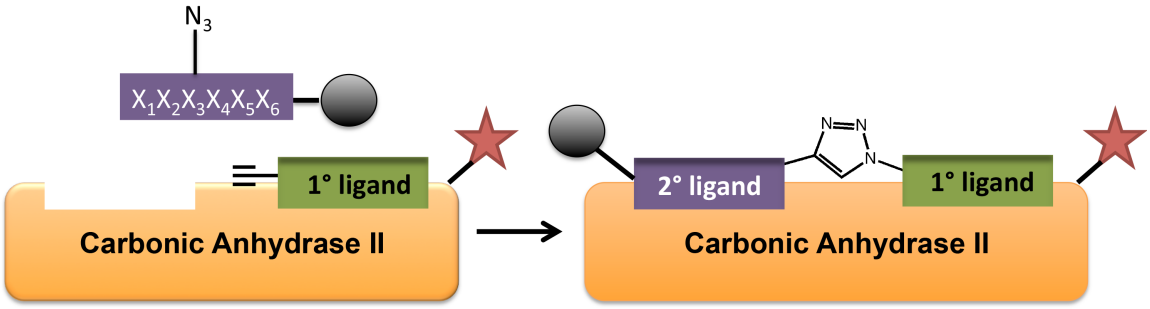


Figure 2.5: Motifs obtained by screening against bovine carbonic anhydrase II in presence of monoligand anchor peptide 37.

Screening against a library with variable number and position of azides, it is observed that the protein favors one azidolysine at X_4. Screens against focused library B yield motifs wkXAz4Xw and vkXAz4Xw. Screen against a further focused library C containing these two motifs yields the final biligand anchor arm k(alloc)-wkv-Az4-lw.

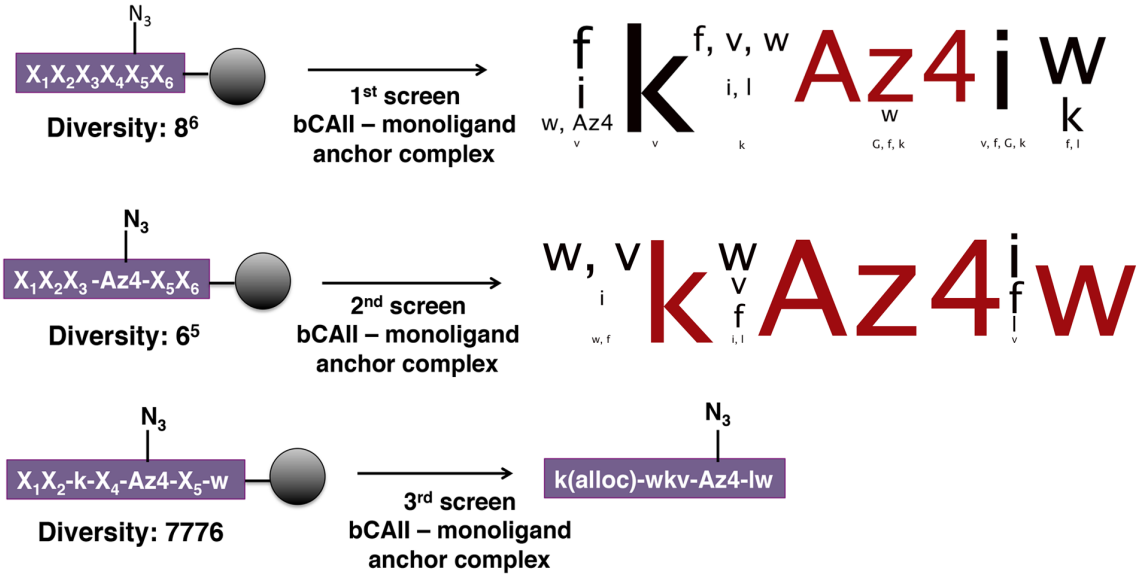
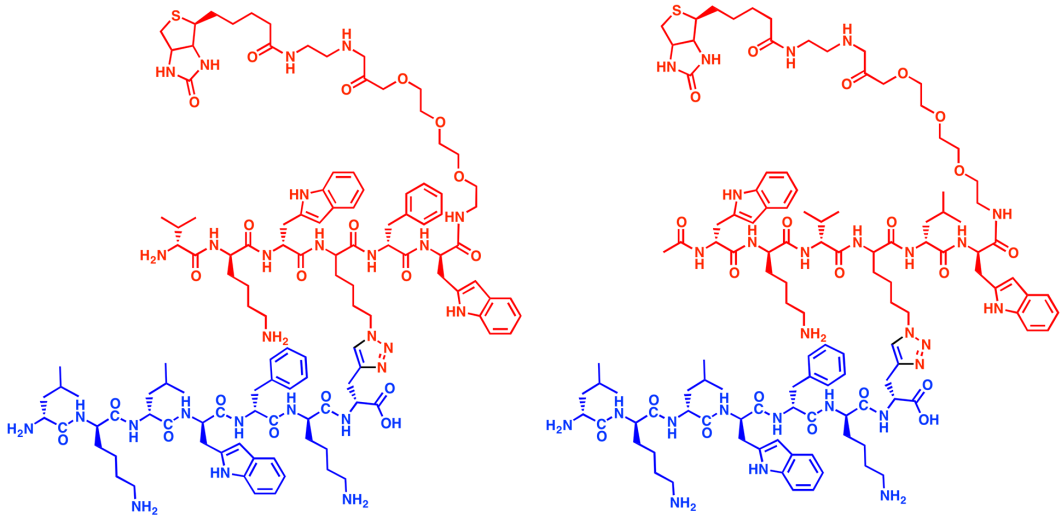
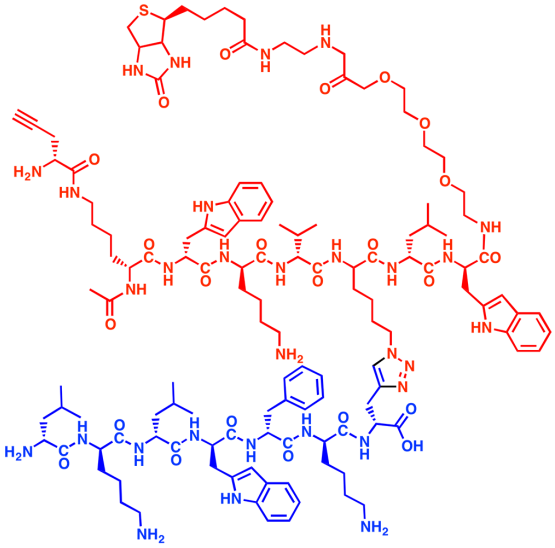


Figure 2.6: Molecular structures of branched biligands 38 and 39 and biligand anchor 40. In the branched biligands **38** and **39**, the 2° arm isolated through screens (in red) is linked by CuAAC chemistry to the monoligand anchor **37** (in blue). An alkyne is appended to the biligand **39** to synthesize biligand anchor **40**. The biotin tag spaced by a polyethylene glycol linker is utilized in assays and in the triligand screen.



38

39



40

2.3.2 Substitution of amino acid in linear ligand to developed branched biligand

An alternative approach to develop a branched biligand would be through amino acid substitution of the developed linear ligand. For this strategy to succeed, one would need to replace amino acids in the linear biligand with an artificial amino acid with similar properties, so as to not disrupt the binding affinity of the ligand. To incorporate a reactive click handle in the reported linear biligand peptide to exploit branched peptides, we synthesized various artificial amino acids containing azide and alkynes in the side chain (Figure 2.7). Hydrophobicity of an amino acid is an important property that can be used to guide replacement of a component amino acid, with minimal disruption in binding properties[22,23]. The Relative Hydrophobicity Coefficient (RHC) is a measure of the hydrophobicity. The RHC values of the naturally occurring as well as the synthesized artificial amino acids were determined by modifying a literature method[21]. In this method, the RHC of an amino acid X is proportional to the retention times of the peptide Ac-X-GLF by HPLC. The RHC values of six of the synthesized amino acids are comparable to those of the hydrophobic naturally occuring amino acids, Leu, Ile, Phe and Trp. Amino acid **21** has RHC value (2.21) lower than those of L-Tyr (**17**, RHC = 2.44) and L-Val (**18**, RHC = 2.61). So **21** can replace L-Tyr or L-Val within a peptide without a big change in hydrophobicity. Note that the same RHC values apply for replacing the D-amino acid with an artificial amino acid within a D-peptide.

For in situ click screens, this strategy was used to replace an amino acid of the D-anchor peptide with an artificial amino acid of similar hydrophobicity index (Figure 2.9). Starting with the earlier developed linear biligand **41** (Figure 2.9) we replaced D-Trp (RHC = 5.74) with the racemic artificial amino acid, Az5 (**25**, RHC = 6.50) to synthesize branched biligand **42**.

The other strategy of amino acid replacement commonly followed in literature is to replace an amino acid with an artificial derivative having similar molecular structure. Following this strategy, D-phenylalanine of the biligand **41** was replaced with 4-azido-D-phenylalanine to synthesize biligand 43**.** The binding affinities of biligands **42** and **43** for bCAII as determined by SPR were similar to the binding affinity of linear biligand **41** for bCAII. So both replacement strategies do not compromise the binding affinity of the modified biligand in SPR measurements (Figure 2.10). However, the yield of **43** was not high due to instability of phenyl azide under the harsh deprotection conditions during peptide synthesis.

Figure 2.7: Synthesized artificial amino acids with side chains containing azide or alkyne functional side chains.

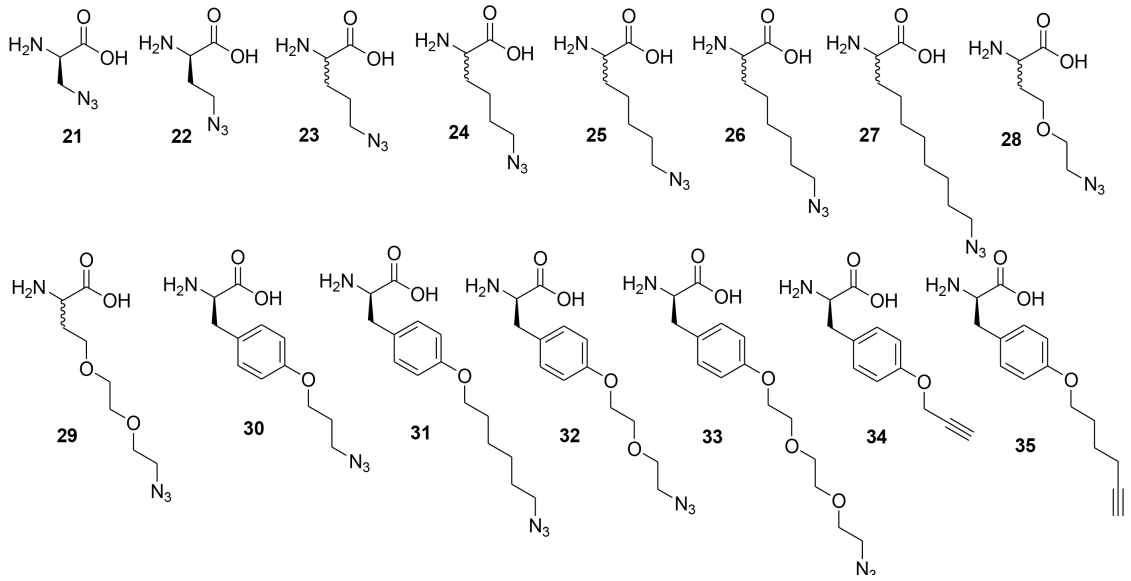


Figure 2.8: Comparison of Relative Hydrophobicity Coefficients (RHC) of natural amio acids with those of synthesized artificial amino acids containing azide or alkyne functional group in the side chain.

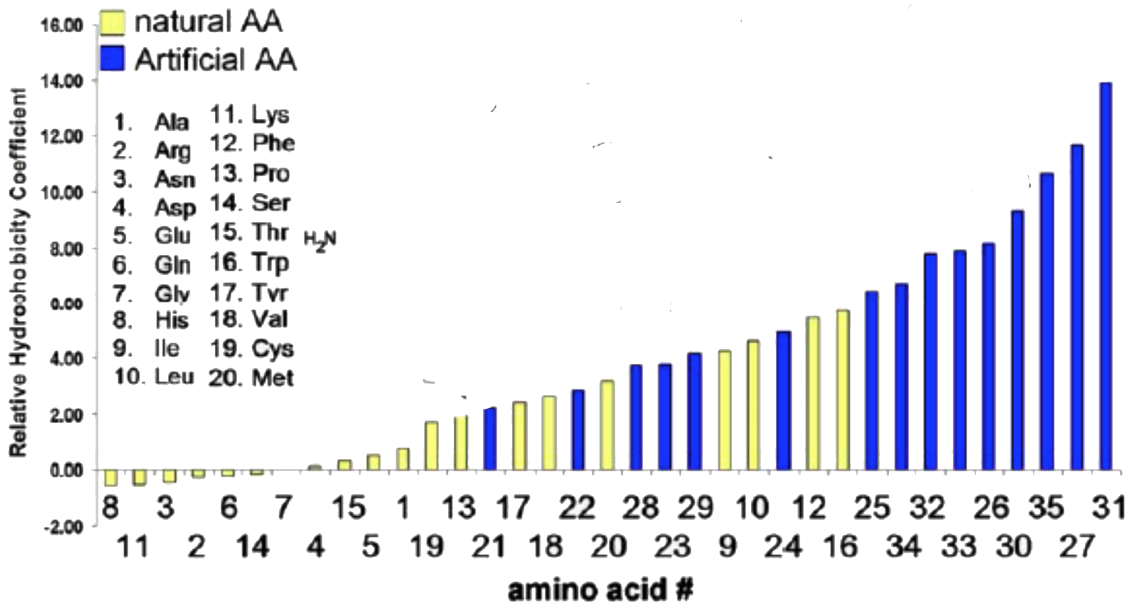


Figure 2.9: Amino acid substitution in the linear biligand to develop branched biligands. The linear biligand peptide **41** developed earlier is modified through amino acid substitutions to yield branched biligands **42** and **43**.

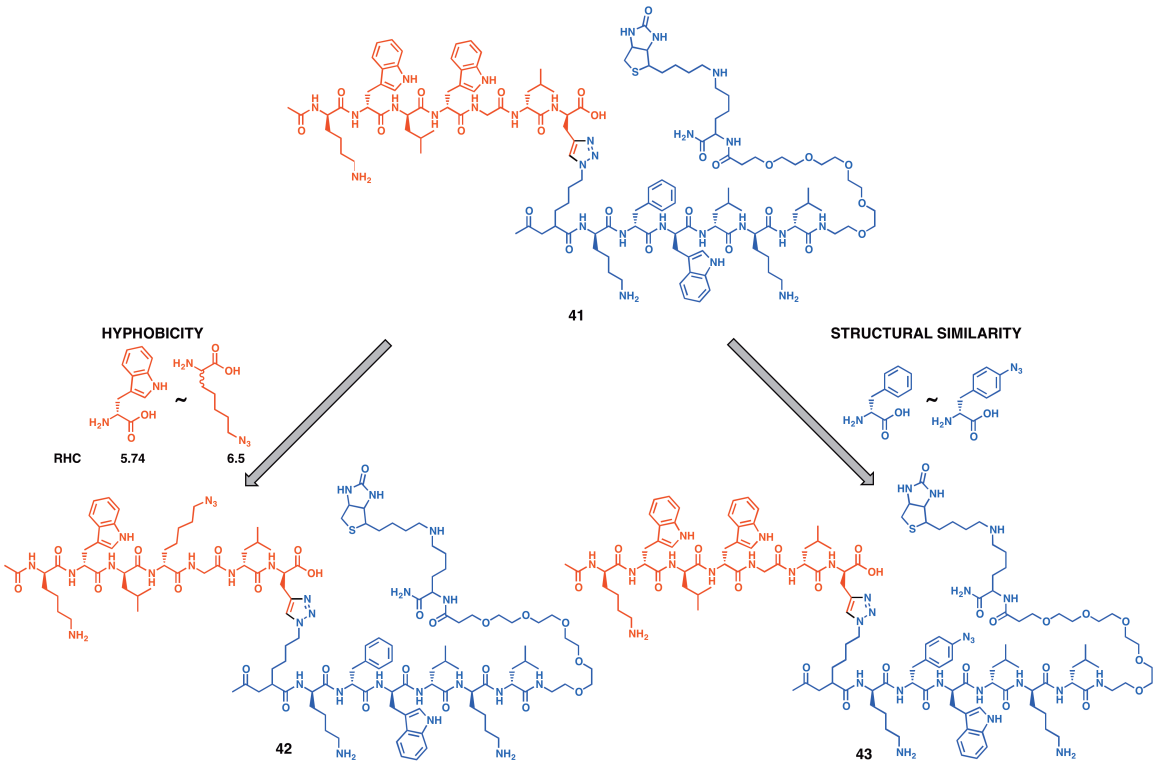
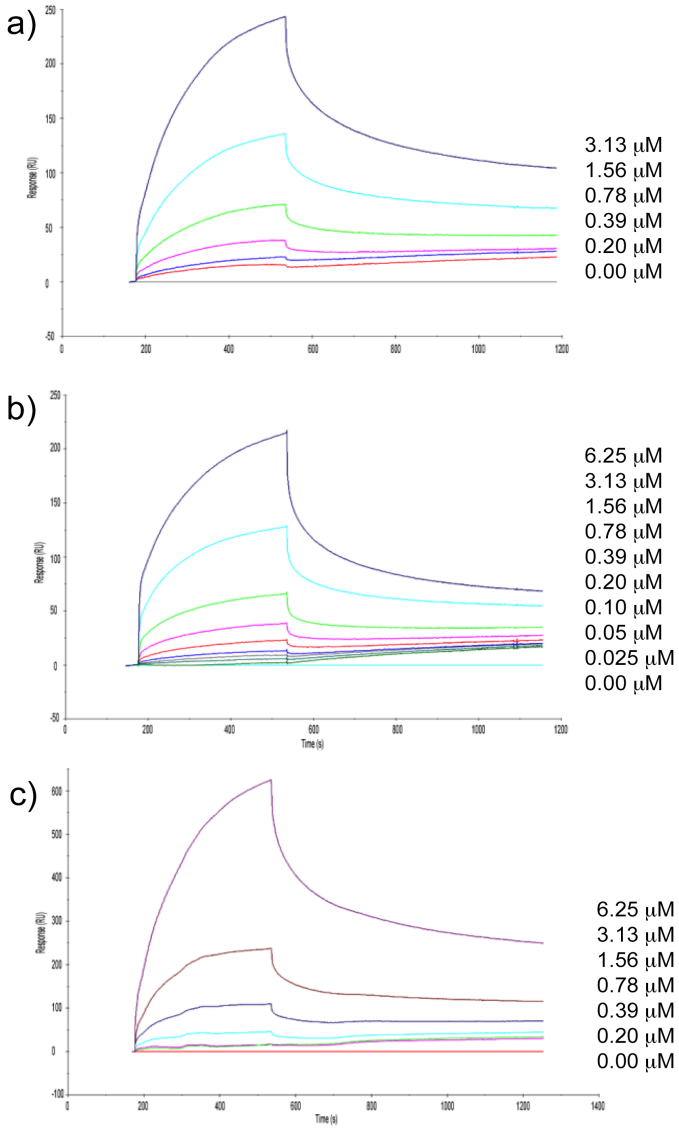


Figure 2.10: SPR response sensograms of the branched biligands developed by amino acid substitution of the linear biligand.

