Chapter 2

Selection of a Multi-ligand Capture Agent for Carbonic Anhydrase II by Iterative In Situ Click Chemistry

2.1 INTRODUCTION

Protein biomarkers comprise an important aspect of *in vitro* diagnostics. Most protein detection methods rely upon antibody-based capture agents.[1] A high-quality antibody exhibits a high affinity and specificity for its cognate protein. However, antibodies are expensive, and can be unstable toward dehydration, pH variation, thermal shock, and many other chemical and biochemical processes.[2,3] In addition, antibodies are not available for many potential protein biomarkers. Thus, a major challenge is to discover an efficient and general approach for producing protein capture agents that display the positive attributes of antibodies, and exhibit a high level of chemical and biochemical stability. This is becoming an increasingly important problem as single protein-based diagnostics are being replaced by measurements of large panels of protein biomarkers.[4]

Several alternative protein capture agents, including oligonucleotide aptamers and phage display peptides, have been reported. Each of them have attributes as well as significant limitations.[5–11] A third alternative is to utilize one-bead one-compound (OBOC) peptide or peptide mimetic libraries.[12–16] An advantage of OBOC libraries is that chemical stability, water solubility, and other desired properties may be achieved by design. However, OBOC libraries are typically only 10^4–10^6 elements, and so significant trade-offs are made between peptide length and library chemical diversity. Phage display methods, by contrast, produce ~10^{12} element peptide libraries. As a result, high-quality protein capture agents can be challenging to identify directly from standard OBOC peptide libraries.

Herein, we combine the chemical flexibility of comprehensive, OBOC libraries of oligopeptides with in situ click chemistry[17–21] to yield a target-guided,[22–24] potentially

general screening approach for building high-affinity protein capture agents. For this selection scheme, the protein target replaces the role of a Cu(I) catalyst for promoting the 1,3-dipolar "click" cycloaddition reaction between azide-functionalized and acetylene-functionalized peptide affinity agents. First, an anchor (1°) ligand, containing acetylene (or azido) functionality, is selected for specific binding to a protein target via standard OBOC methods. Second, the same protein target is utilized to template the covalent coupling between two peptide ligands, the pre-identified 1° ligand and a secondary (2°) ligand, which is selected by the protein target and the 1° ligand from a comprehensive OBOC library of 2° ligands displaying azido (or acetylene) functionality. Synthetic scale-up yields a biligand composed of the 1° and 2° ligands, joined together via the 1,2,3-triazole linker. This biligand can then be used as a new anchor ligand, and the in situ click chemistry selection may be repeated to form a triligand, and so forth. As the number of peptide ligands that comprise the multi-ligand capture agent increases, the binding affinity and specificity rapidly increase.[25,26] Thus, multivalent binding agents can provide a potential shortcut to high affinity.[27]

By instituting *iterative* in situ click chemistry selections with OBOC, we exploit both technologies to produce a triligand capture agent against human and bovine carbonic anhydrase II (hCAII and bCAII, respectively). These two proteins are >80% identical in sequence (PDB ID: 1CA2, 1V9E). Carbonic anhydrase II belongs to a family of metalloenzymes that catalyze the reversible hydration of carbon dioxide. CA II expression is induced in the endothelium of neovessels in melanoma, renal carcinoma, and other cancers.[28] Furthermore, CA II represents a major target antigen for stimulating an autoantibody response in melanoma patients,[29] and is potentially a therapeutic target

for glial tumors.[30] It has served as a model protein to understanding protein-ligand interactions, and is a demonstrated receptor for bivalent ligands.[31–34]

In this chapter, the discovery process for high-affinity protein capture agents is discussed, using the triligand capture agent for b(h)CAII as the prototype. First, the construction of OBOC libraries containing artificial amino acids is detailed. Through *iterative* OBOC and in situ click chemistry selections, specific binders of b(h)CAII are identified sequentially—1° ligands, then biligands, and finally a triligand capture agent which displays ≥20 ng sensitivity for the protein target in dilute serum. The entire screening approach is summarized in Figure 2.1.

