

Chapter 2: Identifying Protein Capture Agent via *In Situ* Click Chemistry

2.1 Introduction

In vitro diagnostic tests can be instrumental in the characterization of disease pathways and mechanisms of cancer, providing more-targeted treatment plans and better survival rates for patients. Quantitative, multi-parameter measurements of proteins in the blood, which monitor the changes in protein levels in a patient, will revolutionize *in vitro* diagnostics. A major challenge in realizing this goal is to find an efficient and general approach for producing protein capture agents for protein-biomarker-based detection that have positive attributes of antibodies, and exhibit a high level of chemical and biochemical stability. Antibodies are proteins and are, thus, unstable towards thermal shock, dehydration, pH variation, degradation and many chemical processes. In addition, developing a high affinity and selectivity antibody is very expensive and time consuming. Therefore, commercial antibodies do not exist for all proteins.¹ This is becoming an increasingly important problem as diagnostic measurements of proteins are quickly moving from single parameter to multi-parameter assays of large panels of biomarkers.²⁻⁴ Despite the drawbacks, antibodies remain the standard protein capture agent used in protein assays because of their high sensitivity and selectivity for their cognate proteins.

Nucleic acid aptamers, small molecules, and phage-display peptides are alternatives to antibodies. Nucleic acid aptamers⁵⁻⁷ are promising alternatives, with an extensive sequence space that can be screened in parallel ($\sim 10^{15}$ elements).⁸ However, the chemical diversity of aptamers is typically limited to 4 chemical constituents rather than 20 amino acids compared to peptides. Although, aptamers have been prepared as protein capture agents ($K_D \approx 10^{-8}$ - 10^{-9} M), the synthetic scale-up can be nontrivial as

aptamers are large (5–25 kDa) oligomers.⁹ Also, aptamer length does not directly translate to high three-dimensional diversity.¹⁰

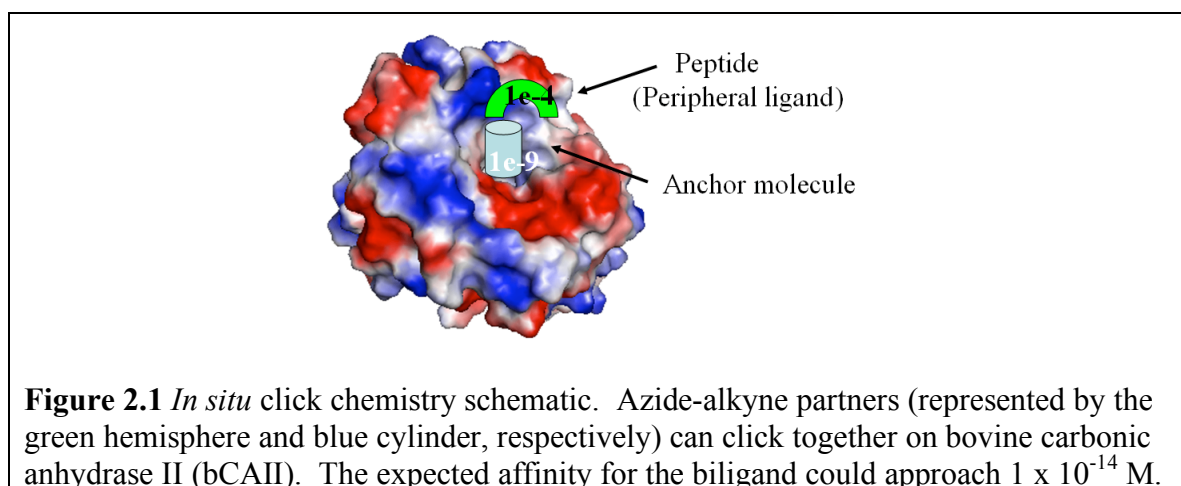
Small molecule inhibitors, while displaying very high-affinity binding ($K_D \approx 10^{-8}$ – 10^{-13} M), bind to just one epitope on the protein of interest, and that epitope may not be unique to the single protein against which the inhibitor is developed. This means that the inhibitor may exhibit cross-reactivity with other related proteins.¹¹ Moreover, small molecule libraries can be time-consuming to synthesize, and such libraries do not represent a generic platform for screening against all proteins.

Peptides selected by phage display methods also can offer reasonably high-affinity binding ($K_D \approx 10^{-8}$ – 10^{-9} M) to a protein target with a relatively low molecular mass.^{4,12} However, for phage display approaches, peptides are limited to linear sequences comprised of naturally occurring L-amino acids that provide poor bioavailability and enzymatic degradation. Other characteristics, such as water solubility, are inconsistent and dependent upon the amino acid composition of the peptide.

Peptide affinity agents can also be identified using one-bead one-compound (OBOC) libraries.^{13–15} This approach allows for the inclusion of broad classes of amino acids, including artificial and nonnatural amino acids,^{16,17} peptoids,¹⁸ and other peptidomimetics.¹⁹ However, serious trade-offs between peptide length and library diversity have to be made, since OBOC libraries are typically only 10^4 – 10^6 elements in size. Phage display, by contrast, produces $\sim 10^{12}$ element libraries. As a result, high quality protein capture agents are rarely identified from OBOC peptide libraries.

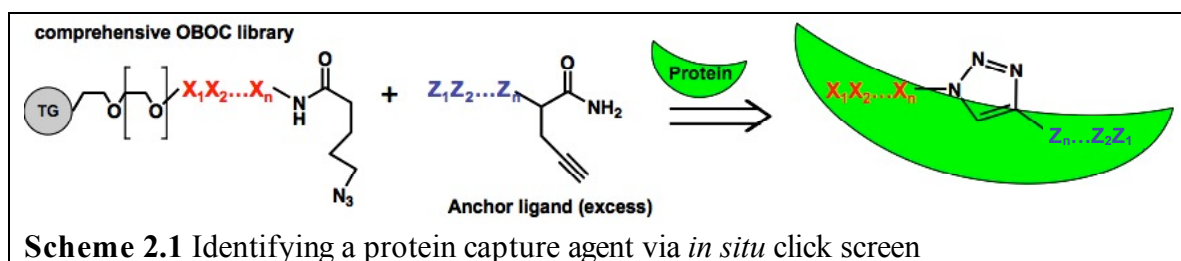
In situ click chemistry,^{20–23} as a target-guided^{24–26} approach, has been utilized previously for the identification of small molecule enzymatic inhibitors. For this method,