Sensograms of a) linear biligand **41** and branched biligands b) **42** and c) **43.** The immobilization level of bCAII is 4000 RU.



2.3.3 Branched triligand development with substituted biligand

The biligand **42** obtained by modifying the linear biligand **41** using the hydrophobicity approach was utilized as a new anchor peptide for a branched triligand peptide development. In the in situ click target screen, the Alexa Fluor 647 labeled bCAII pretreated with biligand **42** was screened against library D. The beads with high fluorescence intensity are automatically sorted using COPAS plus and sequenced by Maldi TOF/TOF[24] (Table 2.6). The 8 most frequently occurring amino acids were used to generate the focused library. Screening this focused library gave highly homologous peptide sequences (Table 2.7). The hit sequences are found to contain into three motifs (denoted by red, green and blue colors in the sequences). One peptide was chosen from each of the three motifs as the triligand arm. Triligand **45** was constructed with the sequence Ac-(D-Pra)fkifvr as the third peptide arm. Other two triligands **46** and **47** were synthesized with Ac-(D-Pra)irlflk and Ac-(D-Pra)kyffrf as the third peptide arm respectively.

Figure 2.11: Schematic representation of the in situ click target screens TRI1 and TRI2 for developing branched triligand.

The Alexa Fluor 647 (denoted by star) labeled bCAII protein is treated with biligand **42** and screened against on bead peptide library.

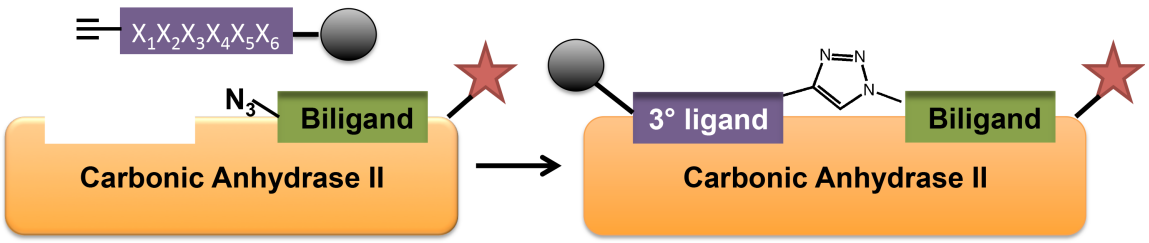


Figure 2.12: Branched triligand development through two generation target screen starting from a comprehensive randomized library.

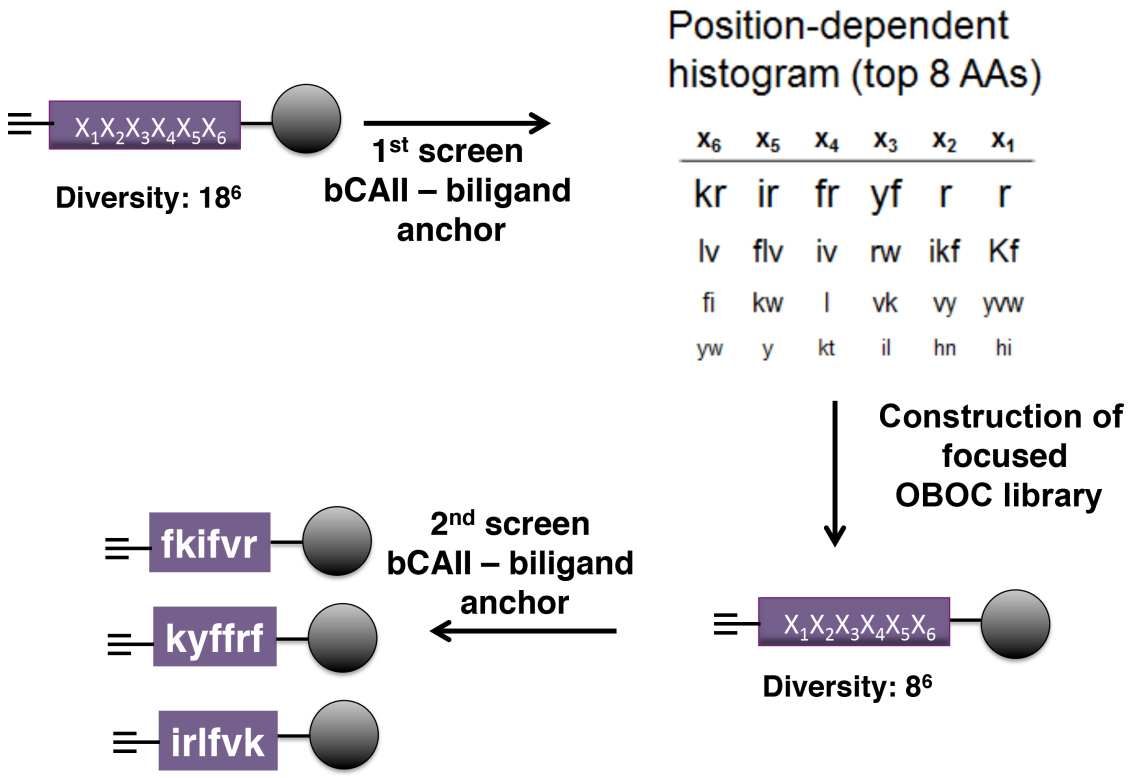
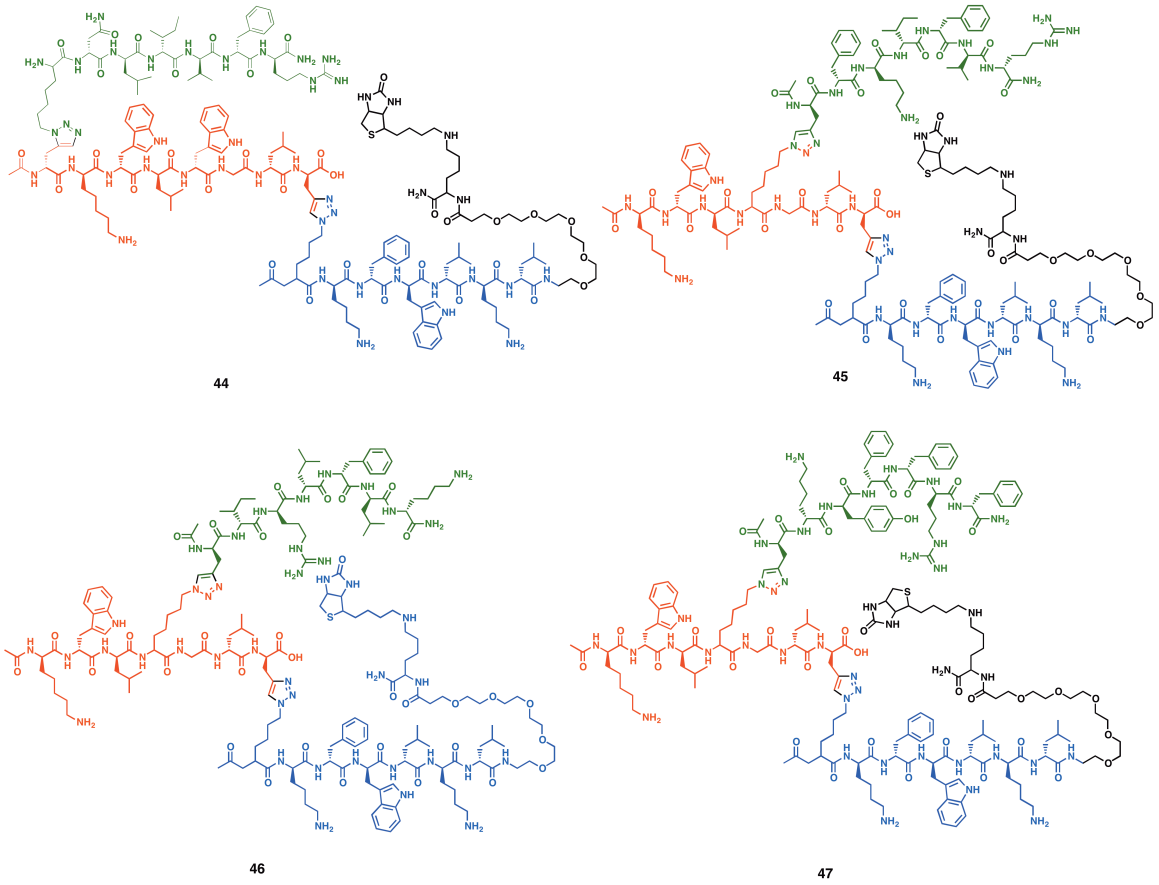


Figure 2.13: Molecular structures of the linear triligand 44 and the three branched triligands 45, 46 and 47 obtained through the target screening process using modified biligand 42 and bovine carbonic anhydrase II protein.



2.3.4 Development of branched triligands using biligand anchor isolated through screens

To select the best triligand binders for bCAII using biligand anchor **40** a product screen was performed. In the earlier target screens, beads with the high fluorescent intensity had been picked as hit beads. These beads contain peptide sequences that bind to the fluorescent protein. As the protein is pretreated with a high concentration of the anchor peptide, it is assumed that the hit sequences bind effectively to the protein in presence of a anchor peptide bound to the protein surface. An alternate method of screening is one that can directly detect the click product formation on the hit beads. This can be achieved by tagging the anchor peptide with the biotin tag and detecting the biotin rather than the protein. After the screen of the OBOC library with the mixture of the protein and the biotin labeled biligand anchor peptide **40**, the protein and peptide binding nonspecifically to the beads are removed by harsh washes with denaturants like guanidium hydrochloride and dimethyl formamide. Only the anchor peptide covalently bound to the on bead peptide via triazole formation remain and are detected by an anti biotin antibody. To eliminate beads binding to the detection antibody, the beads are rescreened. The screen is identical to the first but uses biotin saturated anti biotin antibody for detection. The biotin containing beads remain colorless as the biotin binding site on the antibody is already occupied. The colorless hit beads were picked and sequenced.

The four sequences obtained after the second screen are listed in Table 2.8. Note that the four sequences were obtained as the triligand arms starting from a randomized library containing ~2 million unique sequences, while the sequences for the biligand were obtained from much smaller focused libraries. This process of direct detection of the clicked product is much more stringent than the earlier screen formats and do not need development and screening of consecutively focused libraries. The triligands **48**, **49**, **50** and **51** were synthesized by CuAAC

reaction of the biligand anchor with triligand arm Az4-hdtgf, Az4-hdtfy, Az4-hdegg and Az4-ysqwa, respectively.

Figure 2.14: Scheme for in situ click product screen TRI3 for development of branched triligand.

The click product is directly detected on bead by detecting the biotin tag on the biligand anchor peptide.

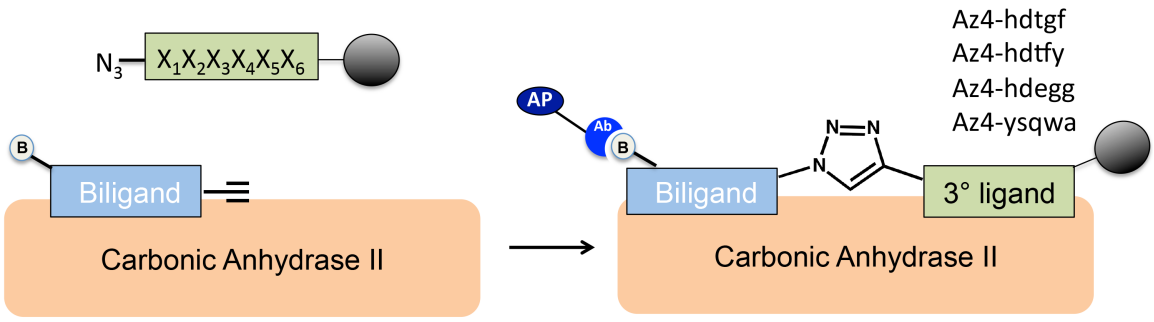
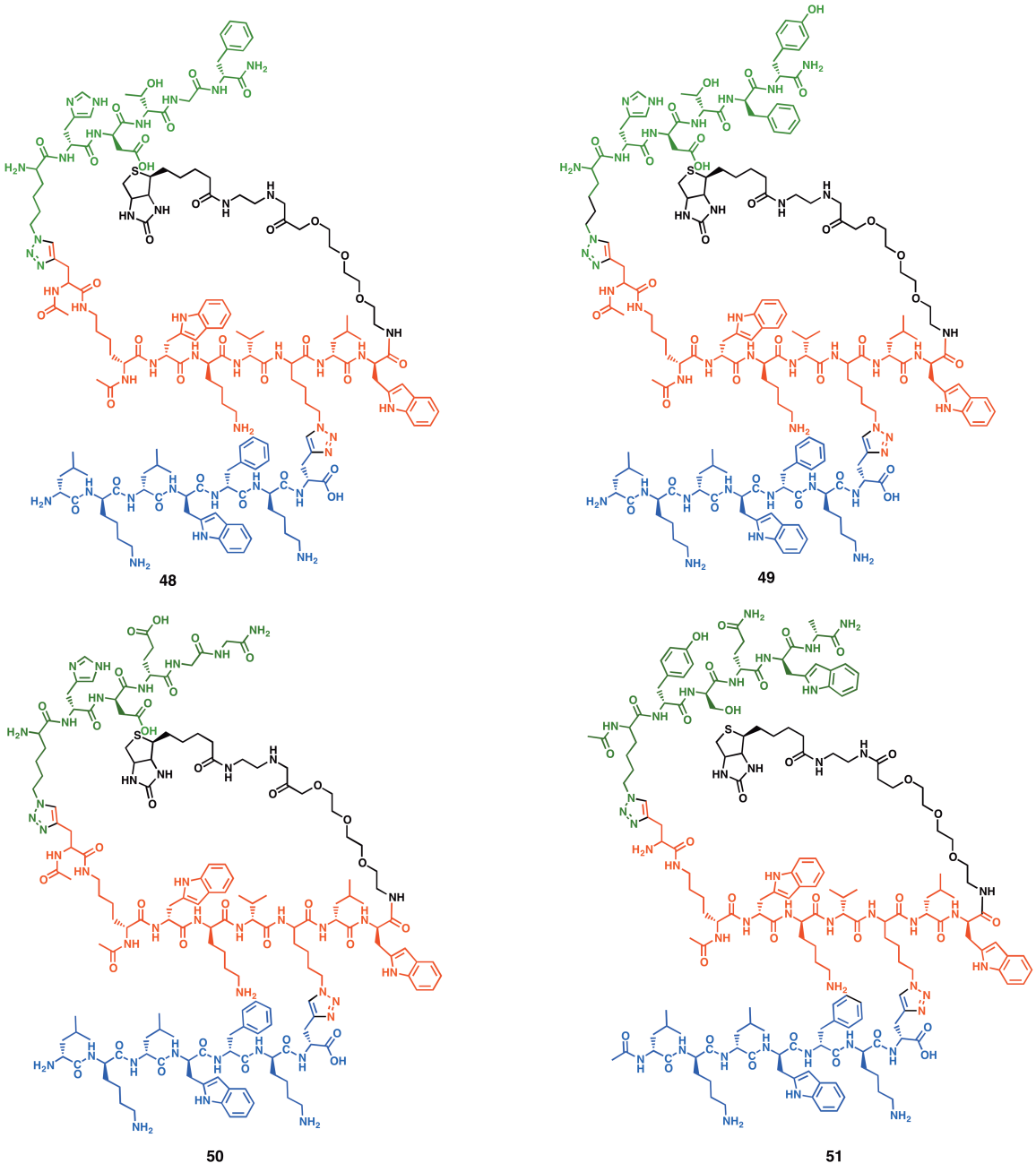


Figure 2.15: Molecular structures of branched triligands developed through product screen against the bovine carbonic anhydrase II.

The triligand arm (in green) is linked to the biligand anchor through CuAAC reaction.



2.3.5 Characteristics of developed capture agents

The branched biligands and triligands developed were tested for their binding affinities, their selectivity and specificty for bCAII. The bovine and human carbonic anhydrase II have high homology in the amino acid sequence and protein structure and it had been noted that the monoligand and linear triligand had similar affinity for both. The same trend was observed for the ligands developed here.

To demonstrate the efficiency of the ligands as detection agents they are used to detect bCAII spotted on a nitrocellulose membrane. The dot blot experiment is performed with a series of concentrations of the protein to determine the minimum amount of protein that can be detected (L.O.D). The L.O.D for the several triligands **45, 46** and **47** is 50 ng, as compared to 100 ng for the linear triligand **44**. The L.O.Ds for all the ligands are noted in the Table 2.9. By nature, the dot blot experiments are dependent on the concentration of the detecting reagent. To cross verify the L.O.D.s for the ligands, two different concentrations of the ligands, 1 μM or 10 nM, are used in different sets of experiments. Although the values are different for the two sets of experiments, the same trend is observed relative to each other.

The use of the developed ligands as capture agents is demonstrated in a series of sandwich ELISA assays. The biotinylated ligands were immobilized on a streptavidin plate and titrated with varying concentrations recombinant human carbonic anhydrase II. The absorbance at 450nm wavelength was proportional to the binding of the capture agent to the protein and was used to calculate the relative affinity curves of the various ligands.

The sandwich ELISA is a more sensitive assay than the dot blot and gives us better insight in the change in binding affinities. In the ELISA experiment we observe that when the D-Trp in the linear biligand **41** was replaced by D,L-azidolysine to create the substituted branched biligand **42**, there was a ~ 2 fold decrease in the binding affinity of the biligand for the protein, despite amino acid **25** and tryptophan having similar RHC values. Thus substitution of an amino

acid in a linear ligand to make it branched did not make it a better binder in the biligand stage. However, when the substituted biligand **42** was used in the next phase of multiligand development process, the resultant triligand candidates **46** (EC_{50} 50 nM) and **47** (EC_{50} 58 nM) have ~3 times higher affinities than the earlier reported linear triligand **44** (EC_{50} 144 nM).

Figure 2.16: Biligands and triligands used as detection agents for bCAII.

Dot blot using biligands for protein detection. The picture on the left specifies the amount of bCAII protein spotted on the membrane.