2.2 MATERIALS AND EXPERIMENTAL METHODS

2.2.1 Materials

Fmoc-D-**X**-OH (Fmoc, fluoren-9-ylmethoxycarbonyl) (**X** = Ala, Arg(Pbf) (Pbf, pentamethyldihydrobenzofuran-5-sulfonyl), Asn(Trt) (Trt, trityl), Asp(O*t*Bu) (*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) and used as received. TentaGel S-NH_2 resins (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

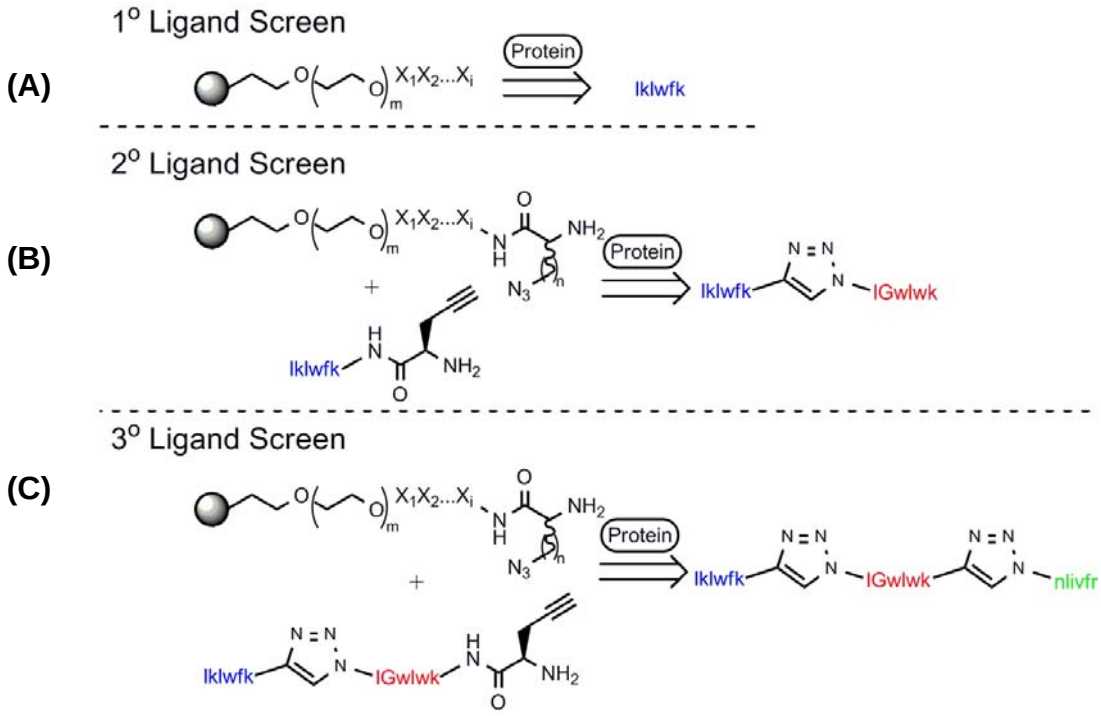


Figure 2.1. A schematic representation of a method for preparing a multi-ligand capture agent. **(A)** In the first step, a plurality of candidate oligopeptides in an OBOC library is contacted with a labeled target to identify an anchor (1°) ligand. **(B)** In the second step, a modified 1° ligand from the first step is contacted with the same OBOC library now appended with an azide linker to identify a secondary (2°) ligand. A biligand, formed by the 1° ligand of the first step and the 2° ligand, can be obtained. **(C)** In the third step, the screen is repeated by employing the biligand formed from the second step as the new primary ligand to allow identification of higher-order multi-ligands.

solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received, unless otherwise noted.

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.

Bovine carbonic anhydrase II (bCAII, C2522), from bovine erythrocytes, lyophilized powder, was obtained (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 a 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-Alexa Fluor 647) was characterized by UV-Vis and mass spectrometry.