the protein target replaces the role of a Cu(I) catalyst for promoting the 1,3-dipolar ‘click’ cycloaddition reaction between two low-affinity molecules into a single high-affinity bivalent ligand or biligand. In these studies, a known small molecule inhibitor to a biological target is divided into parts, each part is expanded into a small library and engineered to have a terminal alkyne or azide functionality. The uncatalyzed click reaction, which is slow at room temperature, is accelerated when the azide and alkyne groups are held within close proximity by the biological target (for example, a protein). Under this condition, the azide and alkyne ‘click’ together irreversibly, creating a bivalent enzymatic inhibitor. This approach has been used to assemble small molecule inhibitors to acetylcholinesterase (AChE), carbonic anhydrase (CA), and HIV-1 protease with sub-pM binding affinities,²⁰⁻²⁶ and we have shown that peptide-based high-affinity lead compounds can be isolated with similar success. Furthermore, when a molecule with a 10^{-4} -M affinity is combined with a molecule with a 10^{-9} -M affinity, the ‘clicked’ product can exhibit an affinity as high as 10^{-13} M (Figure 2.1).²¹ While achieving the full product affinity may be rare, increasing the affinity by 2 to 3 orders of magnitude per additional ligand should be readily achievable.



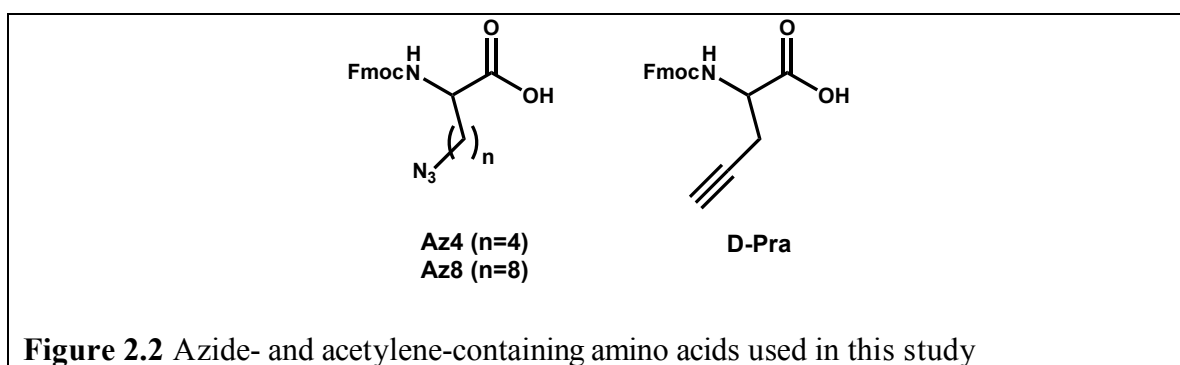
If the achieved affinity and selectivity of the biligand is not sufficient for the desired task, then the biligand itself may be treated as an anchor ligand, and the *in situ* click chemistry process may be repeated as needed to prepare triligands.^{27,28} This process can further be repeated to produce multi-ligands, such as tetraligands, pentiligands, etc., until the desired affinity is achieved. Thus, multi-valent binding agents can provide a potential shortcut to high affinity.²⁹ This approach is used here as a general platform for high-throughput peptide capture agent development.

We have generalized the *in situ* click chemistry strategy for producing protein capture agents by implementing peptides selected from OBOC libraries.¹³⁻¹⁵



OBOC libraries allow thousands to millions of compounds to be rapidly prepared on bead and screened concurrently for specific binding. For many biological targets, recognition of targets is effective using peptides as small as three-to-five amino acids in length.^{30,31} Therefore, penta- to heptapeptide libraries were expected to afford enough diversity to discriminate between weak and strong binders. OBOC libraries offer a distinct advantage in which nonnatural modifications are easily accomplished, allowing for incorporation of almost infinite diversity elements. Natural, nonnatural, and artificial amino acids are assembled together to form peptides using standard coupling chemistries.³² D-amino acids were chosen for the core of the library so that the synthesized peptides have an

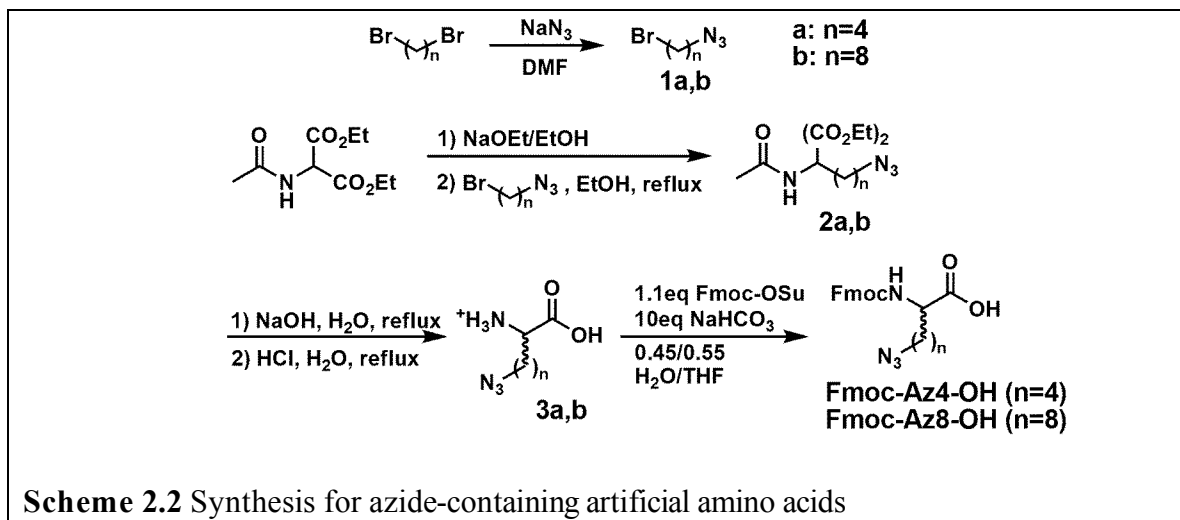
increased resistance to enzymatic degradation when applied to *in vivo* assays. Azide-containing amino acids displaying 4-carbon (**Az4**) or 8-carbon (**Az8**) azidoalkyl linkers and D-propargylglycine (**D-Pra**) are chosen as the azide and alkyne handles, respectively, for click chemistry (Figure 2.2). Azides and alkynes are also chemically attractive because they are synthetically convenient to introduce, compatible with a variety of solvents and species, and tolerant of other functionalities. The OBOC library technique also allows for desirable chemical and physical properties, such as water solubility, to be precisely tuned in the peptide capture agent. Scalability to gram quantities can be done straightforwardly and significantly cheaper than with antibodies.