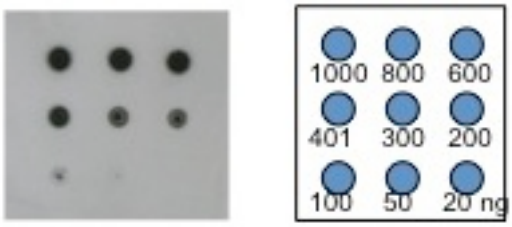
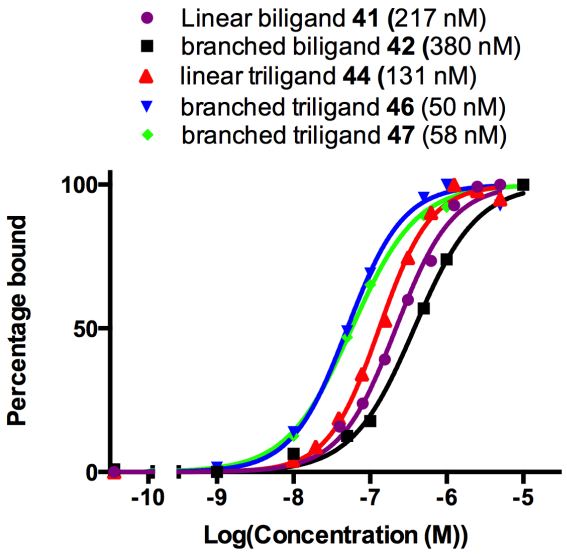


Table 2.9: Table summarizing the limits of detection (L.O.D) of the bovine carbonic anhydrase II protein using the developed multiligands as detection agents.

Ligand	**L.O.D. using 10 nM ligand solution for detection (ng)**	**L.O.D. using 1 uM ligand solution for detection (ng)**
Linear biligand **41**	200	Not determined
Branched biligand **42**	300	Not determined
Linear triligand **44**	100	Not determined
Branched triligand **45**	50	50 ng
Branched triligand **46**	100	50 ng
Branched triligand **47**	50	50 ng
Branched triligand **48**	200	50 ng
Branched triligand **49**	200	50 ng
Branched triligand **50**	200	50 ng
Branched triligand **51**	200	50 ng

Figure 2.17: ELISA demonstrating relative binding affinities of biligands and triligands. Binding of linear biligand **41** (purple), branched biligand **42** (black), linear triligand **44** (red), and branched triligands **46** (blue) and **47** (green) for the CAII protein. The biotinylated ligands are immobilized on a SA plate and treated with different concentrations of the protein. The individual absorbance values are denoted as points and the fits are denoted as solid lines.



2.3.6 Substitution of triazole linkage by triazole mimic

The orientation of the alkyne and azide during in situ protein catalyzed click chemistry dictates the nature of the triazole linkage between the two functionalities. If the protein scaffolds and orients the azide and alkyne is a syn orientation, a 1,5 - disubstituted triazole linkage is formed by the in situ click reaction. If, on the other hand, the protein scaffolds and orients the azide and alkyne is an anti orientation, a 1,4 - disubstituted triazole linkage is formed by the in situ click reaction. A protein, when catalyzing the click reaction, may yield only one triazole product exclusively. So it is important to check both the triazole products for binding affinity to the protein. While the 1,4 substituted triazole linked peptide biligand can be synthesized relatively simply by copper-catalyzed alkyne azide chemistry (CUAAC), it is synthetically

challenging to synthesize the 1,5 disubstituted triazole linked peptide biligand in bulk for different assays. To overcome this difficulty, a mimic of the 1,5 disubstituted triazole linker was synthesized. As proline has been successfully replaced by the 1,5 disubstituted triazole molecule in proteins[25], we reversed the concept to replace 1,5 disubstituted triazole with proline. To recreate the length of the 1,5-triazole link that is formed between L-azidolysine and D-propargyl glycine, as in the in situ protein click reaction, D-Pro and D-Asp was coupled to the acid side chain of L-diaminobutyric acid.

For evaluating the binding affinity of the 1,5-triazole containing ligands as compared to the 1,4-triazole containing ligands for the protein, two branched biligands **38** and **39** were synthesized containing the 1,5-triazole mimic linker. Each of the ligands were immobilized on an ELISA platform and titrated with the protein. For both the branched triligands **38** and **39,** the 1,5-triazole mimic linked ligands have higher affinities for the CAII than the corresponding 1,4 triazole linked ligands. The binding affinity of the 1,5 triazole mimic linked biligand was ~3.3 times the binding affinity of the 1,4 triaozle linked ligand **38**. The binding affinity of the 1,5-triazole mimic linked biligand for protein CAII was ~3 times that for 1,4 triazole linked branched biligand **39.** This indicates that at the biligand stage, the protein catalyzes the formation of the 1,5 triazole linked biligand, where the two peptide arms are turned towards each other rather than the 1,4 triazole linked biligand, where the two peptide arms are placed away from each other.

Figure 2.18: Molecular structure comparison of 1,5 disubstituted triazole linked biligand and its mimic.

A dipeptide D-Pro-D-Asp is coupled to the side chain of L-diaminobutyric acid to mimic the 1,5 triazole linker. The number of atoms in the linker and the orientation of the triazole are maintained. The two peptide arms are syn to each other in the 1,5 mimic.

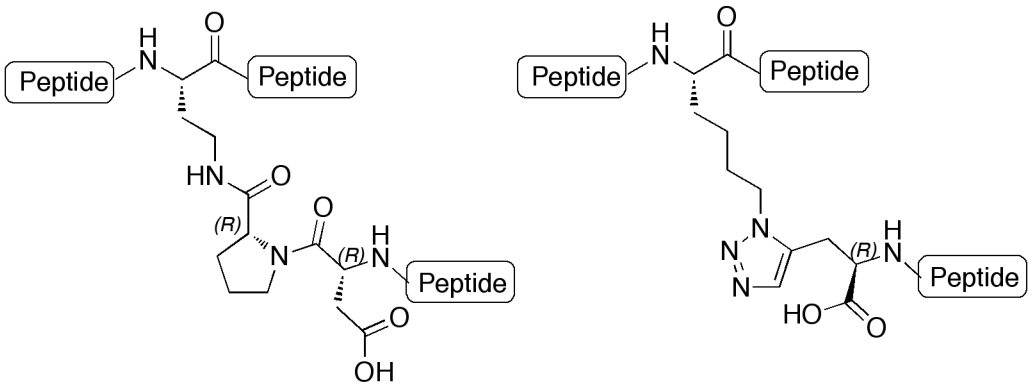
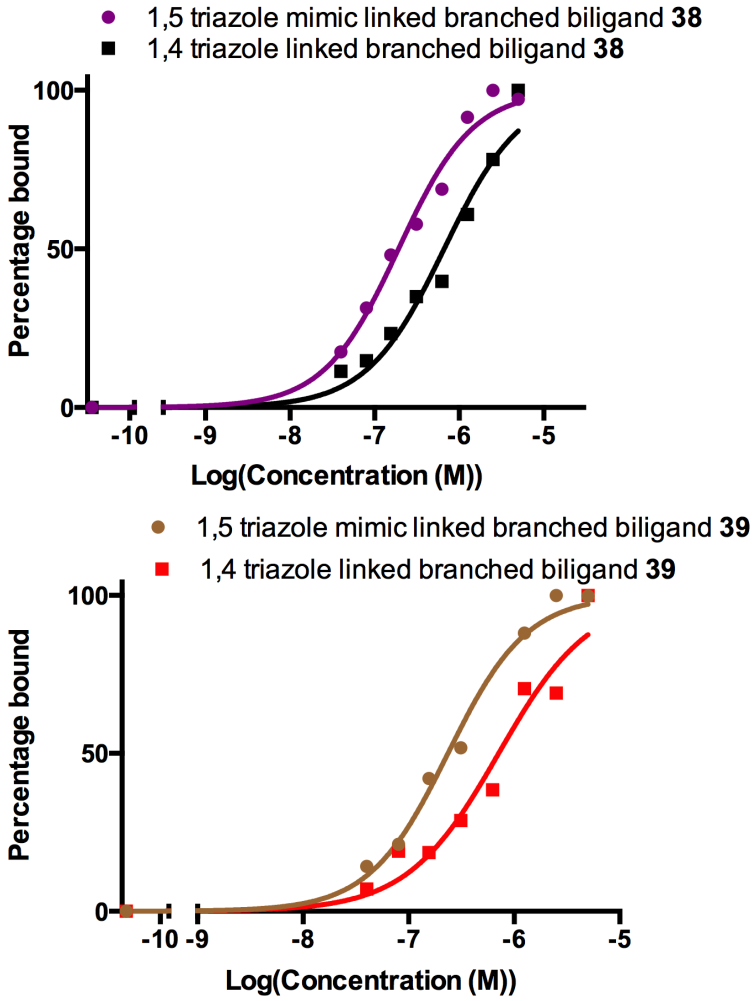


Figure 2.19: Comparison of the binding affinities of 1,4 and 1,5 disubstituted regioisomers of biligands to carbonic anhydrase II protein.



2.4 Conclusions

In this work we evaluate the effect of branching a linear peptide ligand starting with the same weak binding monoligand peptide arm. Two approaches are described. One involves replacing an amino acid in the isolated linear biligand with an amino acid of similar hydrophobicity, to make the biligand branched. The protein, in presence of this branched biligand searches for a third linear peptide arm. The resulting triligands developed are branched. The developed triligands ligands **45**, **46** and **47** have either similar or lower limits of detection than the linear triligand previously developed. The other strategy involves using an OBOC peptide library with random number and position of the branching amino acid azidolysine to find the natural preference of the protein binding in presence of the weak monoligand peptide. Using focused libraries, this pattern is further resolved as wX(Az4)xw or vX(Az4)Xw. The protein in presence of this branched biligand anchor is screened against a linear peptide library to find triligand binders. Of the four ligands isolated though the screens, two triligand arms are found to have a pattern of hdtXX. However despite the high homology of the triligand sequences, their limit of detection are higher than the other branched triligands using the semi quantitative dot blot method. The ligands are also used as capture agents in an ELISA platform. The ELISA assays show the same trend as the dot blots, with branched triligands **46** and **47** having ~3 times higher affinity than the linear triligand **44**.

While the protein might form the 1,4 or 1,5 triazole linkage between the two peptides, where the relative peptide orientations are very different, bulk synthesis and characterizations of ligands are performed with 1,4 triazole linked peptide ligands. Here we determine whether selecting a particular regioisomer for further screens can lead to a change in binding affinity. To circumvent the difficult synthesis of the 1,5 triazole linked peptide, a mimicking strategy is applied that conserves the linker length and triazole cis or trans orientation. All the biligands are

found to favor the triazole cis orientation, particularly the branched biligands developed through screening. This might partially explain why we do not attain the desired affinity of the PCC agent in the triligand stage, as we screened with the 1,4 diastereomer of the biligand with low affinity. In the triligand stage also the 1,5 regioisomers might have better affinities than the 1,4 regioisomers. In the development of future PCC agents, it would be beneficial to check the binding affinity of the different regioisomers of the multiligand from the biligand stage and choose the best binder before proceding to triligand development.

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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_2L-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_α-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_2L-Az4-PEG$_2$-Biotin to phospho-amino acids and a phosphate containing peptide

422 μM solution of Zn_2L-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_2L-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_2L-Az4-PEG$_2$-Biotin* – pSer), (*Zn_2L-Az4-PEG$_2$-Biotin* – pTyr) and (*Zn_2L-Az4-PEG$_2$-Biotin* – Ac-I-pY-GEF)[22]. The pure compound *Zn_2L-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_2L-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_2L-Az4-PEG_2-Biotin*.

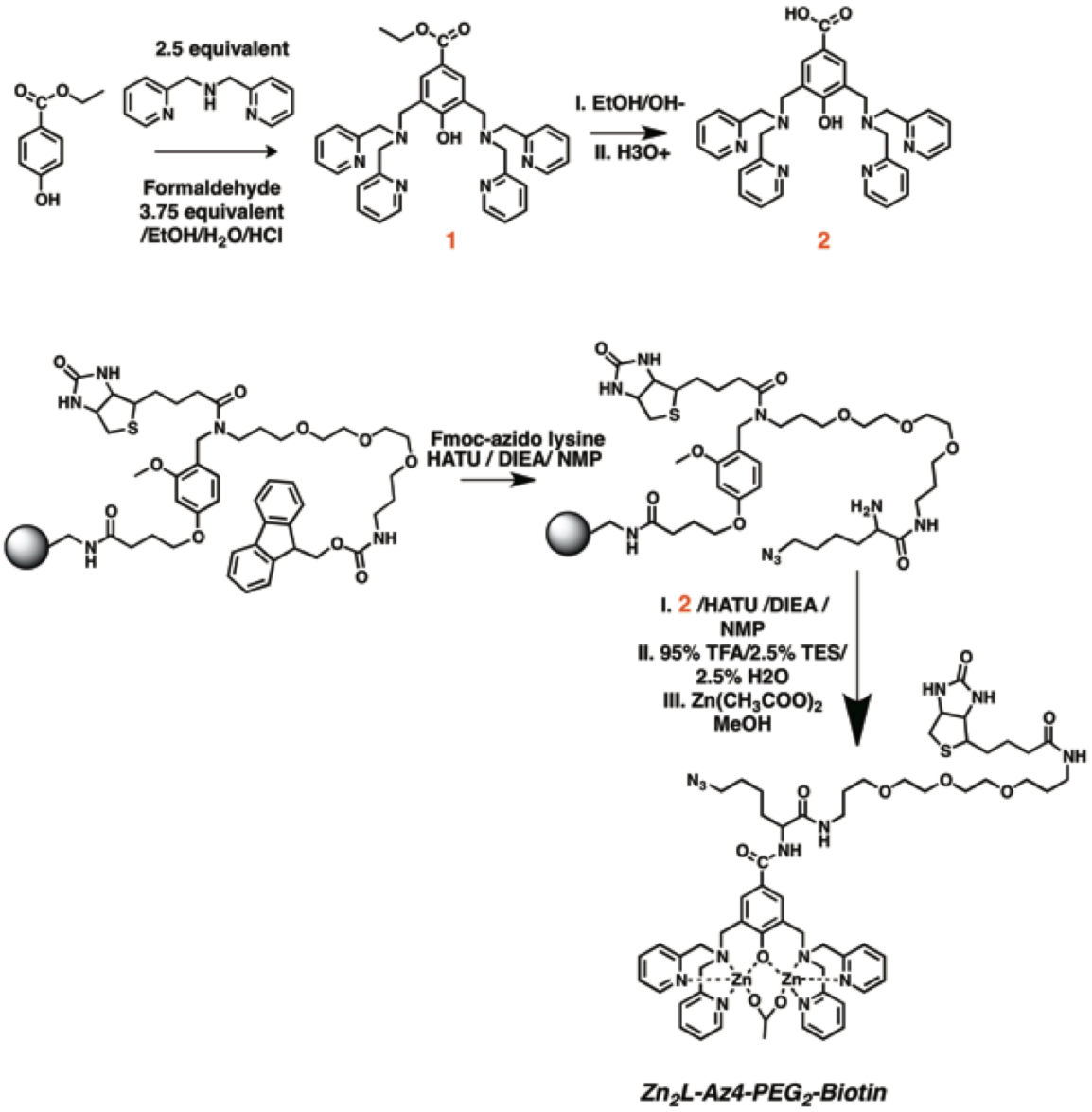
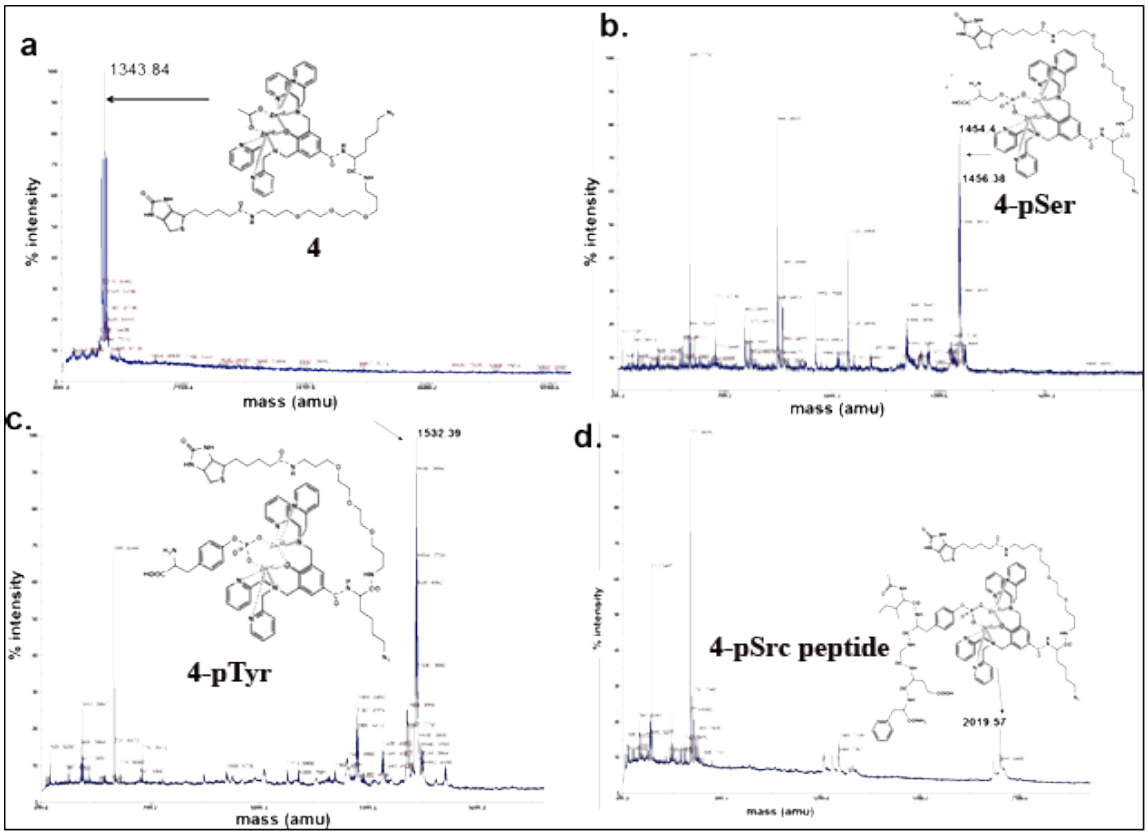


Figure 3.2: Evidence of binding of dinuclear zinc chelator to phospho amino acids and phospho peptide.

Structure and Maldi TOF spectra of the Zn chelator *Zn_2L-Az4-PEG$_2$*-Biotin and complexes of *Zn_2L-Az4-PEG$_2$-Biotin* with phosphorylated amino acids and phosphorylated peptides. Mass spectrum of a) pure *Zn_2L-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_2L-Az4-PEG$_2$-Biotin* - phospho-serine (observed mass =1454.4; expected mass = 1454.43) c) *Zn_2L-Az4-PEG$_2$-Biotin* - phospho-tyrosine (observed mass = 1532.39 ; expected mass = 1532.46). d. Mass spectrum and structure of *Zn_2L-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_2L-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_2L-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*.