Human carbonic anhydrase II (hCAII, C6165), from human erythrocytes, lyophilized powder, was obtained (Sigma-Aldrich; St. Louis, MO) and used in affinity and specificity studies. Both bCAII and hCAII were tested by SDS gel electrophoresis, and confirmed to display a single band corresponding to 29,000 Da.

2.2.2 Artificial Amino Acids

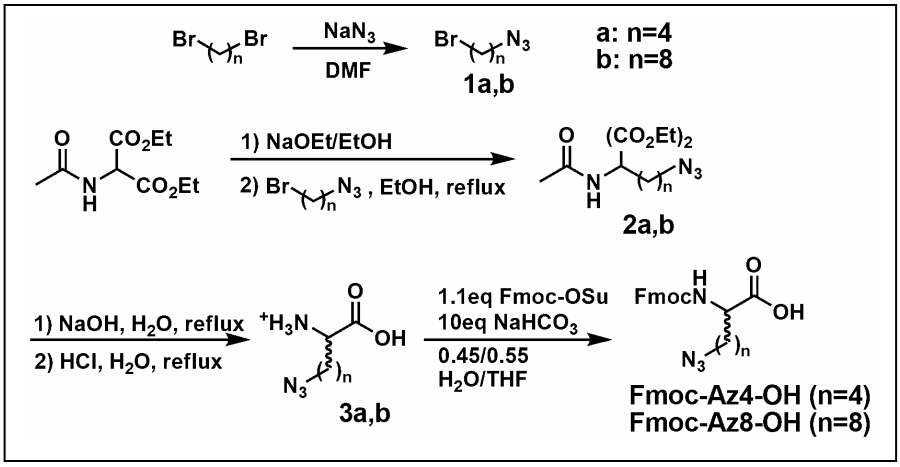
Fmoc-D-propargylglycine (Fmoc-D-Pra-OH) was acquired (Chem-Impex International; Wood Dale, IL) and used as the acetylene handle for construction of

ligands. Azide-containing amino acids Fmoc-Az4-OH (and intermediates 1a-3a) and Fmoc-Az8-OH (and intermediates 1b-3b) were synthesized using a modification of literature protocols (Scheme 2.1).[35–37]

Azidobutylbromide (1a). To a solution of 1,4-dibromobutane (123 mmol), sodium azide (61.5 mmol) was added and stirred overnight in *N,N′*-dimethylformamide (DMF) at 50 °C. The reaction was diluted with ethyl acetate, and the organic layer was washed with water, then brine, and then dried over $MgSO_4$. The crude residue was purified by silica gel chromatography (100% hexanes) to give a product (80%) as a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$): 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-dibromooctane was used as the starting material. ^{1}H NMR (300 MHz, $CDCl_3$): 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.[35] 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 h at room temperature and refluxed for 6 h 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. $NaHCO_3$, water, and brine, and were dried over $MgSO_4$ and then concentrated. Silica gel chromatography (Hex:EtOAc = 1:1) gave a product (63%) as a clear, viscous oil. ^{1}H NMR (300 MHz,



Scheme 2.1. Artificial amino acid synthesis.

$CDCl_3$): 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-(8-azidooctyl)malonate (2b). Similar synthetic protocol as **2a** was adopted, only with azidooctylbromide **1b** serving as the starting material. ^{1}H NMR (300 MHz, $CDCl_3$): 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 methods,[36] the diester **2a** (2.8 mmol) in 25 mL of 10% NaOH solution was heated to reflux for 4 h. 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 h. The solvent was reduced and extraction with MeOH afforded amino acid **3a** as the hydrochloride salt (85%). ^{1}H NMR (300 MHz, CD_3OD): 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. ^{1}H 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.[37] 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). ^{1}H 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. ^{1}H NMR (300 MHz, $CDCl_3$): 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.