Herein, I describe the use of *in situ* click chemistry as a general synthetic approach towards identifying high-quality, inexpensive protein capture agents. This chapter covers the synthesis of the azide-containing artificial amino acids, peptide library construction, *on bead* click reaction, and bead-based library screening procedures. Edman sequencing of lead compounds, including the custom gradient that was designed to allow for elution and verification of the artificial amino acids are also described in this section.

2.2 Artificial Amino Acid Synthesis

Azide-containing amino acids were synthesized and characterized, and used as handles for click chemistry following Scheme 2.2.



Azidobutylbromide (1a). To a solution of 1,4-dibromobutane (123 mmol) in N,N-dimethylformamide (DMF), sodium azide (61.5 mmol) was added and stirred overnight at 50 °C. The reaction was diluted with ethyl acetate, and the organic layer was washed with water, then brine, and dried over MgSO₄. The crude residue was purified by silica gel chromatography (100% hexanes) to give a product (80%) as a clear oil. ¹H NMR (300 MHz, CDCl₃): δ 3.44 (2H, t, *J* = 6.3 Hz), 3.34 (2H, t, *J* = 6.6 Hz), 1.93–1.98 (2H, m), 1.74–1.79 (2H, m).

Azidooctylbromide (1b). Synthesis was carried out as described above, except 1,8-dibromobutane was used as the starting material. ¹H NMR (300 MHz, CDCl₃): δ 3.41 (2H, t, *J* = 6.9 Hz), 3.26 (2H, t, *J* = 6.6 Hz), 1.86 (2H, p, *J* = 6.9 Hz), 1.60 (2H, p, *J* = 8.7 Hz), 1.34–1.55 (4H, m).

Diethyl 2-acetamido-2-(4-azidobutyl)malonate (2a). To a solution of 0.598 g (0.026 mol) sodium metal in 25 mL absolute EtOH, 5.65 g diethyl acetamidomalonate

(0.026 mol) was added, following previously published procedures.³³ The mixture was stirred for 30 min at room temperature. By dropwise addition, azidobutylbromide **1a** (4.82 g, 0.027 mol) was added with stirring. The reaction mixture was stirred for 2 hours at room temperature and refluxed for 6 hours at 80 °C. After cooling overnight, the reaction mixture was concentrated to dryness, and the residue was extracted with diethyl ether. The combined ether extracts were washed with water, sat. NaHCO₃, water, and brine, and were dried over MgSO₄ and then concentrated. Silica gel chromatography (Hex:EtOAc = 1:1) gave a product (63%) as a clear, viscous oil. ¹H NMR (300 MHz, CDCl₃): δ 6.77 (1H, s), 4.24 (4H, q, *J* = 6.9 Hz), 3.26 (2H, t, *J* = 6.9 Hz), 2.31–2.37 (2H, m), 2.04 (3H, s), 1.59 (2H, p, *J* = 7.5 Hz), 1.26 (6H, t, *J* = 6 Hz), 1.16–1.27 (2H, m). ESI-MS *m/e* 315.

Diethyl 2-acetamido-2-(4-azidooctyl)malonate (2b). Similar synthetic protocol as **2a** was adopted, only azidooctylbromide **1b** served as the starting material. ¹H NMR (300 MHz, CD₃Cl₃): δ 6.76 (1H, s), 4.24 (4H, q, *J* = 7.2 Hz), 3.24 (2H, t, *J* = 6.9 Hz), 2.27–2.33 (2H, m), 2.04 (3H, s), 1.56 (2H, p, *J* = 7.5 Hz), 1.25 (6H, t, *J* = 7.2 Hz), 1.06–1.16, 1.2–1.4 (10H, m). ESI-MS *m/e* 371.

2-Azidobutyl amino acid (3a). Following standard methods³⁴, the diester **2a** (2.8 mmol) in 25 mL of 10% NaOH solution was heated to reflux for 4 hours. The solution was then neutralized with concentrated HCl and evaporated. The residue was dissolved in 25 mL of 1 M HCl and heated to reflux for 3 hours. The solvent was reduced and extraction with MeOH which afforded amino acid **3a** as the hydrochloride salt (85%). ¹H NMR (300 MHz, CD₃OD): δ 3.98 (1H, t, *J* = 6.3 Hz), 3.35 (2H, t, *J* = 7.8 Hz), 1.45–1.7, 1.85–2.05 (6H, m). MALDI-MS *m/e* 173.

2-Azidooctyl amino acid (3b). Synthesis was carried out as described above, using diester **2b** as the starting material. ^1H NMR (300 MHz, CD_3OD): δ 3.94 (1H, t, $J = 6.3$ Hz), 3.27 (2H, t, $J = 6.9$ Hz), 1.3–1.52, 1.52–1.62, 1.8–1.98 (14H, m). ESI-MS m/e 229.

Fmoc-2-Azidobutyl amino acid (Fmoc-Az4-OH). The amino acid **3a** (26.3 mmol) was dissolved in 0.45:0.55 H_2O :THF (150 mL), and NaHCO_3 (22.1 g, 263 mmol) was added, following published methods.³⁵ After the mixture was cooled to 0 °C, Fmoc-OSu (9.7 g, 28.9 mmol) was added dropwise over 5 min. The reaction mixture was allowed to come to room temperature and stirred overnight. Evaporation of THF was completed *in vacuo* and the aqueous residue was washed with diethyl ether (2×200 mL). The aqueous layer was then collected and acidified with conc. HCl to pH 2 before extraction with ethyl acetate (4×100 mL). The combined organic layers were washed with brine, dried over MgSO_4 , filtered, and concentrated. The organic residue was purified by column chromatography (2% MeOH in DCM) to yield a white powder (48% yield). ^1H NMR (300 MHz, CDCl_3): δ 7.76 (2H, d, $J = 7.5$ Hz), 7.59 (2H, d, $J = 6.9$ Hz), 7.40 (2H, t, $J = 7.5$ Hz), 7.31 (2H, t, $J = 7.5$ Hz), 5.34 (1H, d, $J = 7.8$ Hz), 4.49–4.59 (1H, m), 4.43 (2H, d, $J = 6.6$ Hz), 4.22 (1H, t, $J = 6.6$ Hz), 3.27 (2H, t, $J = 6.6$ Hz), 1.3–2.0 (6H, m). ESI-MS m/e 395.