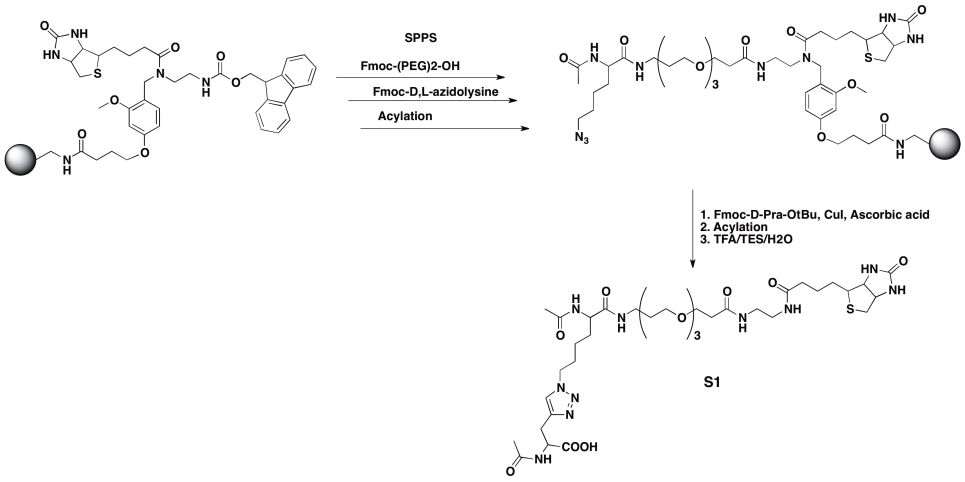
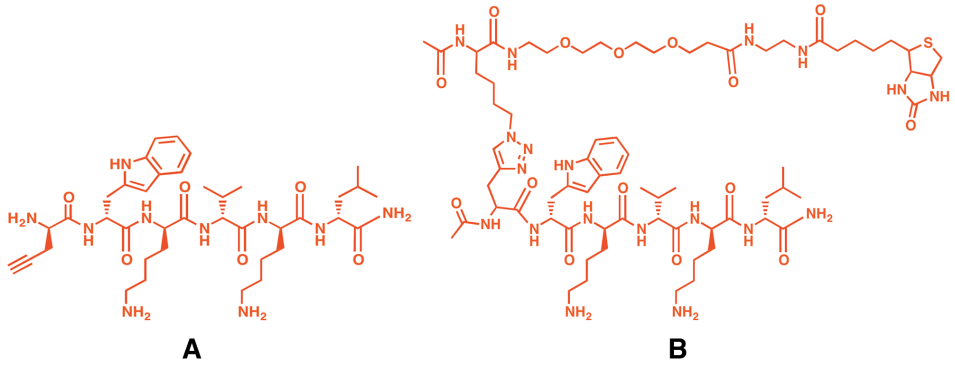


Figure 3.5: Structure of the monoligand peptide *mono-L* developed against the target peptide.

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

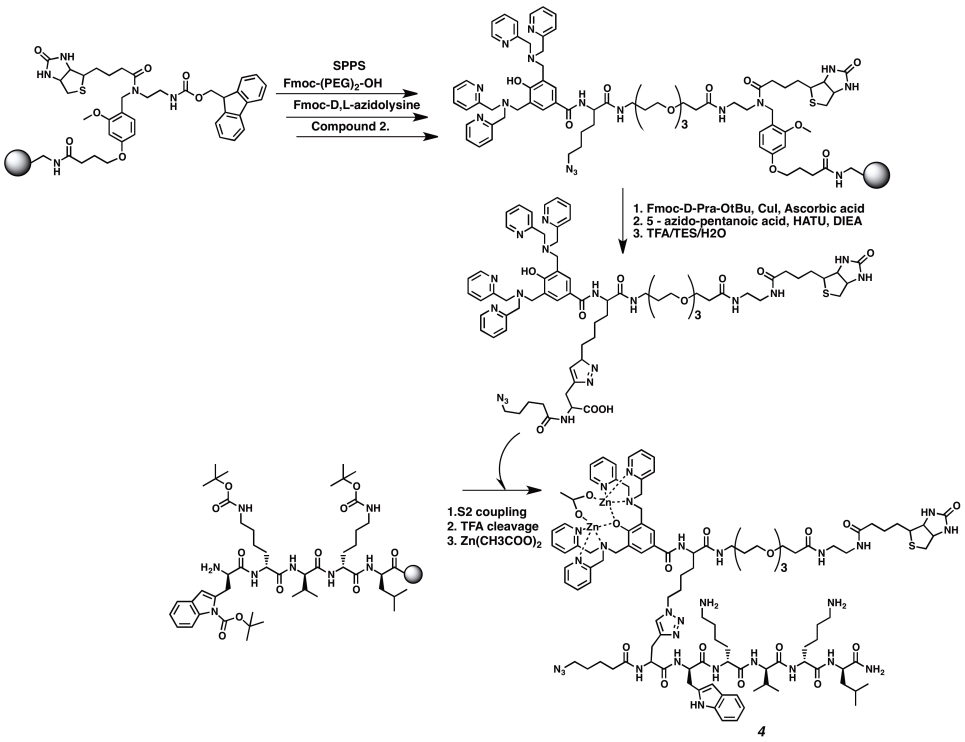


Synthesis of compound 4:

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*.**

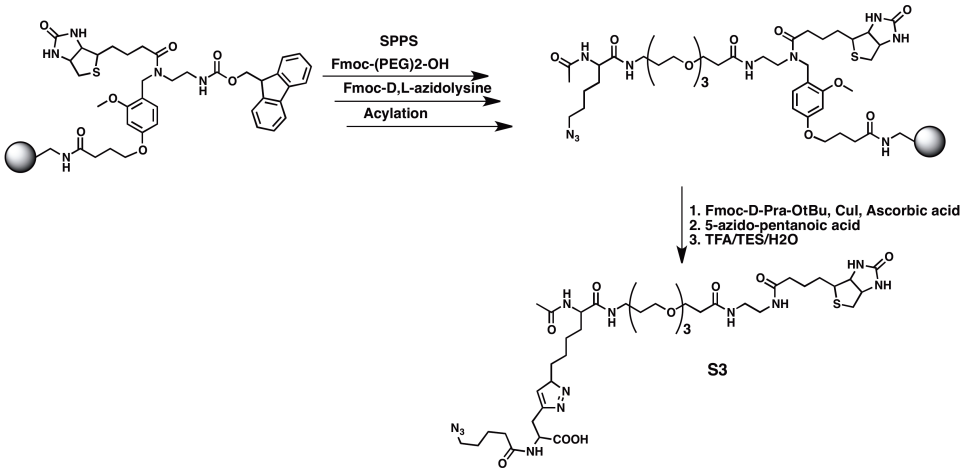
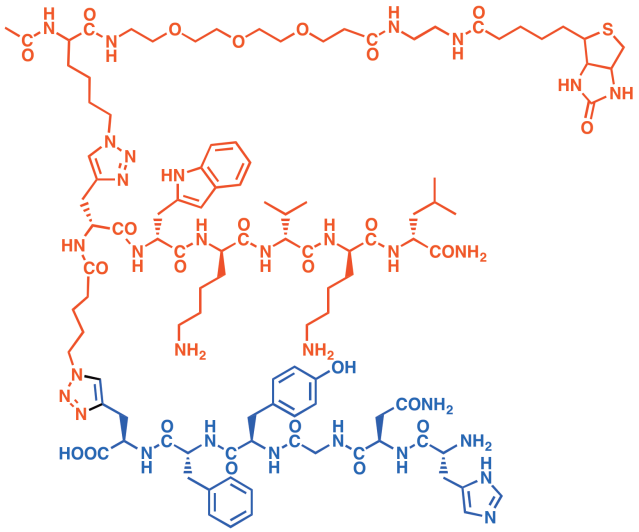


Figure 3.8: Structure of the biligand *bi-L* isolated in the screen against the target peptide.



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.*

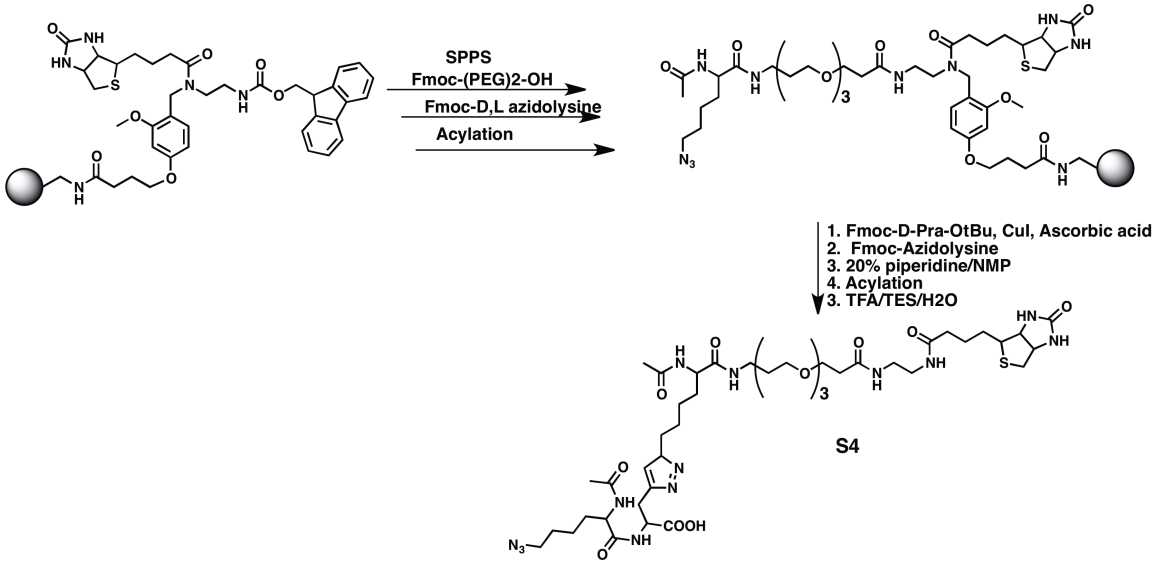
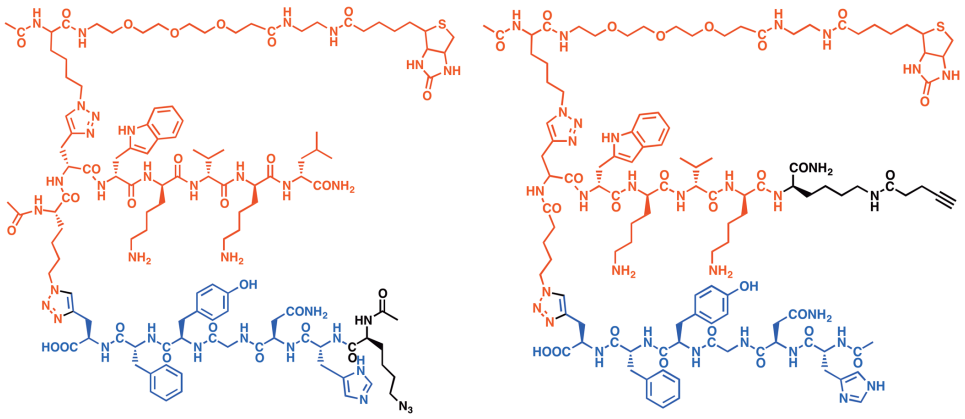


Figure 3.10: Structure of biligand anchor peptides *Anchor-3N* and *Anchor-3C*.

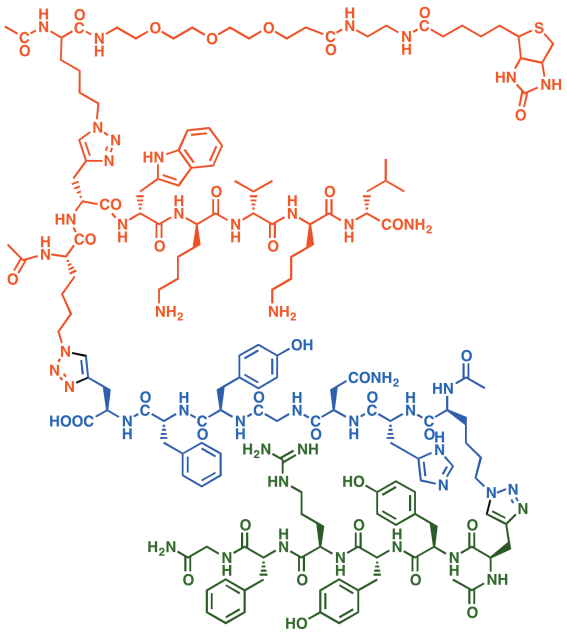
A. *Anchor-3N* is used to the Akt2 protein before screening against a alkyne containing OBOC library A. *Anchor-3C* is used to the Akt2 protein before screening against a alkyne containing OBOC library.



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.*

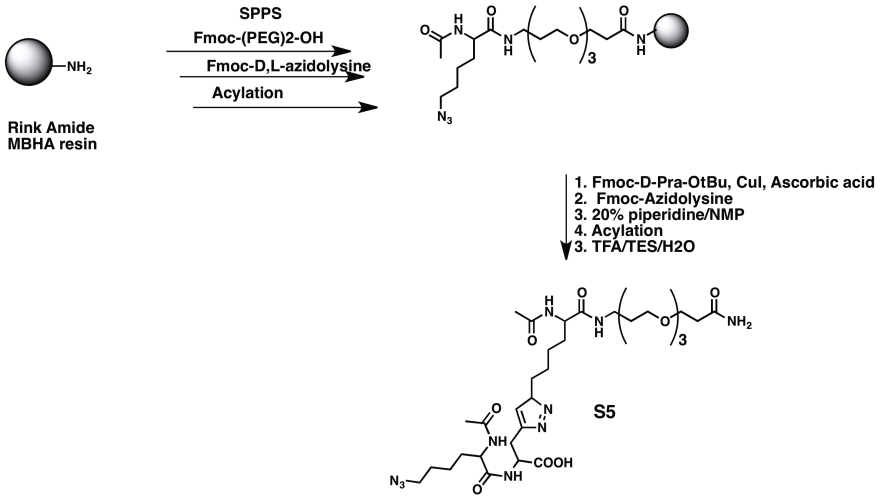
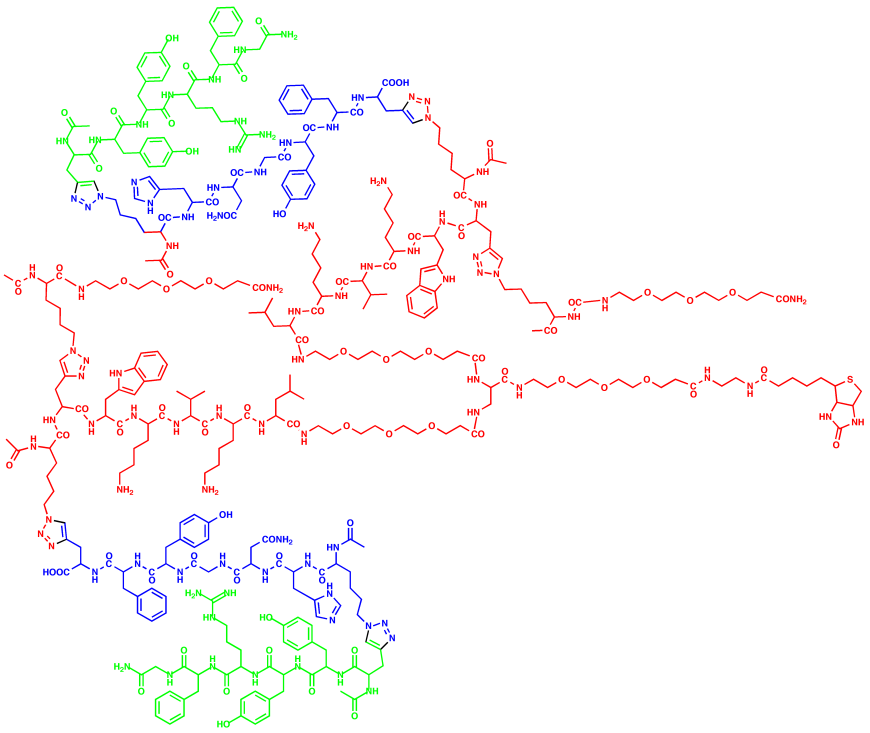


Figure 3.13: Molecular structure of the triligand *N-term-tri-dimer.*



Synthesis of D-Lys (pentyne) amide:

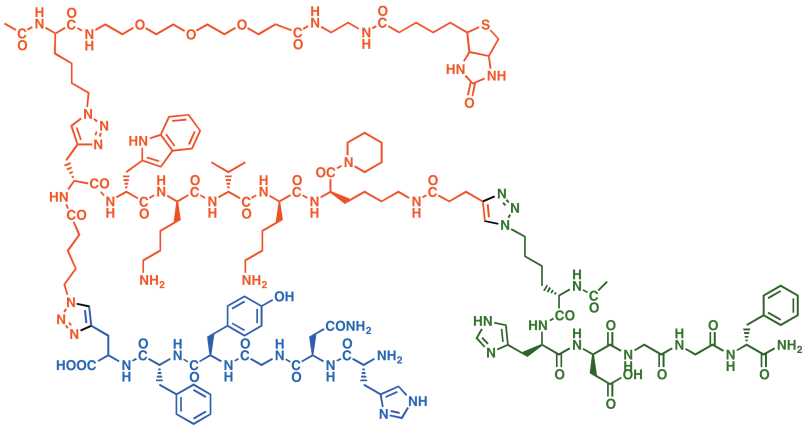
Boc-D-Lys(Fmoc)-OH was coupled with rink amide resin. Then 4-pentynoic acid was coupled with it, after standard piperidine deprotection. The dried resin was cleaved with TFA cocktail and purified using a gradient of water and acetonitrile and 0.1% TFA on the prep-HPLC. Mass calculated: (M+H) 225 Mass 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_2L-Az4-PEG$_2$-Biotin and verification of its binding to phospho amino acids and phosphopeptide

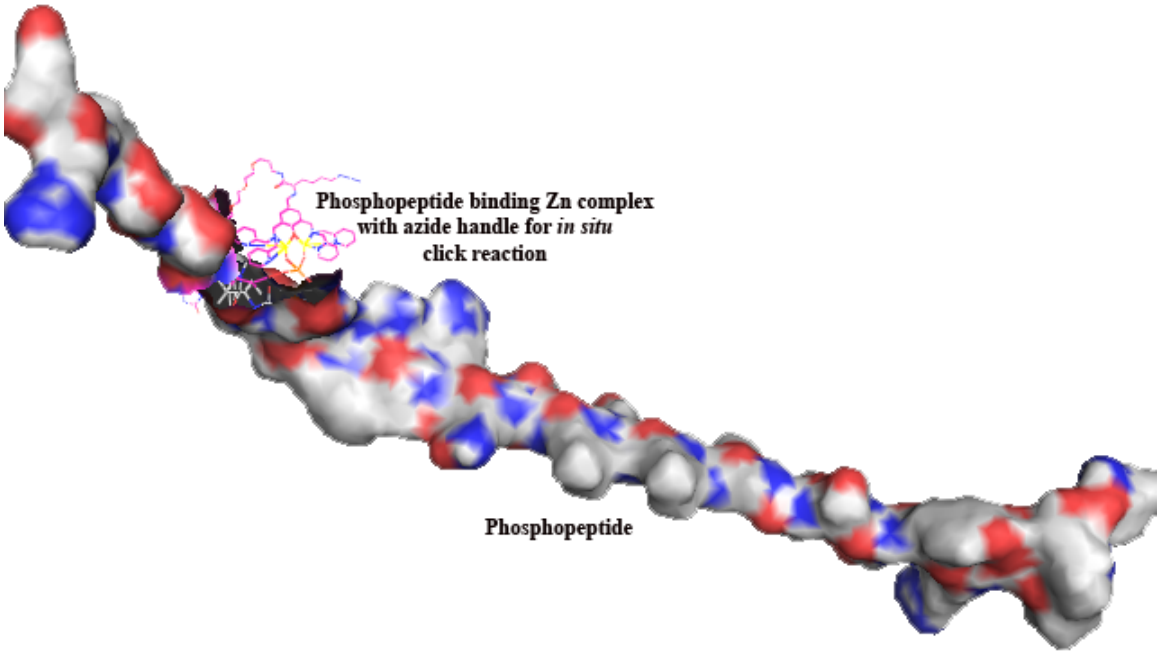
Dinuclear Zn(II)-dipyridylamine (DPA) complexes, due to their avidity for phosphate anions over other anions such as carbonate, sulphate, or chloride, have been used, for example, in the development of fluorescence-based sensors of kinase activity [21], for the detection of bacterial infection sites [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_2L-Az4-PEG_2-Biotin* (Figure 3.1). The property of *Zn_2L-Az4-PEG_2-Biotin* to selectively bind to phospho-serine, phospho-tyrosine and a phospho-peptide (pSrc substrate) was demonstrated by incubating *Zn_2L-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_2L-Az4-PEG_2-Biotin* -pSer, *Zn_2L-Az4-PEG_2-Biotin* -pTyr and *Zn_2L-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_2L-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.