2.2.3 OBOC Oligopeptide 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-NH_2, 0.31 mmol/g, 2.86 × 10^6 beads/g).[12–14] Non-natural D-stereoisomers (denoted by lowercase one-letter amino acid code) were used at every possible position in the peptide sequence to infer intrinsic biochemical stability. At least a 5-fold excess of beads was utilized in each library synthesis to ensure adequate representation of each library element. A standard solid-phase peptide synthesis method with Fmoc chemistry was used.[38] All wash,

deprotection, and coupling steps were facilitated by 180-degree shaking of the resin. The resin was pre-swelled in NMP in a plastic fritted reaction vessel, and was separated into multiple aliquots. Each aliquot was reacted with 2-fold molar excess (relative to resin) of a single N^{α}-Fmoc-amino acid. Amide coupling was initiated by addition of a 2-fold molar excess of HATU and a 6-fold molar excess of DIEA.[39] 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" was performed with a third set of coupling reagents and N^{α}-Fmoc-amino acids (Table 2.1, Libraries D, E, F, and G). Following coupling, the aliquots were thoroughly washed (5 × NMP), mixed together into a single vessel, and deprotected with 20% piperidine in NMP (30 min). The resin was thoroughly washed (5 × NMP), dried (5 × DCM), and re-divided into multiple equal-mass aliquots for the next cycle of coupling. The procedures were repeated until the desired length of peptide was attained.

The amino acid side chain protecting groups were then removed by incubation in trifluoroacetic acid (95%), water (5%), and triethylsilane (2-fold molar excess per protected side chain) for 2 h at 25 °C. The library resin was then neutralized with DMF, and washed thoroughly with DMF (5 ×), water (5 ×), methanol (MeOH, 5 ×), and methylene chloride (DCM, 5 ×),[40] and then dried under vacuum and stored in phosphate-buffered saline [PBS (pH 7.4)] + 0.05% NaN_3 at 25 °C.

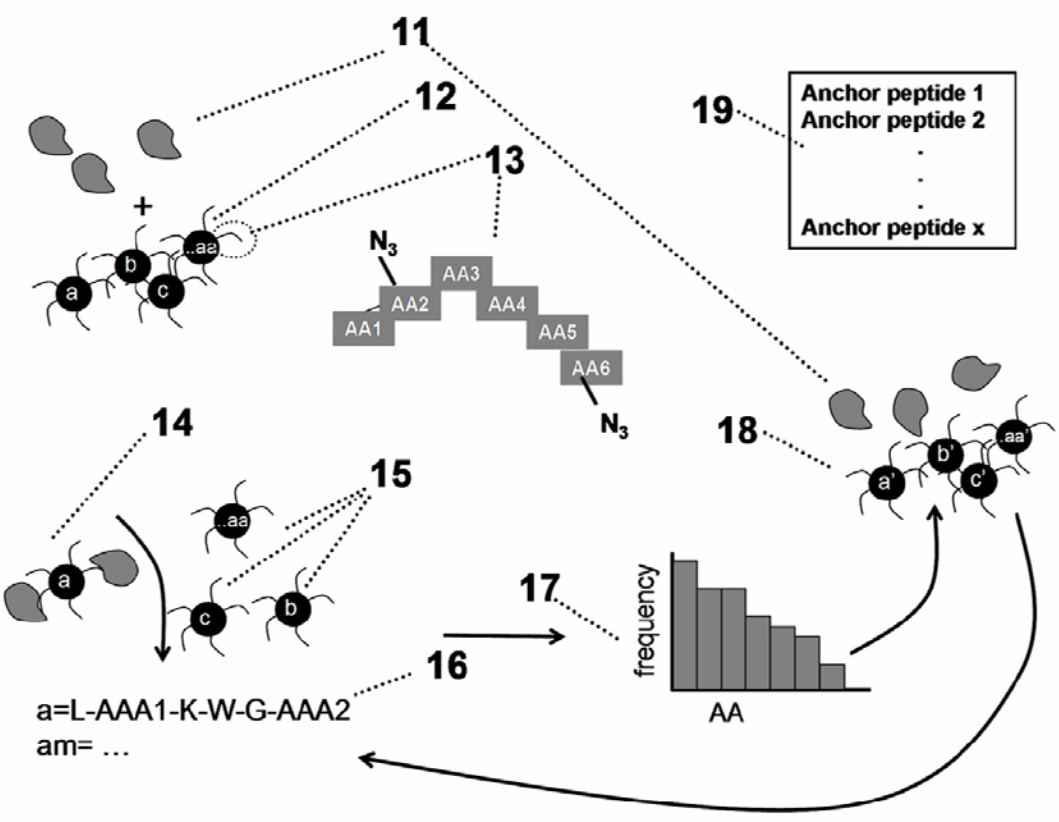
2.2.4 Screening Procedures for Anchor Ligand