Fmoc-2-Azidooctyl amino acid (Fmoc-Az8-OH). The amino acid **3b** was treated to Fmoc protection as described above. ^1H NMR (300 MHz, CD_3Cl_3): δ 7.75 (2H, d, $J = 7.5$ Hz), 7.57–7.61 (2H, m), 7.39 (2H, t, $J = 7.5$ Hz), 7.30 (2H, t, $J = 7.2$ Hz), 5.40 (1H, d, $J = 8.1$ Hz), 4.42–4.52 (1H, m), 4.40 (2H, d, $J = 7.2$ Hz), 4.21 (1H, t, $J = 7.2$ Hz), 3.23 (2H, t, $J = 6.9$ Hz), 1.18–1.98 (14H, m). ESI-MS m/e 450.

2.3 One-Bead One-Compound Peptide Library Construction

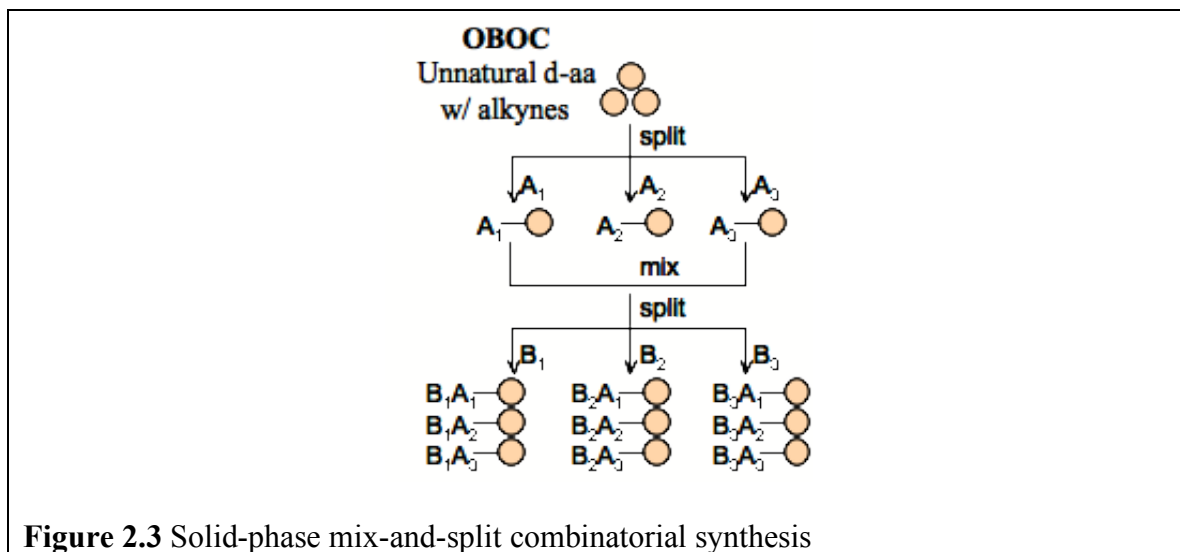
2.3.1 Materials. Fmoc-D-X-OH (Fmoc, fluoren-9-ylmethoxycarbonyl) (X = Ala, Arg(Pbf) (Pbf, pentamethyldihydrobenzofuran-5-sulfonyl), Asn(Trt) (Trt, trityl), Asp(OtBu) (*t*Bu, *tert*-butyl), Glu(OtBu), Gln(Trt), Gly, His(Trt), Ile, Leu, Lys(Boc) (Boc, *tert*-butyloxycarbonyl), Met, Phe, Pro, Ser(*t*Bu), Thr(*t*Bu), Trp(Boc), Tyr(*t*Bu), and Val) were purchased (Anaspec; San Jose, CA). TentaGel S-NH₂ resin (90 μm, 0.31 mmol/g) (Rapp-Polymere; Tübingen, Germany) were utilized for OBOC library construction. Amino acid coupling reactions were performed in 1-methyl-2-pyrrolidinone (NMP, 99%) with HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate, ChemPep; Miami, FL) and *N,N'*-diisopropylethylamine (DIEA). For removal of N^α-Fmoc protecting groups, a solution of 20% piperidine in NMP was used. For final deprotection of the peptide libraries, trifluoroacetic acid (TFA, 98% min. titration) and triethylsilane (TES) were used. All solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

OBOC libraries were synthesized using a 180-degree variable-speed shaker, fitted with small sample adapter (St. John Associates; Beltsville, MD). Fritted polypropylene solid-phase synthesis tubes were used for repeated split-mix cycles. A 24-port SPE vacuum manifold system (Grace, Deerfield, IL) was used for exchanging coupling solutions and washing the resins. Fmoc-D-propargylglycine (Fmoc-D-Pra-OH) was acquired (Chem-Impex International; Wood Dale, IL) and used as the acetylene handle for construction of ligands.

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International; Wood Dale, IL) and used as the acetylene handle for construction of ligands.

2.3.2 Library Construction. Randomized OBOC libraries of penta- to heptapeptides were synthesized manually via standard split-and-mix solid-phase peptide synthesis methods on 90 μm polyethylene glycol-grafted polystyrene beads (TentaGel S-NH₂, 0.31 mmol -NH₂/g, 2.86 x 10⁶ beads/g, Rapp Polymere, Tübingen, Germany)¹³⁻¹⁵. Each bead is functionalized with a unique chemical entity (Figure 2.3). At least a five fold excess of beads was utilized in each library synthesis to ensure adequate representation of each library element. Nonnatural D-stereoisomers (denoted by lowercase one-letter amino acid code) were used at every possible position in the peptide sequence (See Appendix A for the structure of the D-amino acids). Acetylene and azide-containing amino acids are coupled to the N- and C-termini to serve as handles for click chemistry. The distance between the azide and alkyne during the *in situ* click experiment cannot be predicted in advance, so the incorporation of the variable length azidoalkyl linker may improve the assembly of the click product on the face of the protein.



A standard solid-phase peptide synthesis method with 9-fluorenylmethoxycarbonyl

(Fmoc) chemistry was used.³⁶ All wash, deprotection, and coupling steps were facilitated

by 180-degree shaking of the resin. The resin was pre-swelled in *N*-methylpyrrolidinone (NMP) in a plastic disposable reaction vessel with fritted ends. The resin was separated into multiple aliquots and each aliquot was reacted with two fold molar excess (relative to resin) of a single N^{α} -Fmoc-amino acid. Amide coupling was initiated by addition of a two fold molar excess of HATU and a six fold molar excess of *N,N'*-diisopropylethylamine (DIEA). The coupling reaction was run for 15 min. Another 2 equiv N^{α} -Fmoc-amino acid, 2 equiv HATU, and 6 equiv DIEA were added, and allowed to react for 15 min (“double coupling”). In some cases, “triple coupling” with a third set of coupling reagents and N^{α} -Fmoc-amino acid was performed (Table 3.1, Libraries D, E, F, and G).