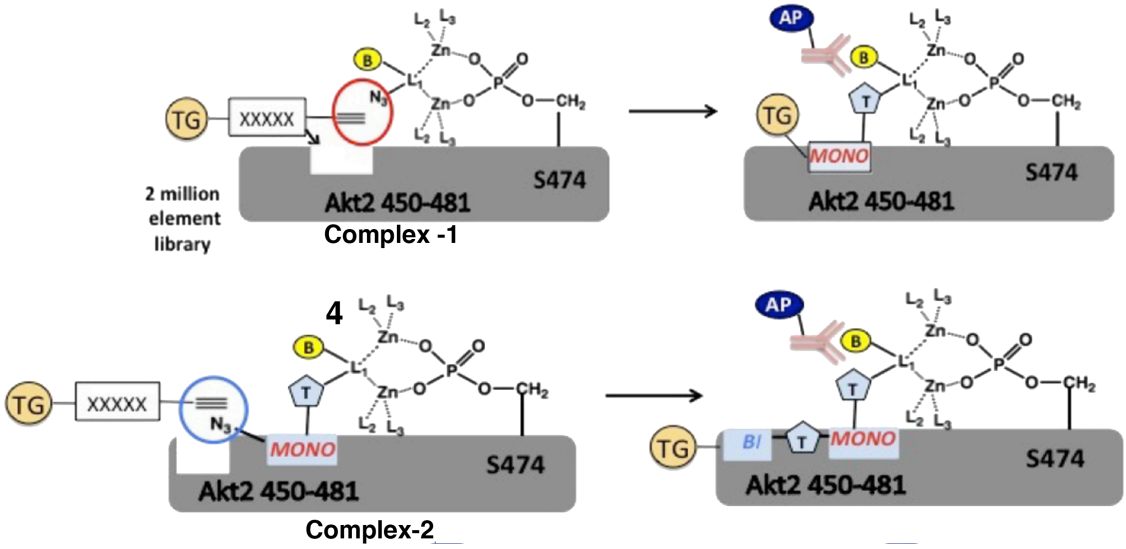


3.3.3 Identification of a 1^{o} ligand targeted against Akt2 C terminal fragment

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_2L-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

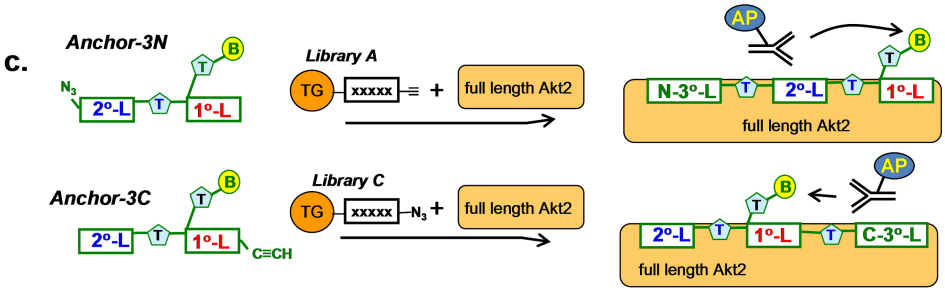
We used a similar strategy to identify candidate 2^o ligands. The Zn chelator *Zn_2L-Az4-PEG_2-Biotin* was modified with peptide wkvkl and an azide on the N terminal to form compound **4**, which was then reacted with the C-terminal 32-mer polypeptide fragment of Akt2 containing p-S474 to form *Complex-2* (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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Chapter 4: Characterization of protein capture agents developed against Akt2

4.1 Introduction

In chapter 3, we discussed the chemical epitope targeting strategy and the development of multivalent ligands against the Akt2 protein through consecutive in situ target catalyzed click reaction. During the development of the various peptide arms from the 1° ligand to the 3° ligand, the resulting ligand is validated in at least one assay and the peptide arm showing the best affinity and specificity is further used in the next step of multivalent ligand development. In this chapter we describe the assays used to choose the peptide arms, and characterize the final developed ligands in additional assays. We also explore the effect of the ligands on the kinase activity of the protein. The relative binding affinities of the ligands for Akt2 were determined through ELISA assays. The triligand candidates had low nanomolar binding affinities. The values are in agreement with the absolute values obtained through SPR experiments. Selectivity or specificity of each ligand were determined against the epitope and against the full protein. We demonstrate that the ligands bind selectively to the target C-terminal 32-mer polypeptide fragment of Akt2. Finally, we explored if the developed ligands have any effects on the kinase activity of the Akt2 protein. Remarkably, the two triligands, sharing the same biligand fragment, were found to have opposite effects on the kinase activity of the Akt2 protein. While one of the ligands enhances the kinase activity of the protein, the other ligand inhibits the kinase activity.

4.2 Materials and Methods

4.2.1 Materials

Mouse anti biotin monoclonal antibody- Horse Radish Peroxide conjugate was purchased from Cell Signaling Technology. Anti His_6 mouse monoclonal antibody, Akt2 phospho S474 antibody and goat anti mouse IgG-Horse Radish Peroxide conjugate were bought from Abcam. Anti Akt (pan) rabbit antibody (11E7) and anti rabbit antibody- Horse Radish Peroxide conjugate,

used in Western blot and dot blot experiments, were purchased from Cell Signalling Technology. Biacore chip, reagents and buffers used in the SPR experiment were bought from GE Healthcare. High capacity Strepatvidin coated 96 well plates were obtained from Thermo Scientific. 96 well plates were bought from Nunc. TMB substrate for HRP, used in ELISA assays, was bought from KPL.

4.2.2 Methods

4.2.2.1 Peptide synthesis

Synthesis of His_6 tagged target phosphopeptide and off target peptide sequence:

The phosphorylated version of the target sequence, amino acids 450-481 of Akt2, with pS474, and the 33mer off target peptide (Akt2 amino acids 346-378) were synthesized on Rink Amide MBHA resin, using standard Fmoc based SPPS protocols. 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/ddH_2O, precipitated in cold ether and purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Target phospho-peptide: Calculated mass: 4858.2 Observed mass: 4858.1 Scrambled phospho-peptide: Calculated mass: 5071.5 Observed mass: 5071.5

4.2.2.2 ELISA for *mono-L* and *bi-L*

On a nunc 96 well Immunosorp plate, 1 μg of active Akt2 per well was immobilized for an hour in carbonate buffer (pH 9.6). The wells were blocked with ethalonoamine for two hours and then with SuperBlock blocking buffer in PBS (Thermo Scientific) overnight at 4°C. The wells were washed with PBST. A half dilution series of the ligand (concentrations from 50 nM to 10 μM) were made in the buffer (1% Superblock/ 0.05% Tween 20/PBS). The immobilized protein was shaken with the ligand solution series for 6 hours at 4°C, then washed five times with binding buffer. The wells were treated with Antibiotin-HRP antibody (1:10,000 dilution of 1 mg/ml stock) in binding buffer for an hour at 4°C. After four washes with PBS and one wash with

PBST (0.05% Tween-20/PBS), the wells were treated with TMB substrate. The reaction was quenched with 0.5 M H_2SO_4. Absorbance was measured at $\lambda = 450$ on a 96-well plate reader. The Net A450 was obtained by subtracting the respective absorbance values for no immobilized protein from the values obtained for the ligand protein interaction. These values were normalized and the data fitted by non-linear regression in GraphPad Prism software.

4.2.2.3 ELISA for *N-term-tri-L* and *C-term-tri-L*

200 nM solutions of the biotinylated triligand in binding buffer (1% Superblock, 0.05% Tween 20/ TBS pH 7.5) were added to the wells of a High Capacity Streptavidin 96 well plate (Thermo Scientific) and shaken for two hours at room temperature. The plate was blocked with 1% BSA/ 0.05% Tween 20/ TBS pH 7.5. The wells were washed with binding buffer five times. Active His_6 tagged Akt2, from 1.15 nM to 450 nM concentrations, was added and the plate shaken overnight at 4°C. The wells were washed three times with the binding buffer, then treated for an hour with a 1:1000 dilution of anti His_6 mouse monoclonal antibody. A 1:10,000 dilution of goat anti mouse antibody-Horse Radish Peroxide conjugate (Abcam) in binding buffer was added to the wells for an hour. The plate was washed, treated with TMB and quenched with H_2SO_4, and the absorbance measured as described in the previous paragraph. The Net A450 (subtracting the absorbance value for no immobilized ligand) was fitted by non-linear regression in GraphPad Prism software.

4.2.2.4 OVCAR3 lysate pulldown assay

OVCAR3 cells were grown in RPMI-1640 media containing 10% fetal bovine serum and 0.01 mg/ml bovine insulin. Passage five 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 µL of 1 mM ligand stock (DMSO) to 10 µL of the swelled Streptavidin-agarose resin. After shaking overnight at 4°C, 50 µM 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 150 µL OVCAR3 cell lysate (0.8 mg/ml) and binding buffer was added to a final volume of 250 µL. The tubes were shaken at 4°C for 18 hours. The beads were then 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 40µL of 2X SDS-PAGE sample loading buffer (BioRad) and heating at 95°C for 10 minutes. 10 µL of each sample was loaded on a 12% SDS-PAGE gels (BioRad) and run for 50 minutes at 120 volts. The gel was transferred to a nitrocellulose membrane, blocked for 2 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 (11E7, Cell Signaling Technology) in 0.5% non-fat milk. The membrane was washed and treated for an hour with a 1:10,000 dilution of monoclonal mouse anti-rabbit- HRP secondary antibody (Cell Signaling) in 0.5% milk. After five washes of five minutes each with TBST and one wash of five minutes with TBS, the blot was developed with West Dura ECL substrate (Thermo Scientific) and imaged on film.

4.2.2.5 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 Signalling. Serial dilutions of 2 mM ligand solution were made in filtered 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 µL reaction mixture containing 400 ng Akt2, 400 ng GST-GSK-3α/β fusion protein, 500 mM ATP and 0.5 µL of peptide solutions in DMSO or DMSO only. Reactions were allowed to proceed for 30 minutes at 30°C. The reactions were quenched by addition of 2.5 µL of a 500 mM DTT/ 20% SDS solution.

For dot blot, 2 µL of each of the reaction mixtures were spotted in triplicate on 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-3α/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.

For the Western blot, an additional 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. This reaction mixture was treated similar to the other kinase reaction mixtures. After 30 minutes the reactions were quenched with addition of 3X SDS sample buffer. The solution was heated at 95°C for five minutes. 10 µL from each sample was loaded on a 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, treated with Phospho GSK-3α/β Ser (21/9) rabbit antibody and anti-rabbit –HRP antibody as described in the previous paragraph. 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.

4.2.2.6 Epitope Targeting Selectivity Assay

1.25 μM 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 μM solutions of the His_6 tagged target phosphopeptide epitope Akt2 amino acids 450 – 481 or the His_6 tagged off target peptide Akt2 amino acids 346-378 (His_6 – PEG – off-target) 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 His_6 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 H_2SO_4. 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.

4.2.2.7 Protein Isoform Selectivity Assay

The selectivity assay with full length Akt1, Akt2 and Akt3 is performed following the protocol in section 4.2.2.6, using 25 nM protein instead of 2.5 μM His_6 tagged peptide epitope.

4.2.2.8 Measurement of binding affinity of N-term-tri-L and N-term-tri-L-dimer 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 (450 nM to 1 nM) of the Akt2 protein were made in HBSP+ buffer for the experiment with the immobilized *N-term-tri-L*. For the *N-term-tri-L-dimer*, serial dilutions of Akt2 from 45 nM to 0.0086 nM were made. The solution was flowed over the chip at 50 μL/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 corrected using an unmodified reference flow cell. Biacore Evaluation software was used to calculate the dissociation constants.

4.3 Results and discussion

4.3.1 Characterics of PCC ligands developed against Akt2

Biligand candidates were prepared from the 1^{o} ligand and each of the 2^{o} ligands using the Cu(I)-catalyzed click reaction,[28,29] and the biligands were then compared in an immunoprecipitation assay for their ability to pulldown Akt2 from OVCAR3 cell lysate. The highest performing peptide biligand (*bi-L*) exhibited superior performance to *mono-L* (Figure 4.2), and was selected for further characterization. The affinity of *bi-L* was estimated, from a direct ELISA involving the full Akt2 protein, to be 1.3 μM. The *N-term-tri-L* exhibited the best performance in the selectivity immunoprecipitation assays from OVCAR3 cell lysate (Figure 4.1). The affinity of the *N-term-tri-L* and *C-term-tri-L* were estimated from single component ELISA assays to be 19 nM and 124 nM, respectively (Figure 4.2). We note that the 3^{o} ligand on the *C-term-tri-L* is remarkably similar in sequence to the 2^{o} ligand arm, indicating that both arms may be competing for the same spot on the protein for this triligand.

We carried out selectivity assays on *bi-L* and both triligands to determine the level of epitope selectivity. To this end, we performed an ELISA assay, comparing the binding of the ligands to either the C-terminal 32-mer polypeptide fragment of Akt2, or an off target 32-mer

polypeptide Akt2 fragment (corresponding to residues 346 through 378) that is similarly surface exposed and unstructured in the full length protein. All the three capture agents exhibited a high selectivity for the C-terminal 32-mer relative to the other polypeptide fragment (Figure 4.3)

The selectivity of the *N-term-tri-L* and *C-term-tri-L* for Akt2 over its isoform Akt1 and Akt3 was determined at the epitope level and against the full-length protein (Figure 4.4). One of the triligands, *C-term-tri-L* is highly selective for the peptide epitope of Akt2, and bind significantly less to the highly homologous C terminal epitopes of isomeric proteins Akt1 and Akt3. The same trend is observed in the binding of the ligands to the full-length Akt isoform proteins.

The absolute affinities of the high affinity ligands, *N-term-tri-L* and *N-term-tri-L-dimer* were measured by Surface Plasmon Resonance (SPR), by immobilizing the biotinylated ligand on the SPR chip and measuring the responses for various concentrations of the analyte, the full length active Akt2 protein. The absolute affinity of *N-term-tri-L* was obtained as 10 nM from steady state fit on Biacore T100 evaluation software, which is very close to the affinity measured in the ELISA (19 nM). The *N-term-tri-L-dimer* for Akt2, having two binding sites on the ligand, could not be fitted to the simplest 1:1 Langmuir binding model. The sensograms, when fit to the more appropriate model of 1:1 binding with mass transfer[1], yielded a K_D of 6.25 nM (Figure 4.5).

Figure 4.1: Immunoprecipitation and Western Blot assays showing the performance of the various PCC Agents.

Akt2 is pulled down from the lysate of an ovarian cancer cell line Acetyl-glycine-biotin is used as the control.

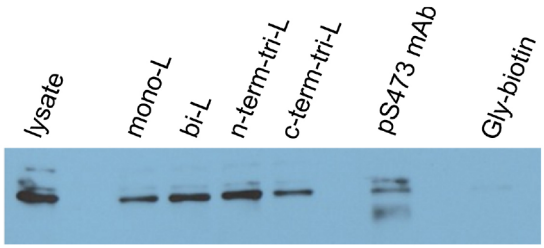


Figure 4.2: ELISA assays demonstrating use of PCC Agents.

PCC agents used as surface-localized capture agents (for *N-term-tri-L, C-term-tri-L)* or as detection agents (for *mono-L, bi-L)*. This type of assays provides relative affinity measurements, which are given as EC_{50} values in the key.

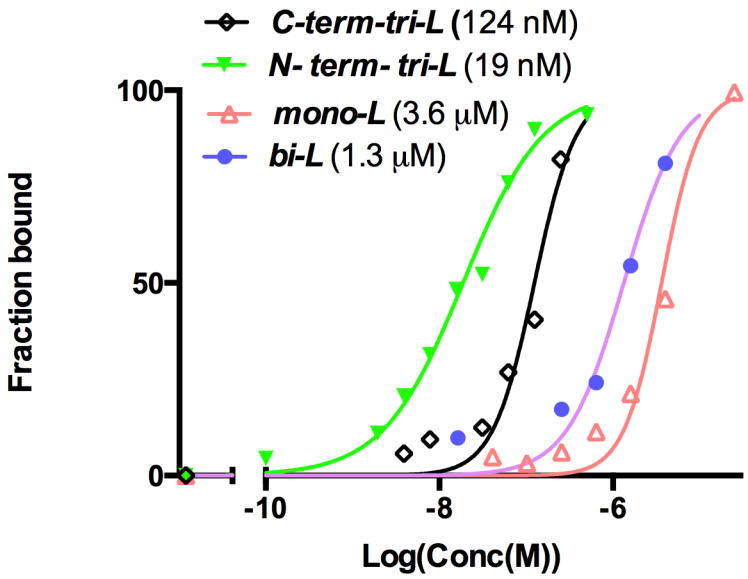


Figure 4.3: Epitope specificity assay.

The various triligand PCC agents are used as capture agents for the peptide epitope. The biotinylated capture agents are immobilized on a Streptavidin plate, and then probed with the His_6 tagged target or control peptides. The target peptide (Akt2 450-481) with pS474 shows a distinct binding affinity for the capture agents compared to the control peptide (Akt2 346-378). The ***C-term-tri-L*** also significant selectivity for the Akt2 epitope compared to the corresponding regions in Akt1 (449-480) and Akt3 (448-479).

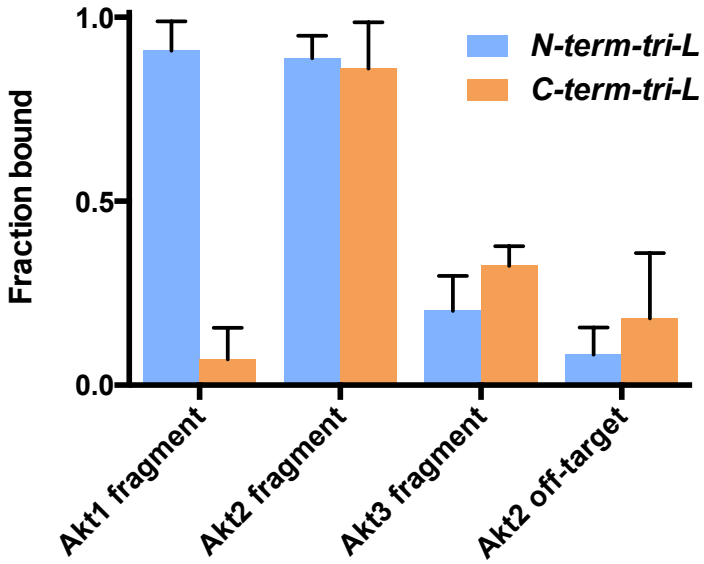


Figure 4.4: Selectivity of triligands for the full length Akt isoform proteins.

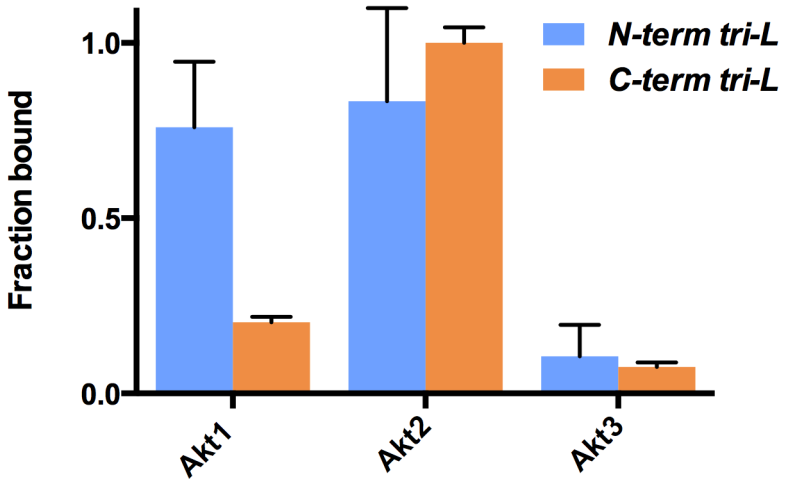
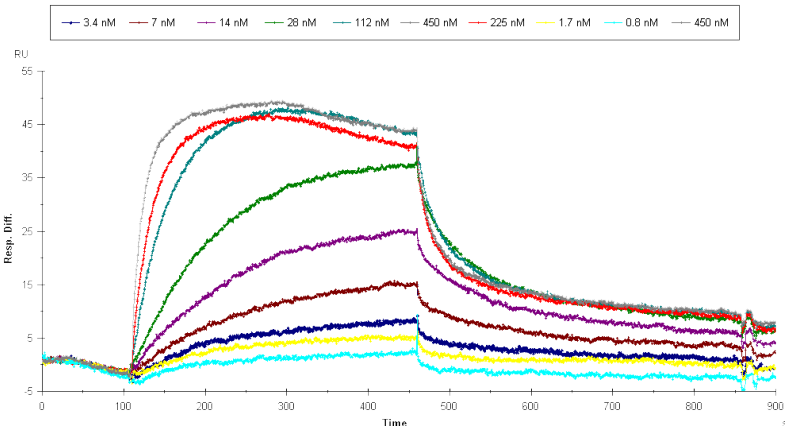


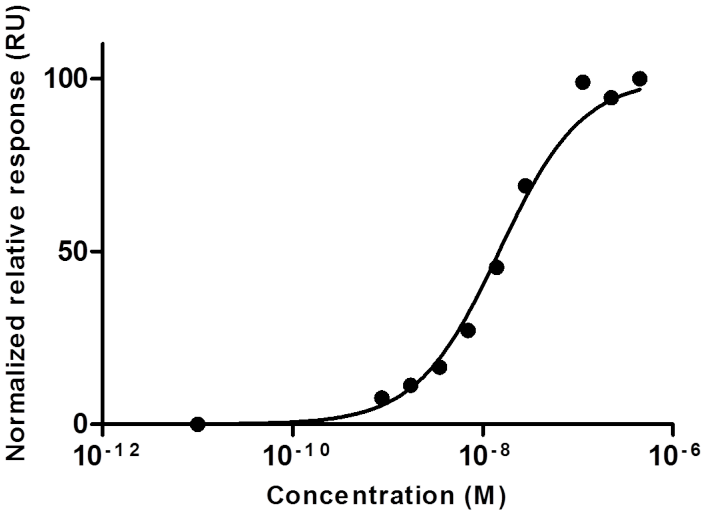
Figure 4.5: SPR measurements of triligands.

A. Sensograms from Surface Plasmon Resonance (SPR) experiment, immobilizing *N–term-tri-L* on SA chip and using Akt2 protein as the analyte. B. Steady state dissociation constant is measured by taking an average of the RUs in steady state over 3 time points, 150s, 200s and 250s, for each concentration of the protein. C. Sensograms from Surface Plasmon Resonance (SPR) experiment, immobilizing *N –term-tri-L-dimer* on SA chip and using Akt2 protein as the analyte. Because of the dimeric nature of the ligand, the sensograms fit best with the heterogenous ligand parallel model.

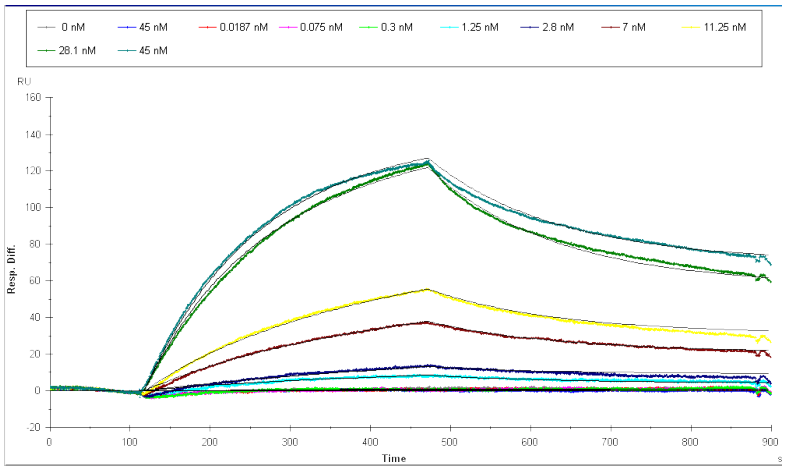
A.



B.



C.



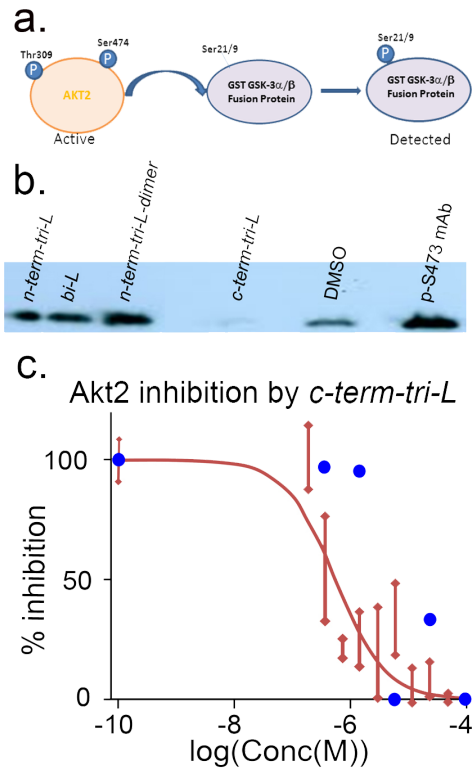
4.3.2 Inhibitory Characteristics of the Akt2 Capture Agents

Phosphorylation of Akt2 at Thr309 activates Akt2, but further phosphorylation at Ser474 increases its enzymatic activity four to ten fold [2,3]. In cancer cell lines[4,5] and tissue samples[6] the constitutively active PKB is phosphorylated at S473. Overexpression and/or activation of Akt have been found to increase resistance of tumors to chemo[7] or radio[4] therapies. The importance of Akt as a therapeutic target has prompted several approaches towards selective Akt inhibition[8]. Akt small molecule kinase inhibitors are classified into two general classes: type I ATP competitive inhibitors, and type II allosteric inhibitors that bind to the interface between the membrane docking pleckstrin homology domain (PHD) and the kinase domain[9]. These allosteric inhibitors exhibit no influence on the enzymatic activity of Akt when the PHD is removed[8]. We recently reported on an peptide based allosteric inhibitor ($K_{inhibition}$ ~3 μM) of the kinase domain of Akt [10]that did not compete with either the ATP or peptide substrate binding sites, but appeared

to overlap at least partially with a commercial mAb that was directed at the C-terminus of the protein. As the phosphorylated hydrophobic motif around S474 (near the C-terminus) of Akt2 has been identified to act as an allosteric activator, we hypothesized that a ligand that was targeted near S474 would influence the enzymatic activity of Akt2. In this section, we explore the inhibitory characteristics of the various ligands developed here.

The Akt kinase in vitro non-radioactive assay kit (Cell Signaling Technology) was used to estimate the influence of the ligands on the kinase activity of Akt2. In this assay, the active Akt2 is combined with its downstream effector GSK-3α/β substrate (GST-GSK3α/β fusion peptide substrate), ATP, and one of the peptide ligands (or dimethylsulfoxide (DMSO) for a control). The level of phosphorylation of GSK-3α/β, as measured by spotting the reaction mixture on a nitrocellulose membrane or by Western Blot, provides a readout for the Akt2 kinase activity (Figure 4.6). The *bi-L* and *N-term-tri-L* capture agents were found to activate Akt2, as did the commercial anti-p-Ser473 mAb (Figure 4.6). However, the *C-term-tri-L* inhibits Akt2, with an IC_{50} (~500 nM) (Figure 4.6). Thus, the enzymatic activity of Akt2 is clearly hypersensitive to perturbations near the C-terminus, whether it is phosphorylation of S474, or structural perturbation of that (phosphorylated) terminus via ligand binding. This hypersensitivity apparently works in both directions; perturbations of the ATP binding site can influence phosphorylation at the C-terminus. Mechanistic studies of traditional ATP-competitive small molecule Akt inhibitors have highlighted this intricate structure/activity balance; ATP-competitive inhibitors like A-443654 cause paradoxical hyperphosphorylation of Akt at both Thr308 and Ser473, and thus lead to reactivation of the protein after inhibitor dissociation[11].

Figure 4.6: Influence of the PCC Agents on the enzymatic activity of the Akt2 kinase.
A. The activity of Akt2 directly influences the phosphorylation state of GSK-3a/b, and detecting p-GSK-3a/b provides an assay readout, as a function of varying the nature or the concentration of a potential p-Akt2 inhibitor. B. Western blot analysis of p-GSK-3α/β levels as the various PCC Agents are added to influence the activity of p-Akt2. DMSO is used as the control. The p-GSK-3α/β levels for the *N-term-tri-L*, the *bi-L*, the *N-term-tri-L-dimer*, and the commercial p-S473 mAb all imply that these agents activate p-Akt above baseline, with the *N-term-tri-L-dimer* and the mAb being the strongest activators. The *C-term-tri-L*, however, serves as an inhibitor. C. Quantitation of the inhibitory characteristics of the *C-term-tri-L* PCC Agent on p-Akt2. Red points with error bars are from a dot blot analysis of p- GSK-3α/β, while the blue points are from a Western Blotting analysis (which only yielded a limited dynamic range in terms of % inhibition detectable). The red line is a fit is to the dot-blot data points. The assays yield similar IC_{50} values of 500 nM (dot blot) and 2 μM (Western).



4.4 Conclusions

We report here on an epitope targeting strategy that, when combined with sequential in situ click chemistry, permitted the development of a series of peptide multi-ligands (called PCC Agents) that are targeted near the key activating phosphorylation site of Akt2. The strategy relies on the use of dinuclear Zn(II)-dipyridylamine complex that binds to the phosphorylated residue[12] of interest, and presents both a biotin label and an azide functionality near that site. An advantage of the strategy is that it can be initiated using only a polypeptide fragment that represents the phosphorylated epitope of the target. This permits stringent chemical characterization of all of the relevant chemical species used in the process.

The strategy is utilized to identify an initial PCC Agent monoligand, a PCC Agent biligand and two PCC Agent triligands. One of those triligands is further expanded into a dimer structure. All of affinity agents are effective at detecting total Akt from ovarian cancer cell lysate, with improved performance being increasingly observed through the biligand and triligand stages. The best triligand (and triligand dimer) exhibits an affinity in the 10 nM range, and epitope specificity is shown for the biligand and triligand PCC Agents.

The p-Ser474 region near the C-terminus of Akt2 was explored here because phosphorylation of this hydrophobic motif leads to allosteric activation of Akt2, and so provides an interesting therapeutic epitope on the protein. However, the epitope itself is not obviously amenable towards the development of traditional small molecule inhibitors. Indeed, we find that PCC Agents developed against this epitope exhibit both inhibitory and activating characteristics, depending upon the detailed structure of the PCC Agent. An obvious next step is to develop approaches for delivering these PCC Agents into live cells, and to further explore their potential as therapeutic inhibitors of Akt2. Such studies are currently underway.

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Chapter 5: Development of CuAAC cyclized one bead one compound peptide libraries and their application in the epitope targeting strategy

5.1 Introduction

Cyclic peptides have been frequently used for drug discovery[1,2], as molecular probes[3], and as a protein detection agent. Whereas small molecules bind to proteins that have well developed hydrophobic pockets, a significant amount of protein interaction surfaces are relatively flat and distributed over a large contact surface area. So for surface binding, peptides perform better than small molecules. However, for peptide protein interactions to be high affinity, it is essential to have structural constraints in the peptide[4]. Cyclizing a peptide provides this constraint, at the same time increasing the protease resistance of the peptide[5].

For cyclic ligand development against proteins, phage display libraries have been frequently used. But a limitation of the phage display method is in incorporating unnatural amino acids such as D amino acids in the library. While innovative approaches, such as mirror image phage display [6], have been explored to overcome this disadvantage, it is by no means a trivial to introduce unnatural amino acids into phage display libraries. One Bead One Compound (OBOC) peptide libraries[7,8] overcome this disadvantage, allowing easy incorporation of unnatural amino acids and other chemical modifications. However on bead screening and sequencing processes are not compatible with several categories of cyclization processes[9-11].

Here we describe the development of CuAAC cyclized peptide libraries that are compatible with OBOC screening. The developed cyclic library is used in epitope targeting strategy. In the epitope targeting strategy, we are trying to detect peptide-peptide interaction, which is typically weaker and tougher to detect than peptide-protein interaction. The property of cyclic peptide to have higher affinity than its linear one is particularly attractive for optimizing of the epitope targeted PCC agent development process. To demonstrate of this process cyclic ligands were developed against a peptide epitope that has already been demonstrated to be good for developing peptide based ligands. This epitope is the C terminal fragment from the protein Akt2, amino acids 450-481, that contains pSer474.

Another motivation of this study is to study the cell penetrating properties of the isolated cyclic peptide inhibitor. Along with the robustness and compatibility of the cyclization procedures with on bead peptide chemistry, an important characteristic of the developed cyclic libraries is the nature of the cyclization linker. It has been found that an all hydrocarbon cross-linker stabilizes the peptide and increases the cell penetrating property of the peptide[12,13]. For the triazole cyclized library, the hydrocarbon part, like the stapled peptide, should have a positive effect on the intracellular uptake of the cyclized peptide. The triazole unit acts as an amide bond[14], and may or may not hinder the uptake of the cyclic peptide. The triazole, is, moreover, not susceptible to protease cleavage as amide bonds are. We have preliminary data that suggest the branched multiligand developed against Akt, as described in chapter 3 and 4, can be effective inside cells when tagged with the HIV derived TAT peptide sequence. However the ideal situation would be to have a peptide based drug that has significant cell permeating property by itself. CuAAC cyclized peptides are promising in this respect. Among the four cyclic ligands isolated, one peptide Cy(yytytTz4) exhibits selective binding to both the peptide epitope and the Akt2 protein. We have preliminary data that the ligand acts as an inhibitor of kinase activity.

5.2 Materials and Methods

5.2.1 Materials

Materials as described in sections 3.2.1 and 4.2.1 are used.

5.2.2 Methods

5.2.2.1 Synthesis of randomized peptide library and on bead cyclization

Three comprehensive linear peptide libraries A, B and C were synthesized on Tentagel-S-NH_2 resin using standard SPPS library synthesis method. Each library was synthesized at a 10-fold excess to ensure adequate representation of each library element. After the synthesis of each

library, all the beads in the linear library were subjected to an on bead CuAAC reaction, for 6 hours at room temperature with 1.5 equivalents of CuI, 2.5 equivalents of ascorbic acid in 20% piperidine in DMF (Figure 5.1). After washes to remove the adsorbed Cu, the library was washed with DMF, methanol and DCM and dried. Random beads were picked from the library to be sequenced. The rest of the library was stored in NMP. The structure and details of the three libraries are shown in Table 5.1.

Figure 5.1: Peptide cyclization on bead by the Cu catalyzed Azide Alkyne Cycloaddition (CuAAC) reaction.