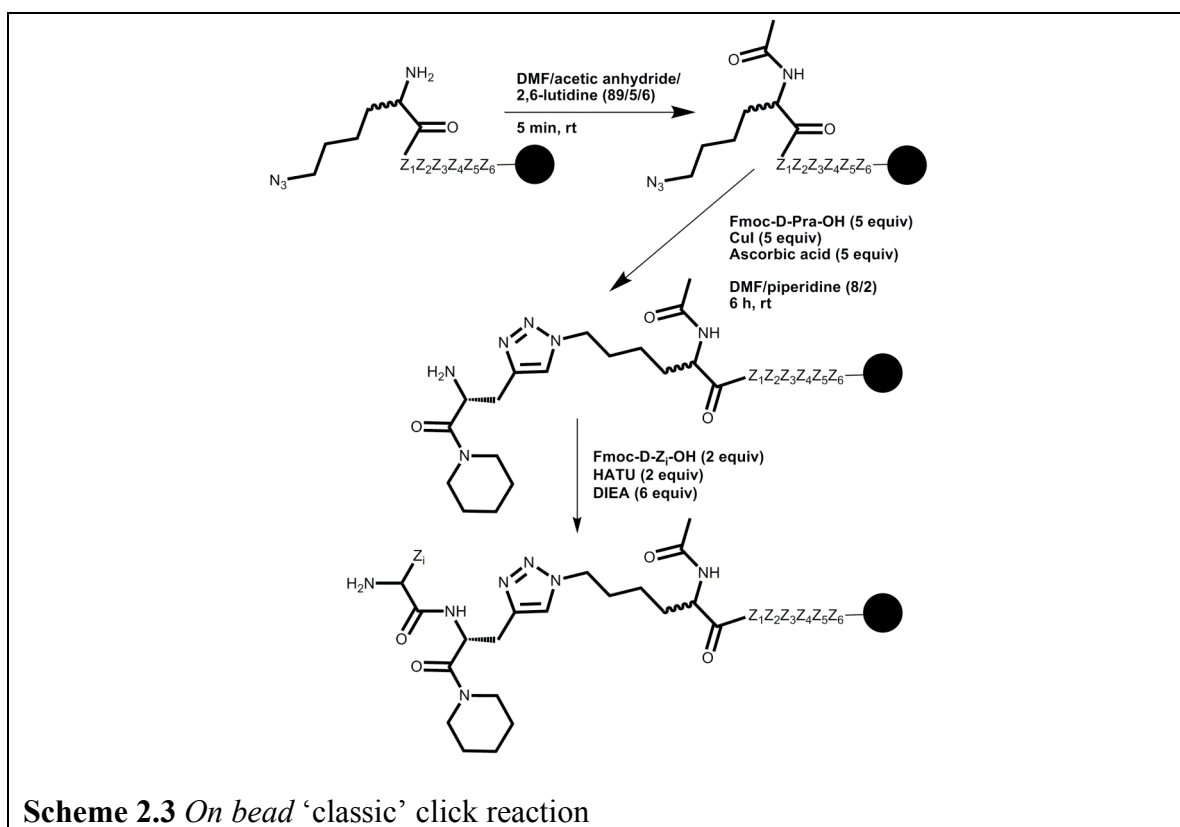
After coupling, the aliquots are thoroughly washed (5 x NMP), mixed together into a single vessel, and deprotected with 20% piperidine in NMP (30 min). The resins are thoroughly washed again (5 x NMP), dried (5 x DCM), and redivided into multiple aliquots for the next cycle of coupling. This second cycle of coupling adds another single N^{α} -Fmoc-amino acid to the growing peptide chain. The procedures of couple, wash, mix, deprotect, and split iterate until the desired length of peptide is attained.

The amino acid side-chain protecting groups are removed by incubation in 95% trifluoroacetic acid (TFA), 5% water, and triethylsilane (two fold molar excess per protected side chain) for 2 hours. The library resin was then neutralized with DMF, and washed thoroughly with DMF (5 x), water (5 x), methanol (MeOH, 5 x), and methylene chloride (DCM, 5 x)³⁷ and then dried under vacuum and stored in phosphate-buffered saline [PBS (pH 7.4)] + 0.05% NaN₃ at 25 °C.

2.4 On Bead Click Reaction

2.4.1 Materials. For peptide biligand and triligand synthesis, acetylation reagents (acetic anhydride, 2,6-lutidine, and *N,N*-dimethylformamide (DMF)) were purchased from Sigma-Aldrich (St. Louis, MO). For the *on bead* Cu(I)-catalyzed click reaction, copper(I) iodide, L-ascorbic acid, and sodium diethyldithiocarbamate trihydrate were purchased from Sigma-Aldrich (St. Louis, MO).

2.4.1 Click Reaction. For preparing biligand and triligand candidates, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) on bead was completed in four general steps: (1) anchor ligand synthesis, (2) acetylation, (3) click reaction, and (4) addition of 2° ligand sequence. Scheme 2.3 illustrates these reactions (*Z* = any amino acid).



We found that the azide moiety needed to be attached to the bead and that the alkyne molecule needed to be in solution for the click reaction to work efficiently. For the

acetylation, the fully protected TentaGel S-NH₂ bead-bound anchor ligand (0.420 g, 0.13 mmol) was capped by a solution of acetic anhydride (1 mmol) in DMF, containing a catalytic amount of 2,6-lutidine.³⁸

The acetylated peptide was reacted with Fmoc-D-Pra-OH (0.218 g, 0.65 mmol) in the presence of copper(I) iodide (0.124 g, 0.65 mmol), L-ascorbic acid (0.114 g, 0.65 mmol), and DMF/piperidine (8/2) at 25 °C for 6 hours.³⁹ The resin was washed with 5 × 5 mL sodium diethyldithiocarbamate trihydrate (Et₂NCSSNa•3H₂O, 1% w/v), containing 1% DIEA (v/v) in DMF to remove excess coordinated copper.⁴⁰ Following the click reaction, the next N^α-Fmoc-amino acid was added to the peptide chain.

2.5 Bead-Based Library Screening Procedures

2.5.1 Proteins. Bovine carbonic anhydrase II (bCAII, C2522), from bovine erythrocytes, lyophilized powder, was obtained from Sigma-Aldrich (St. Louis, MO) and used as received. To prepare the protein for screening, dye-labeling was accomplished with the Alexa Fluor 647 Microscale Protein Labeling Kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol for low degree of labeling (DOL). Protein (100 μg) was incubated with 6 mol equiv Alexa Fluor 647 succinimidyl ester for 15 min at 25 °C. Excess dye was removed by BioGel P-6 size exclusion resin (Bio-Rad, Hercules, CA). The labeled protein (bCAII-Alexa647) was characterized by UV-Vis and mass spectrometry.

2.5.2 Screening. A typical peptide library screen against bCAII is described as follows. The peptide library is screened not all at once, but in several mg portions. Approximately 10–20 mg dried peptide-bead library is incubation in PBS (pH 7.4) +

0.1% Tween 20 + 0.1% bovine serum albumin (BSA) + 0.05% NaN₃ (PBSTBNaN₃) for 1 hour, with shaking, to block nonspecific protein binding.⁴¹ The library was then washed with 3 x 5 mL PBSTBNaN₃ only. *On bead* multi-ligand screens were conducted at an appropriate bCAII-Alexa Fluor 647 dilution (Table 2.1), and then washed with 3 x 5 mL PBSTBNaN₃, 3 x 5 mL PBS (pH 7.4) + 0.1% Tween 20, and finally 6 x 5 mL PBS (pH 7.4). All *in situ* screens contained an additional 2 hours preincubation of bCAII-Alexa Fluor 647 with anchor ligand (≥ 2000 equiv, relative to protein), after which the bead library was added to this mixture and the screen was continued (Table 2.1). Following *in situ* screening, beads were washed with 3 x 5 mL PBSTBNaN₃, 3 x 5 mL PBS (pH 7.4) + 0.1% Tween 20, and then 6 x 5 mL PBS (pH 7.4). We found that longer incubation times, up to 24 hours, select for more specific tight-binding events and promote dissociation of the weakly bound peptides.

Table 2.1 Screening summary; pH=7.4 and T=25°C, unless otherwise noted

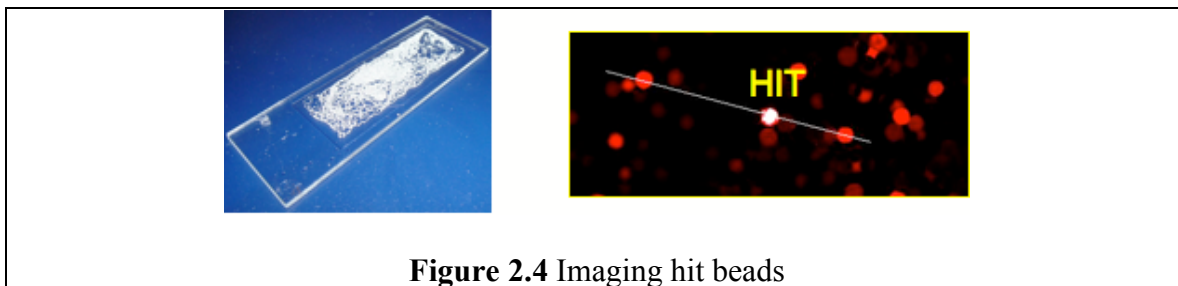
Screen	Library	[bCAII-AF647]	Time(h)	% hit beads	Buffer	Other components
An1	A	100 nM	1 h	0.02%	PBS	
An2a	B	50 nM	1 h	0.09%	PBS	
An2b	B	8 nM	24 h	2 hits	PBS	
Bi1	C	50 nM	2 h; 37° (no beads) + 48 h; 37°	0.007%	PBS + 1% DMSO (v/v)	100 μ M of Iklwfk-(D-Pra)
Bi2a	D	50 nM	17 h	0.07%	PBSTBNaN ₃	
Bi2b	D	10 nM	17 h	0.008%	PBSTBNaN ₃	
Tri1	C	10 nM	2 h (no	0.007%	PBSTBNaN ₃ +	100 μ M of (D-Pra)-kwlwGI-

			beads) +15 h		1% DMSO (v/v)	Tz1-kfwlkl
Tri2	E	10 nM	17 h	0.008%	PBSTBNaN ₃	
TriX	A	10 nM	17 h	0.007%	PBSTBNaN ₃ + 1% DMSO (v/v)	100 μM of (D-Pra)-kwlwGI- Tz1-kfwlkl
Tri3	F	0.5 nM	2 h (no beads) +18 h	0.005-0.01%	PBSTBNaN ₃ + 1% DMSO (v/v)	100 μM of (D-Pra)-kwlwGI- Tz1-kfwlkl
Tri4	G	0.25 nM	18 h	0.005-0.01%	PBSTBNaN ₃	

Anchor ligand screens (**An1**, **An2a**, **An2b**) were conducted using **Libraries A** and **B**. Following screening, the beads were washed with 3 x 5 mL PBS (pH 7.4), then 7 x 5 mL water.

In situ biligand and triligand screens were carried out utilizing **Libraries C** and **F**. *On bead* biligand and triligand screens were performed using **Libraries D**, **E**, and **G**. Screening conditions are provided in Table 2.1, and further details will follow.