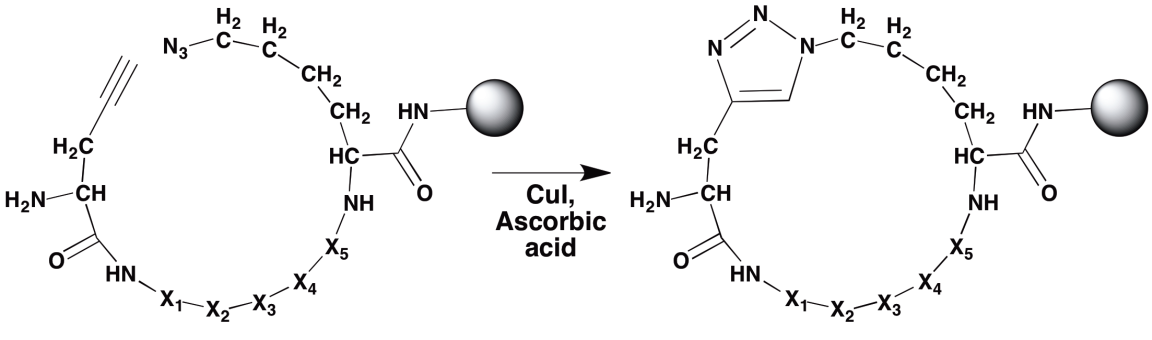
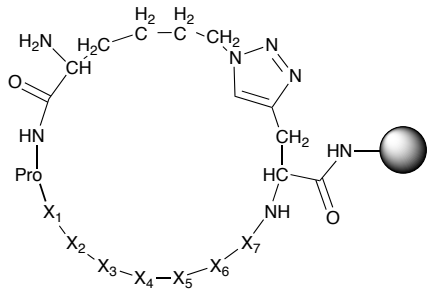
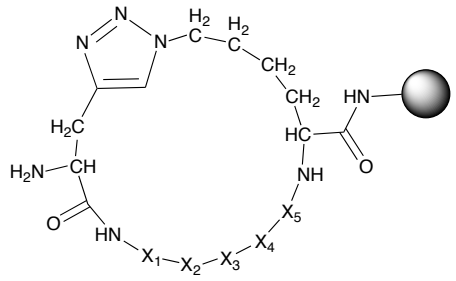
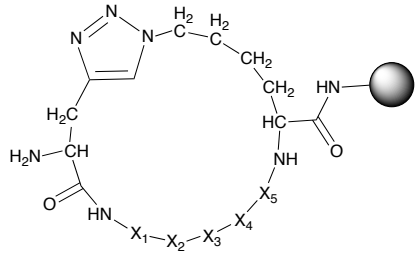


Table 5.1: One Bead One Compound (OBOC) cyclic peptide libraries used in screens

Library (notation)	**Sequence**	**Amino acids used**	***Number of unique sequences***
A Cy(Pro-$X_1X_2X_3X_4$ $X_5X_6X_7$–Tz4)		X_i =D-Ala, Gly, D-Leu, D-Ile, D-Val, D-Phe, D-Trp, D-Arg, D-His, D-Lys, D-Asp, D-Glu, D-Asn, D-Gln, D-Ser, D-Thr, D-Tyr, D-Pro.	612,220,032
B Cy($X_1X_2X_3X_4$ X_5–Tz4)		X_i =D-Ala, Gly, D-Leu, D-Ile, D-Val, D-Phe, D-Trp, D-Arg, D-His, D-Lys, D-Asp, D-Glu, D-Asn, D-Gln, D-Ser, D-Thr, D-Tyr, D-Pro.	1889568
C Cy($X_1X_2X_3X_4$ X_5–Tz4)		X_i =L-Ala, Gly, L-Leu, L-Ile, L-Val, L-Phe, L-Trp, L-Arg, L-His, L-Lys, L-Asp, L-Glu, L-Asn, L-Gln, L-Ser, L-Thr, L-Tyr, L-Pro.	1889568

The linker containing the amino acid azidolysine clicked to L-Pra in a 1,4 disubstituted orientation is referred to as Tz4.

5.2.2.2 Synthesis of scrambled phospho-epitope and target phospho-epitope

Akt2 450-481 (ITPPDRYDSLGLLELDQRTHFPQF(pS)YSASIRE) is used as the target phospho-epitope. The scrambled non-phosphorylated epitope has sequence ITPPDRYDSLGLLEL-QRTHYFFASQPSSIRE. For screening both peptides are synthesized on Rink Amide MBHA resin with a polyethylene glycol spacer (PEG_2) and biotin on the N terminal.

5.2.2.3 IR spectra

HPLC purified lyophilized peptides were ground with KBr powder to make pellets. Measurements were made on a Vertex 70 FT-IR spectrometer (Bruker Optics). Scans of air were served as the background and were subtracted from the spectra.

5.2.2.4 Screening cyclic OBOC library with peptide epitope

For isolating cyclic ligands against the peptide epitope, three consecutive libraries A, B and C were screened against the phospho-peptide. Each of the screens followed the following protocol: 500 mg of library resin were dried with DCM and afterwards, swelled and shaken overnight in binding buffer (25 mM trisCl, pH = 7.5, 150 mM NaCl, 0.1% BSA, 0.05% Tween20) at room temperature. Beads were incubated overnight with a solution of 500 nM of the scrambled phosphopeptide in binding buffer. The beads were washed three times, for fifteen minutes each, with the binding buffer. They were then treated for 1 hour at room temperature with a 1:1000 dilution of mouse monoclonal Anti-biotin antibody conjugated with Alkaline Phosphatase (mAb-AP) in screening buffer. Following the screen, the beads were successively washed three times with each of the following buffers: 25 mM trisCl, pH = 7.5, 150 mM NaCl, 0.1% BSA, 0.05% Tween20; 25 mM trisCl, pH = 7.5, 150 mM NaCl, 0.05% Tween20; 25 mM trisCl, pH = 7.5, 150 mM NaCl; 100 mM Tris-HCl, pH 9.0, 150 mM NaCl, 1 mM $MgCl_2$) (AP buffer). The library was treated with a 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (33 uL BCIP in 10 mL AP buffer) for fifteen minutes. The AP reaction was stopped using 0.1 M HCl, which denatures AP protein. Blue background hits were removed from clear ones using a

pipette. Clear beads were combined and transferred to a peptide synthesis vessel. The beads were shaken in 7.5 M of guanidine-HCl pH = 2, which serves as a common protein denaturant, for 2 hours at room temperature. Afterwards, the library was washed with deionized water and treated with NMP to decolorize the blue beads. Following a wash with DCM, the clear beads were dried, then shaken overnight in binding buffer.

The beads were screened overnight with a solution of 500 nM of the target phospho-peptide in binding buffer. Following washes with the binding buffer, the beads were treated with a solution of mAb-AP. The same procedure as in the prescreening was conducted to obtain blue click hits. The beads were manually picked, washed with 7.5 M guanidium hydrochloride (pH 2) and water, and sequenced using the Edman Sequencer.

Table 5.2: List of sequences obtained from screen against target epitope using cyclic library C.

X_1	X_2	X_3	X_4	X_5	X_6	X_7
-	Y	V	Y	K	S	Tz4
–	V	F	A	K	V	Tz4
–	I	R	Y	Y	S	Tz4
–	Y	Y	T	Y	T	Tz4

5.2.2.5 ELISA for cyclic peptide Cy(yytytTz4) against full length Akt2 protein

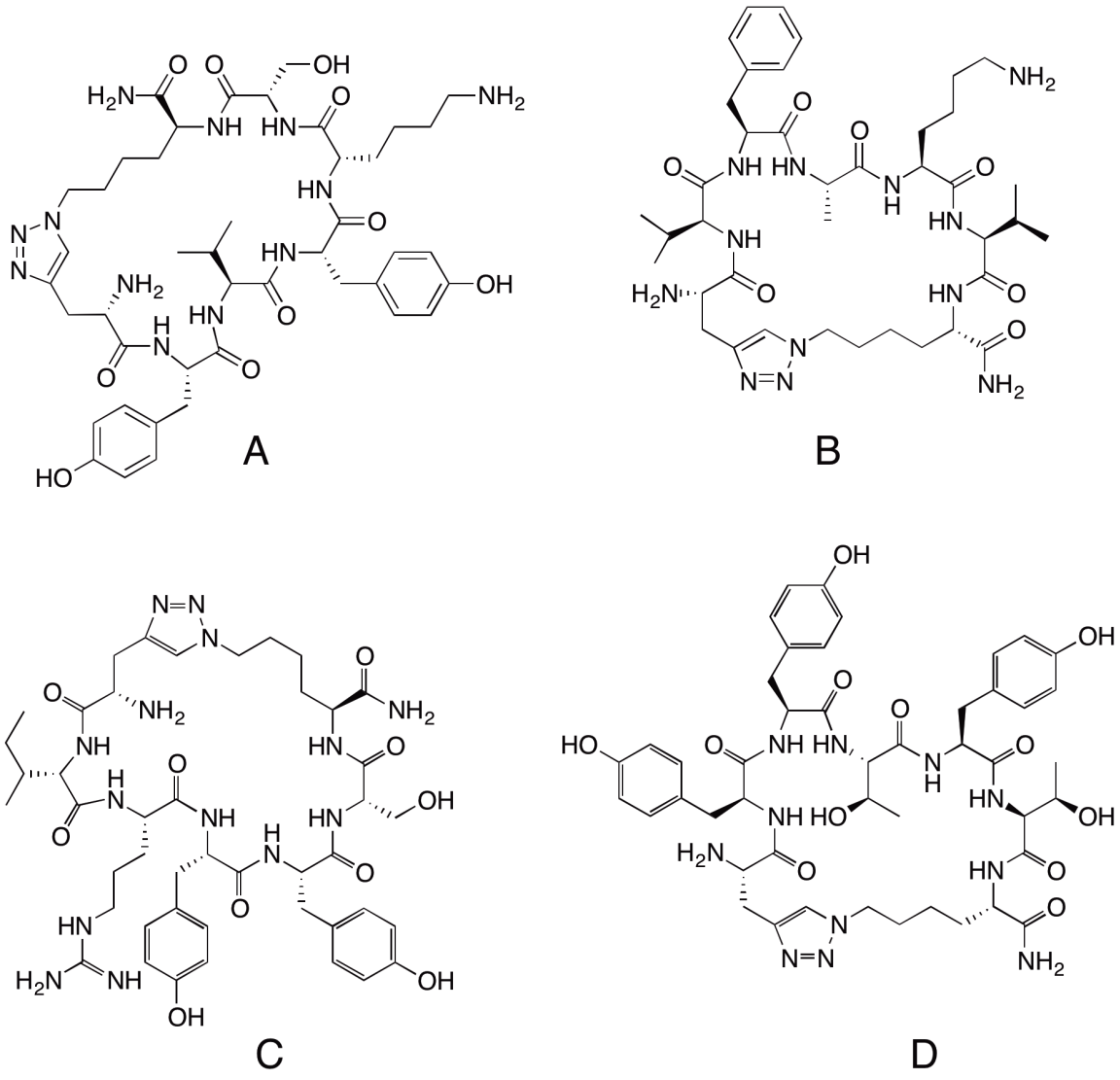
2 μM solution of the biotinylated cyclic peptide was prepared in binding buffer (TBS pH 7.5, 0.05% Tween 20/0.1% BSA. 100 μL of the solution was added to each well of a High Capacity Streptavidin 96 well plate (Thermo Scientific) and the plate shaken for two hours at room temperature. The wells were blocked with 1% BSA / TBS pH 7.5 / 0.05% Tween 20. The

wells were washed with binding buffer five times. Serial dilutions, from 1.15 nM to 450 nM, of active His_6 tagged Akt2 (Abcam) were made in the binding buffer. 100 µl of each solution was added per well the plate shaken overnight at 4°C. The wells were washed three times with the binding buffer, then treated for an hour with a 1:1000 dilution of anti His_6 mouse monoclonal antibody. A 1:10,000 dilution of goat anti mouse antibody-Horse Radish Peroxide conjugate (Abcam) in binding buffer was added to the wells for an hour. The plate was washed, treated with TMB and quenched with H_2SO_4, and the absorbance at 450 nm wavelength measured. From the A450 value of each protein concentration, the corresponding blank A450 (with no ligand, same amount of protein) was subtracted. All the A450 values were normalized w.r.t the maximum A450 value. They were then fitted by non-linear regression in Graphpad Prism 6.

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

To evaluate if the cyclic ligand had any effect on the kinase activity of Akt2, we performed a preliminary non-radioactive kinase assay with a single concentration of the cyclic ligand. The protocol as described in A solution of 400 ng Akt2, 400 ng GST-GSK-3α/β fusion protein, 500 mM ATP and 0.5 µL of 90 mM peptide solution or DMSO (control) was made in kinase buffer (25 mM trisHCl (pH 7.5), 10 mM $MgCl_2$, 0.01% Triton-X, 1X Roche Phosstop phosphatase inhibitor, 1X COMplete protease inhibitor), so that the final concentration of the peptide in the reaction mixture was 1.8 mM. The kinase assay protocol as described in Chapter 4, subsection 4.2.2.5 was followed.

Figure 5.2: Cyclic peptide ligands isolated by screening against target peptide epitope.
A) Cy(YVYKS-Tz4), B) Cy(VFAKV-Tz4), C) Cy (IRYYS-Tz4), D) Cy(YYTYT-Tz4)



5.3 Results and discussion

5.3.1 Verification of on bead cyclization by Edman Peptide Sequencing

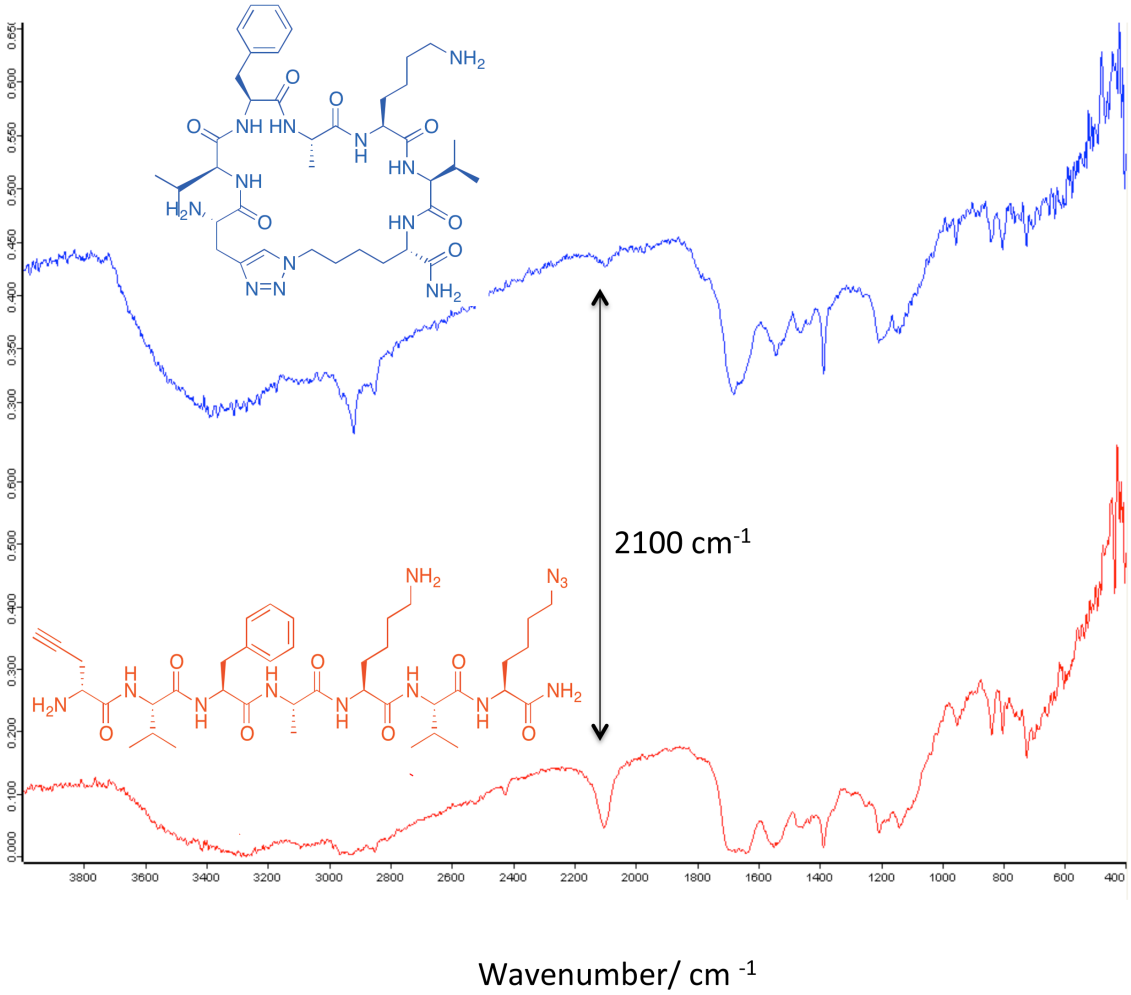
A single bead of tentagel resin from the library was loaded on a cartridge of the Edman Sequencer for sequencing. A peptide from library A or C, on Edman sequencing, is expected to

generate a particular pattern of signals. During the first sequencing cycle, the amino acid with triazole in the side chain should be converted to its PTH derivative. Since the amino acid is still attached to the peptide on bead, there should be no peak in cycle 1. From the 2nd cycle onwards, the amino acids of the peptide should get sequenced like in a linear peptide. The 7th cycle will give the peak corresponding to the triazole side chain derivative. It will cleave as a diphenylthiohydantoin derivative. The diphenylthiohydantoin derivative of D-propargylglycine amino acid elutes at 25 minutes using the standard amino acid calibration curve. Several random beads were picked from library A and sequenced on the Edman. All the random beads from the library yielded this pattern, with no peak in cycle 1, then after sequencing 5 amino acids, there is a characteristic peak on the 7th cycle. This indicates that the cyclization of peptides on bead by CuAAC is a viable and robust and that an on bead CuAAC cyclized library can be readily synthesized.

5.3.2 IR verification of on bead cyclized peptide formation by CuAAC reaction

The MBHA resin, with higher molar capacity than the Tentagel-S-NH_2 resin, was used in this synthesis, so that any issues of dimerization, which could happen from the on bead cyclization, would be more apparent in this case. The cyclized and linear peptides on HPLC purification showed one major peak each, which on mass spectral analysis, showed the correct mass for the monomeric compound. These pure compounds were analyzed by solid phase IR and the spectra compared. While the linear peptide had a distinct peak at ~ 2100cm^{-1} corresponding to azide triple bond rotation, the cyclized peptides did not have this characteristic peak. Thus it was demonstrated that the azide on the bead is efficiently cyclized during the click reaction.

Figure 5.3: IR spectra of linear peptide L-Pra-VFAKV-L-Az4 and the cyclic peptide Cy(VFAKVTz4).



5.3.3 Using on bead cyclic peptide library for ligand discovery

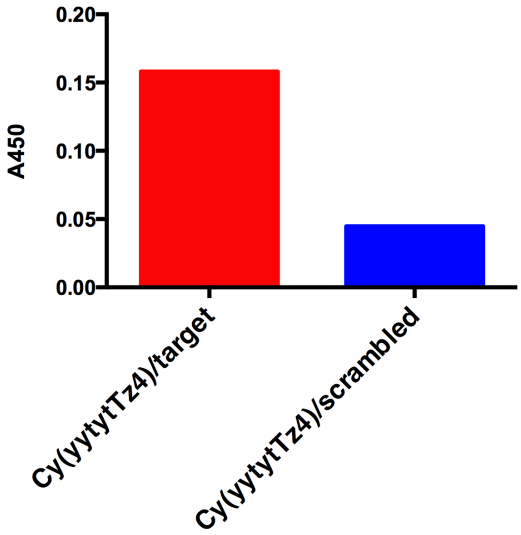
To eliminate binding to a different region than the phosphorylation site and to prevent background binding to the detection antibody, each library was screened against the scrambled phospho-epitope, a peptide that does not contain the phosphate and is scrambled in the AGC kinase specific motif. In the prescreen the beads binding to the detection antibody and the peptide regions other than the hydrophobic motif are detected by the coloration on BCIP treatment. These blue beads are separated from the rest of the library that remains colorless. The colorless beads

are washed with protein denaturants and then screened with the target phospho-peptide epitope. Libraries A and B on screening in this protocol did not yield any hit beads against the target phospho-epitope. Only library C yielded four hits. The hits are listed in Table 5.2.

5.3.4 Determination of best cyclic binder to the target Akt2 C terminal fragment

The four peptide candidates were tested for their capacity to capture the target peptide in a sandwich ELISA platform. Biotinylated capture agents were immobilized on Streptavidin plates and titrated with 1 μM of either the His_6 tagged target peptide or the scrambled peptide. Binding was detected by mouse anti His_6 tag antibody. Only peptide Cy(YYTYT-Tz4) showed binding to the target peptide and no binding to the scrambled peptide.

Figure 5.4: Selectivity of the cyclic peptide Cy(YYTYT-Tz4) for the target peptide.

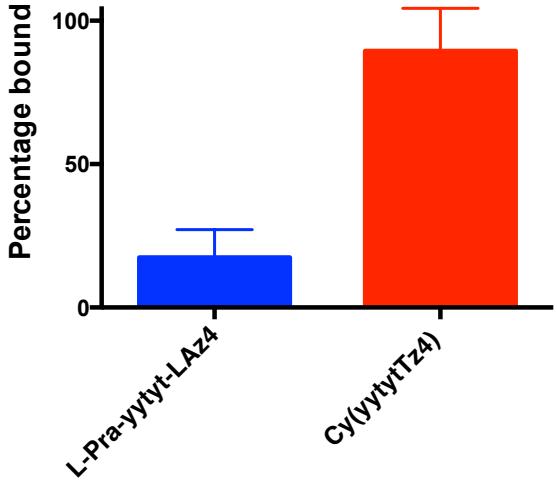


5.3.5 Effect of cyclization on binding specificity

To determine whether the cyclization of the peptide binder was vital to its binding to the

target, the binding of both the linear peptide L-Pra-YYTYT-L-Az4 and the cyclic peptide Cy(YYTYT-Tz4) of the peptide ligand with the target were determined. The linear peptide exhibited low binding to the target peptide, thus demonstrating that the cyclization of the peptide ligand was significant for getting specificity in binding.

Figure 5.5: The cyclized ligand Cy(YYTYT-Tz4) shows significant binding to the epitope compared to its linear version L-Pra-YYTYT-L-Az4.



5.3.6 Effect of ring size on binding

To determine whether the ring size of the cyclic peptide binder is an important factor in its specificity for the target peptide, the linear peptides L-Pra – YYTYT – L-Az1 and L-Pra – YYTYT– L-Az2 were synthesized on resin and subjected to the CuAAC reaction overnight. The peptide L-Pra – YYTYT – L-Az1 failed to cyclize on bead. The peptide L-Pra –YYTYT– L-Az2 could be cyclized. This cyclic peptide of smaller ring size did not exhibit significant binding to the target peptide, and thus it could be demonstrated that the ring size of the cyclic peptide is an important criteria for binding the target peptide.

Figure 5.6: Ring size affects cyclization efficiency.

L-Pra – YYTYT– L-Az2 cyclizes on bead as the major product while L-Pra – YYTYT– L-Az1 does not cyclize efficiently on bead

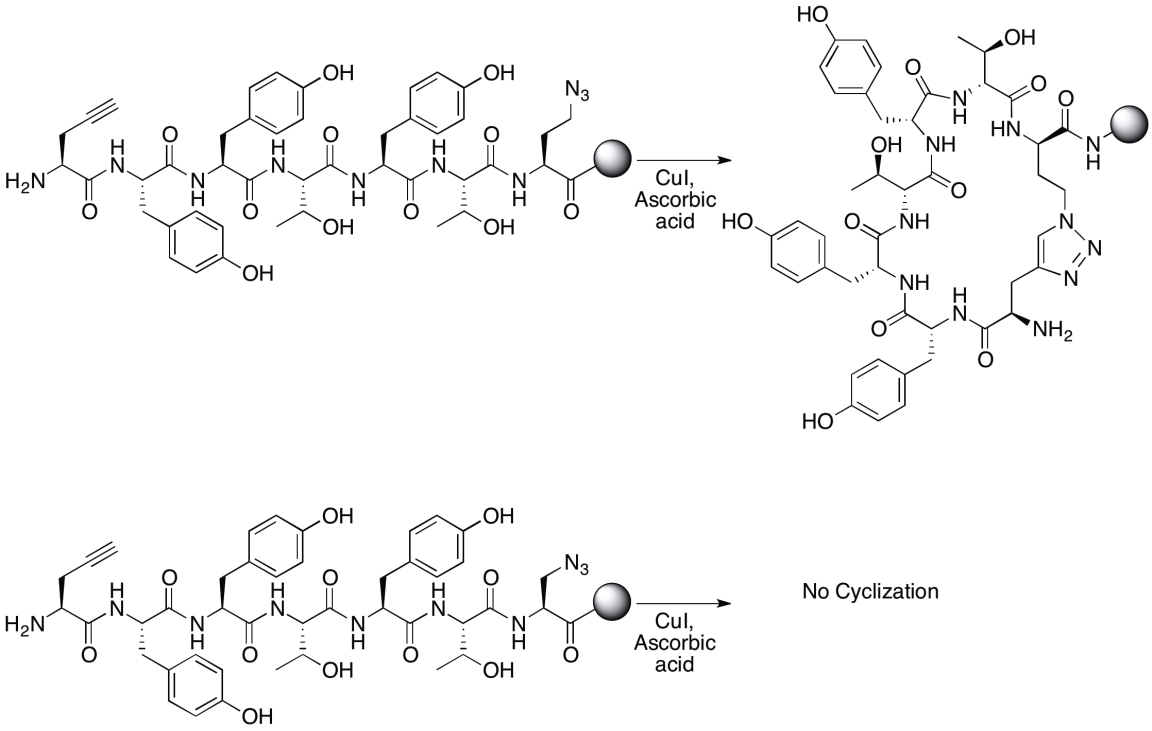
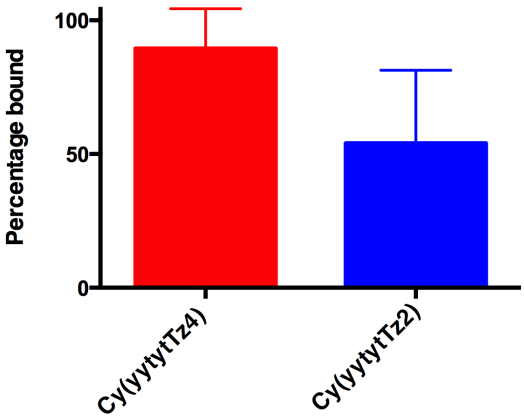


Figure 5.7: Effect of ring size on epitope selectivity.

Cy(YYTYT-Tz4) is a better binder than Cy(YYTYT-Tz2)



5.3.7 Characterization of the cyclic ligand Cy(YYTYT-Tz4)

To determine the affinity of the cyclic monoligand for full length Akt2 protein, the ligand was titrated against immobilized protein. The ligand acts as a detection agent and has a low nanomolar (124 nM) binding affinity compared to the low micromolar affinity (3.6 μM) of the linear monoligand previously developed against Akt. Thus there is a ~ 30 fold improvement in the binding affinity using a cyclic instead of linear peptide monoligand for Akt2. It will be interesting to see if the same improvement can be attained in the biligand stage using a cyclic peptide as the 2 °arm.

We demonstrate the selectivity of the cyclic monoligand for Akt2 protein by comparing the binding of the ligand to the proteins Akt1, Akt2 and Akt3. As the proteins are highly homologous, even in the targeted C terminal region, it is not surprising that binding to all the three proteins is observed to some extent. However there is a distinct preference for Akt2.

Figure 5.8: Determination of binding affinity of cyclic ligand Cy(YYTYT-Tz4) for Akt2 protein

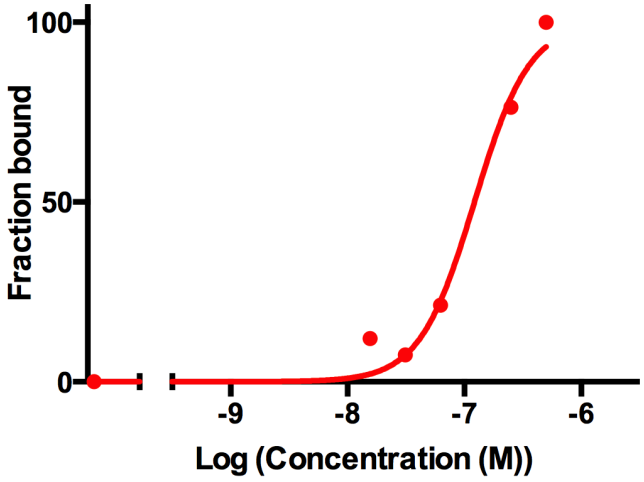
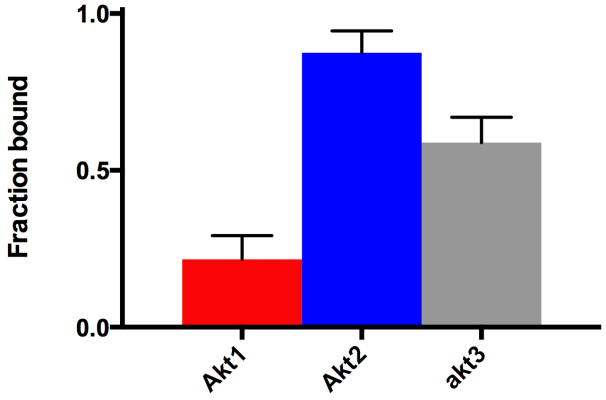


Figure 5.9: Cy(YYTYT-Tz4) is selective for the Akt2 protein over the Akt1 and Akt3 proteins.



5.3.8 Effect of cyclic monoligand on Akt2 kinase activity

To evaluate if the cyclic ligand has any effect on the kinase activity of the Akt2 protein, we performed the non-radioactive kinase assay described in Chapter 4. Briefly, the protein is treated with the ligand, ATP and an enzyme substrate, in this case, GST-GSK-3α/β fusion protein. The reaction mixture is analyzed using western blot to detect the amount of substrate phosphorylated. Under normal conditions (as in the DMSO control) the GST-GSK-3α/β fusion protein will be phosphorylated by Akt2. If, however, the ligand acts as an inhibitor, it will decrease the formation of phospho-Ser21/9- GST-GSK-3α/β fusion protein. The cyclic monoligand, Cy (YYTYT-Tz4) inhibits the phosphorylation of the substrate. As this was a preliminary experiment, we used a high concentration (2mM) of the cyclic ligand. Further verification with lower concentrations of the cyclic peptide is required.

Figure 5.10: Cyclic ligand Cy(YYTYT-Tz4) inhibits Akt kinase activity.

5.3.9 Optimization of monoligand anchor

To introduce an azide handle on the monoligand to screen against an OBOC library containing Alkyne handle, 6- azido hexanoic acid is coupled to N terminal of cyclic peptide to develop the monoligand anchor. The protein is incubated with the monoligand anchor and screened against library D. There was very little background binding to the antibody in the preclear screen, and in the actual screen no hits were obtained. During the purification of the monoligand anchor peptide on the HPLC using a standard gradient, we observed a significant difference in the elution time. While the monoligand eluted at 50 minutes, the monoligand anchor eluted at 65 minutes using the same gradient. The significant change in elution times on addition of a single amino acid to a peptide probably implies that this addition is altering the nature of the peptide. It is possible that the click handle in the monoligand anchor is not available for reaction with the on bead alkyne because the azide containing side chain is binding to the cyclic part of the peptide. It is observed that use of an alternative version of the monoligand anchor with a glycine and azidoalanine (instead of the 6-azidohexanoic acid) decreases the gap in HPLC elution, eluting close to the monoligand. This is chosen as the monoligand anchor candidate for the biligand screen.

Figure 5.11: Monoligand anchor candidate peptides developed for biligand screen.
The length of the azide side chain is same for both

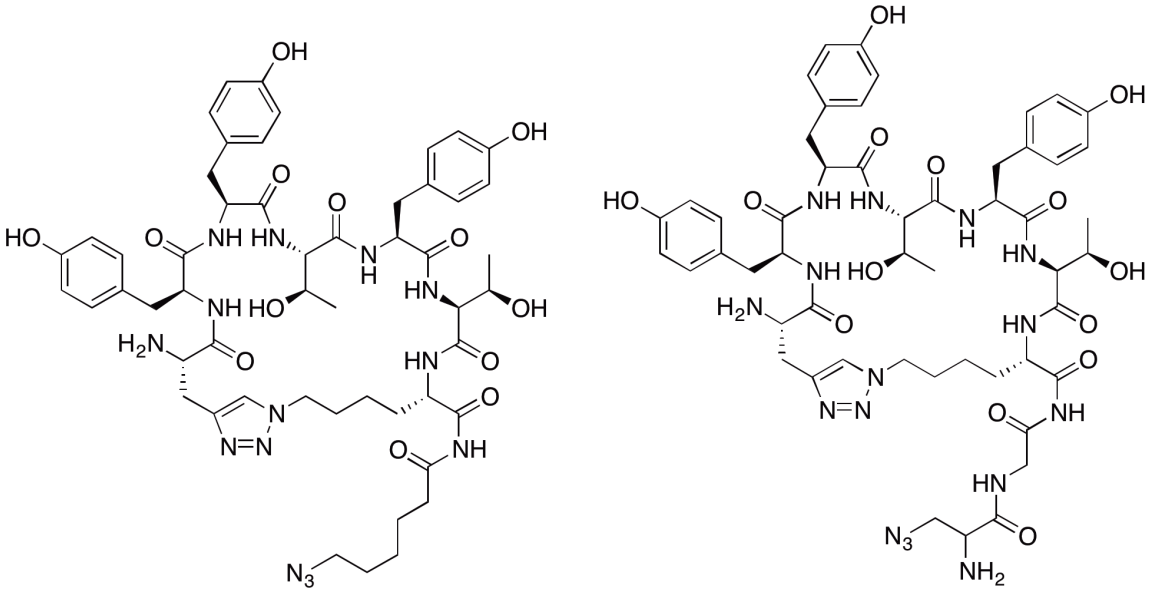
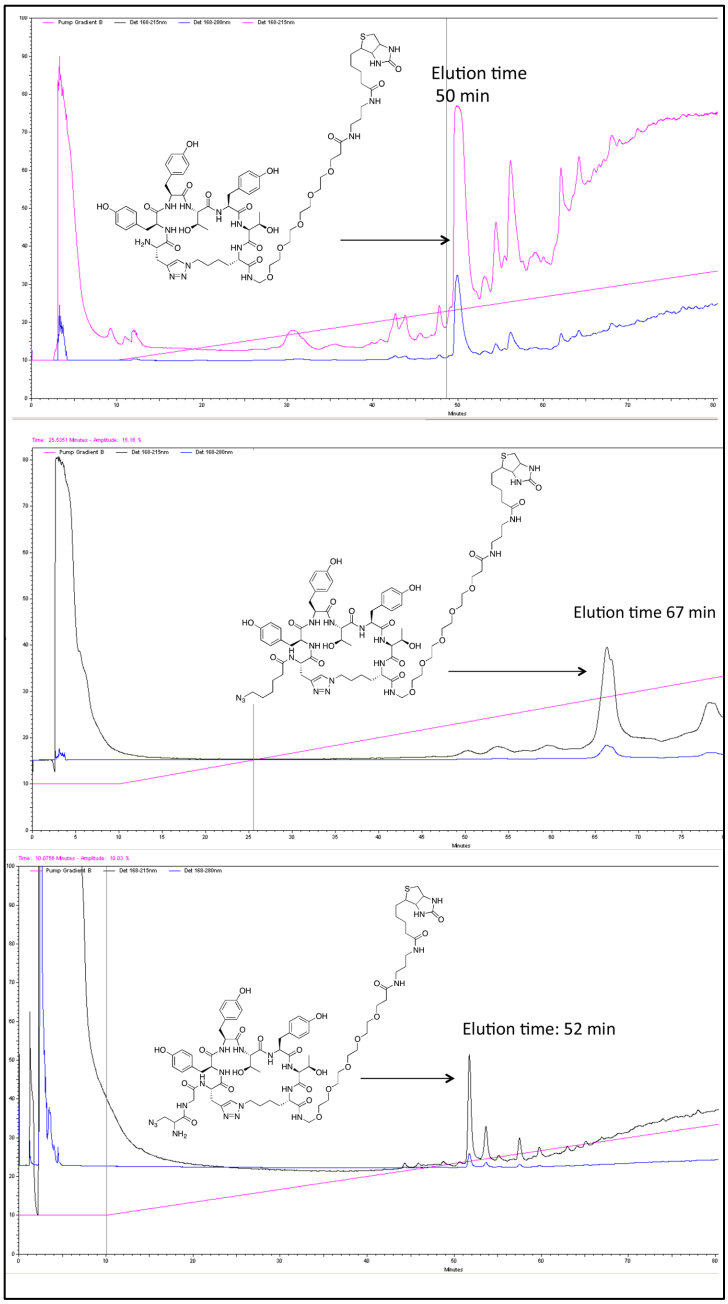


Figure 5.12: Comparison of HPLC traces to determine best monoligand anchor candidate. Monoligand anchor A is significantly different in character from the monoligand Cy(YYTYT-Tz4) while monoligand anchor B is similar in character.



5.4 Conclusion

In this chapter we have described the development of a Cu catalyzed cyclic library and demonstrated its use in OBOC screening. Although on bead cyclization of some specific peptide sequences through CuAAC have been demonstrated, we are not aware of use of CuAAC cyclized comprehensive libraries in OBOC screening. The libraries were developed with a specific aim in mind, isolating monoligands with high affinity to render the epitope targeting strategy more effective. This aim has been fulfilled. We were able to demonstrate the binding of the PCC agent to the epitope in the monoligand stage itself. The cyclic monoligand has a significantly higher binding affinity for the protein compared to the linear monoligand developed using the peptide epitope. It is also selective enough to distinguish between various Akt isoforms. Finally, we have preliminary data that the cyclic ligand is an inhibitor of the kinase activity of the Akt2 enzyme. We have optimized the monoligand anchor to be used in developing the biligand. The biligand will be bicyclic, and hopefully, will have affinity and specificity similar to a monoclonal antibody.

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