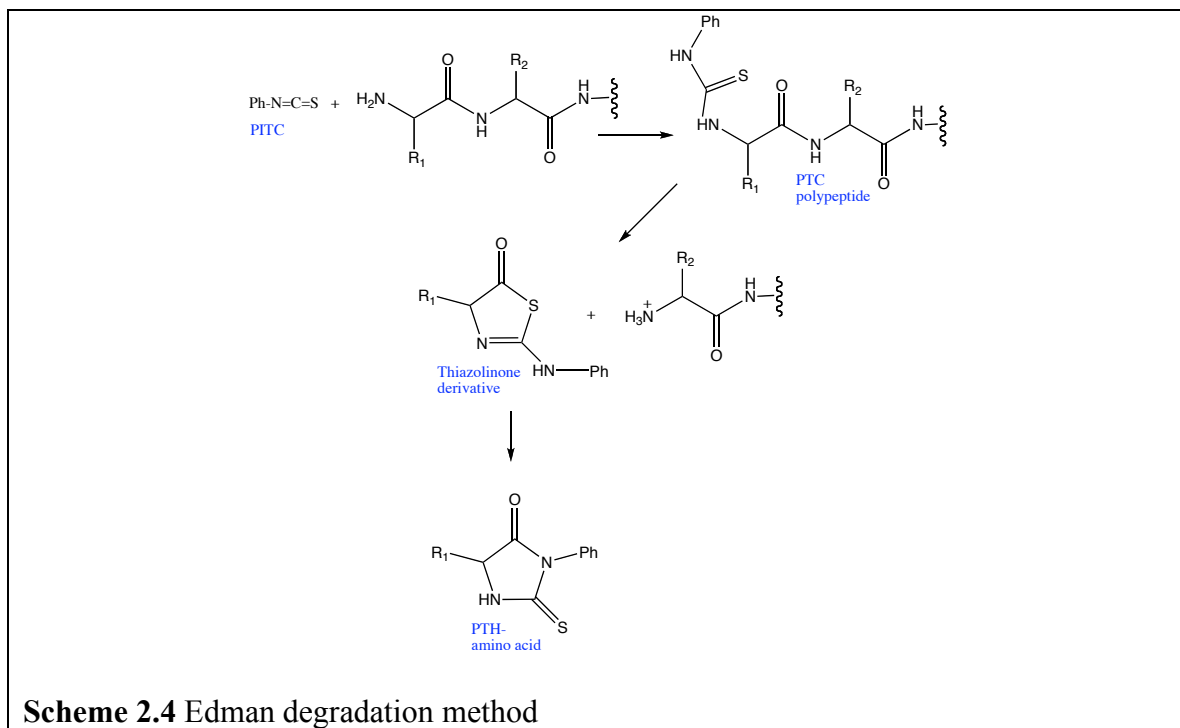
After incubation, the peptide-bead library and protein screening solution is transferred into a clean polyethylene-fritted vessel. The beads are filtered *in vacuo*, then washed with 0.1% Tween20/PBS (3 x 1 ml) followed by PBS pH 7.4 (3 x 1 ml), followed by H₂O (7 x 1 ml). The washed peptide-bead library sample is transferred onto a glass microscope slide and immediately imaged using a GenePix 4200 array scanner ($\lambda_{\text{ex}} = 635$ nm). These individual hit beads, which are white due to saturation of the PMT gain, are manually selected using a glass micropipette.



To remove bound proteins, each hit bead was incubated in 7.5 M guanidine hydrochloride (pH 2.0) for 1 hour, followed by ten rinses with water. These hits are considered lead compounds, and their sequences are analyzed by Edman degradation.

2.6 Analysis of Lead Compounds by Edman Degradation

2.6.1 Method. Edman sequencing of single hit beads was carried out on a Model 494 Procise cLC Sequencing System (Applied BioSystems, Foster City, CA). Edman sequencing requires that the peptide have a free N-terminus on α -amino acids for determination of a peptide's sequence. Edman degradation works in the following way: Phenylisothiocyanate (PITC) is reacted with an uncharged terminal amino group, under mildly alkaline conditions, to form a cyclic phenylthiocarbamoyl derivative. Then, under acidic conditions, this derivative of the terminal amino acid is cleaved as a thiazolinone derivative. The thiazolinone amino acid is then selectively extracted into an organic solvent and treated with acid to form the more stable phenylthiohydantoin (PTH)-amino acid derivative that can be identified by high-pressure liquid chromatography (Scheme 2.4). This procedure can then be repeated again to identify subsequent amino acids in the peptide sequence.



Iterative N-terminal chemical degradation cycles yielded direct positional amino acid information. Each degradation cycle produced one PTH-amino acid (PTH = phenylthiohydantoin) product that was analyzed by C_{18} HPLC and identified by retention time as compared with PTH-amino acid standards.

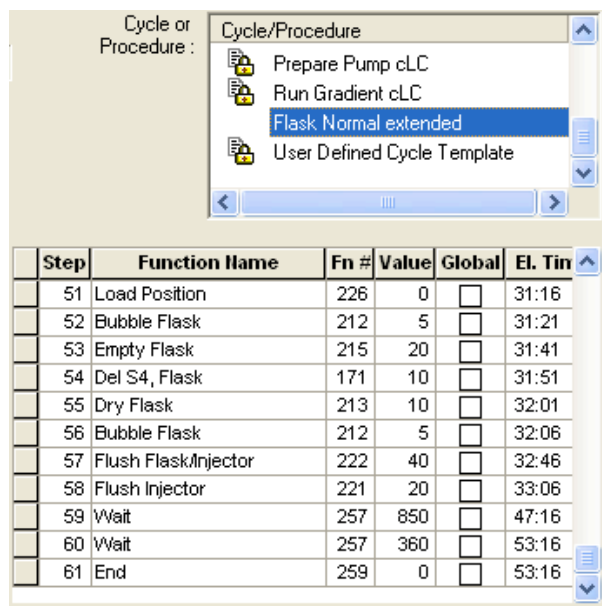
2.6.2 Custom Edman Degradation. A custom gradient was designed to allow for elution of the artificial amino acids, characterization of retention times, and confirmation of their incorporation in the *in situ* library. To allow for resolution of artificial azide-containing amino acids by Edman degradation, the Pulsed-Liquid cLC extended method was utilized (Figure 2.5). It includes a modified gradient, Normal 1 cLC extended (Figure 2.6), and a flask cycle extended by 5 min (Figure 2.7).

Cycle #	Cartridge Cycle	Flask Cycle	Gradient
Default	Cart-PL 6mmGFF cLC	Flask Normal extended	Normal 1 extended
1	None	Prepare Pump cLC	Prepare Pump cLC
2	None	Flask Blank cLC	Normal 1 extended
3	Cart Begin cLC	Flask Standard cLC	Normal 1 extended

Figure 2.5 Pulsed-Liquid cLC extended method

Time	%B	uL/min	Event	Cum. Volume A	Cum. Volume B
0.0	8	40	12	0.00	0.00
0.4	12	40	1	14.40	1.60
4.0	20	40	1	135.36	24.64
22.0	45	40	1	621.36	258.64
34.0	60	40	1	849.36	510.64
35.0	90	40	1	859.36	540.64
39.0	90	60	0	883.36	756.64
40.0	50	20	0	889.36	770.64

Figure 2.6 Normal 1 cLC extended gradient



Cycle or Procedure :

Cycle/Procedure

- Prepare Pump cLC
- Run Gradient cLC
- Flask Normal extended
- User Defined Cycle Template

Step	Function Name	Fn #	Value	Global	El. Tin
51	Load Position	226	0	<input type="checkbox"/>	31:16
52	Bubble Flask	212	5	<input type="checkbox"/>	31:21
53	Empty Flask	215	20	<input type="checkbox"/>	31:41
54	Del S4, Flask	171	10	<input type="checkbox"/>	31:51
55	Dry Flask	213	10	<input type="checkbox"/>	32:01
56	Bubble Flask	212	5	<input type="checkbox"/>	32:06
57	Flush Flask/Injector	222	40	<input type="checkbox"/>	32:46
58	Flush Injector	221	20	<input type="checkbox"/>	33:06
59	Wait	257	850	<input type="checkbox"/>	47:16
60	Wait	257	360	<input type="checkbox"/>	53:16
61	End	259	0	<input type="checkbox"/>	53:16

Figure 2.7 Final steps of flask normal extended flask cycle

The Edman traces corresponding to elution of Az2, Az4, Az6 and Az8 are shown in Figure 2.8 and demonstrate a 6 min retention time increase for every two methylene

units added to the azidoalkyl side chain. Fmoc-Az2-OH was synthesized according to literature protocol,⁴² while Fmoc-Az6-OH was synthesized according to Scheme 2.2.

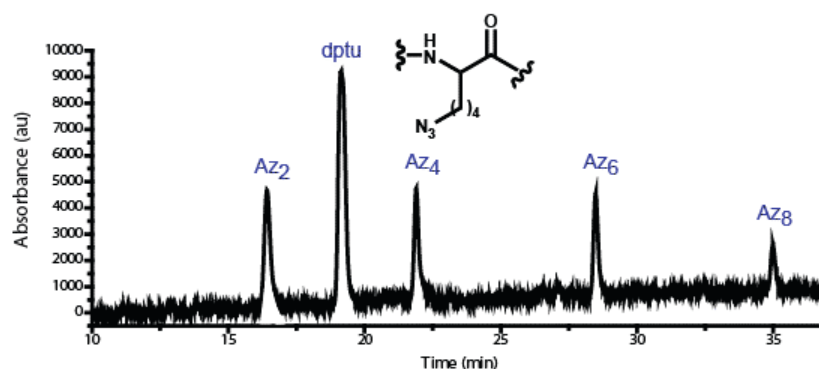


Figure 2.8 Edman traces for artificial azide-containing amino acids

Also, we performed a sequencing calibration for the *in situ* click hit using a commercially available 4-azidophenylalanine (Phe-N₃) and an alkynyl inhibitor against bCAII. The shift in retention is indicative of the click conjugation of the azide and alkyne species (Figure 2.9). Note that dptu is a side product of Edman sequencing.

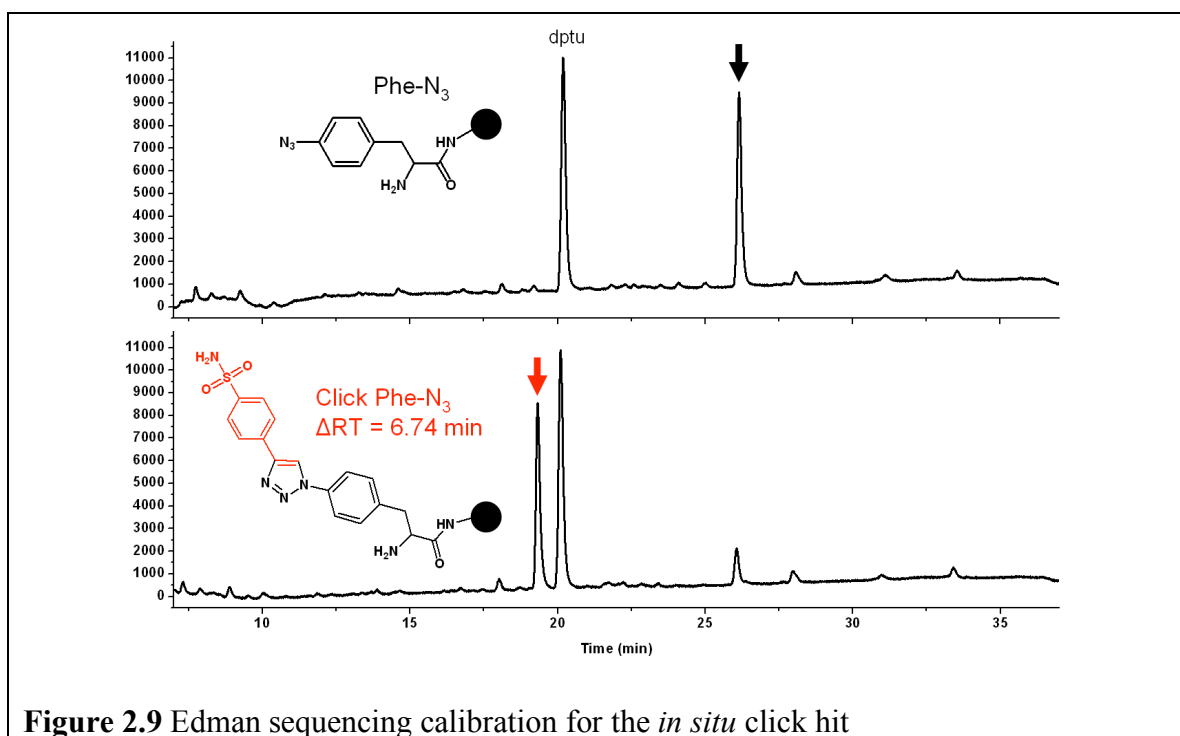


Figure 2.9 Edman sequencing calibration for the *in situ* click hit

2.7 Bulk Peptide Synthesis

Bulk synthesis of hit peptide sequences was performed on either Fmoc-Rink amide MBHA or 2-chlorotrityl chloride resins, on a typical resin scale of 0.3 g per sequence, using standard coupling chemistry. Hits were re-synthesized to contain the appropriate artificial amino acid (azide/acetylene) linkers at their termini to make them suitable for click chemistry. Biligand and triligand were synthesized in bulk and preassembled using Scheme 2.3. Crude peptides were precipitated with ether, and then purified to > 98% by HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector, Fullerton, CA) on a C₁₈ reversed phase semi-preparative column (Phenomenex Luna 10 μm, 250 × 10 mm). The pure peptides were used for screens, affinity measurements, and binding assays. Hit peptide sequences were also re-synthesized on TentaGel S-NH₂ on a similar resin scale, and used for *on bead* binding assays.

2.8 Conclusion

Protein capture agents are constructed from peptide ligands, each of which is constructed from a unique set of amino acids, including nonnatural amino acids and artificial amino acids. Artificial amino acids are synthesized so peptide ligands can be chemically functionalized with an azide (-N₃) or acetylene (-C≡C-H) group serving as handles for click chemistry. All of these amino acids are synthetically incorporated into peptides or polypeptides on beads using standard amino acid coupling chemistries. Using standard screening methods, the anchor, biligand, and triligand hits can be obtained and decoded by Edman degradation as shown in the last section of this chapter.

2.9 References

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