A method for identifying an anchor (1°) ligand is schematically illustrated in Scheme 2.2. In particular, in the illustration of Scheme 2.2, a fluorescently labeled protein of interest (11) is screened against an OBOC library of peptides (12). Each bead

Table 2.1. Libraries used in selecting a triligand capture agent for bCAII.[a]

	Formula	Components	# of unique sequences
A	$x_1x_2x_3x_4x_5$	x_i = 19 D-amino acids (no D-Cys)	2,476,099
B	$x_1x_2x_3x_4x_5x_6$	x_i = r, k, l, w, f, h, y	117,649
C	**Az$_n$**-$x_2x_3x_4x_5x_6$-**Az$_n$**	x_i = 19 D-amino acids (no D-Cys) **Az$_n$** = 1/3 **Az4**, 1/3 **Az8**, 1/3 nothing	22,284,891
D	$x_1x_2x_3x_4x_5x_6$-**Tz1**-kfwlkl	x_i = k, l, w, f, i, G, v	117,649
Tz1 = triazole formed between **Az4** (on terminal k) and **D-Pra** (on x_6)			
E	$x_7x_6x_5x_4x_3x_2$-**Tz2**-kwlwGl-**Tz1**-kfwlkl	x_i = d, r, s, w, G, f, l	117,649
Tz1 = triazole formed between **Az4** (on terminal k) and **D-Pra** (on l) **Tz2** = triazole formed between **Az4** (on terminal x_2) and **D-Pra** (on k)			
F	**Az4**-$x_2x_3x_4x_5x_6x_7$	x_2 = r, n, l, i; x_3 = w, f, l, i; x_4 = r, w, f, l, i; x_5 = w, f, v, l; x_6 = r, w, f, l, k; x_7 = f, r	3200
G	$x_7x_6x_5x_4x_3x_2$-**Tz2**-kwlwGl-**Tz1**-kfwlkl		3200

[a] Randomized positions are denoted by x_i (for D-amino acids) and **Az$_n$** (for azide-containing artificial amino acids).



Scheme 2.2. Selection of anchor ligands by OBOC screen.

contains a unique peptide (13) comprised of non-natural amino acids (D-stereoisomers) or artificial amino acids (displaying azide or acetylene functionalities). The protein (11) and the library (12) are incubated for a period of time at a particular protein concentration (Table 2.2, Screen **An1**), and the "hit" beads (14) are identified by their fluorescence using a GenePix 4200 array scanner (λ_{ex} = 635 nm). Typically 0.1% or less of the beads are identified as hit beads, and are separated manually from the non-hit beads by micropipette (15). The protein is removed from the beads by incubation with 7.5 M guanidine hydrochloride (GuHCl, pH 2.0) for 1 h, and the peptides on single hit beads are sequenced using Edman degradation[41] (Procise cLC Sequencing System, Applied BioSystems, Foster City, CA; see Appendix C) or MALDI-TOF/TOF mass spectrometry.[42]

Once the hit peptide sequences (16) are identified, a histogram (17) that correlates the amino acid frequency vs. amino acid identity is prepared. A second, more focused library (18) that uses those most commonly identified amino acids can then be prepared and re-screened against the protein (11) (Table 2.2, Screens **An2a** and **An2b**). This focused library can contain slightly longer peptides, and the screening process can involve a lower concentration of the protein (11). This process can then be repeated until the desired affinity of peptide anchor ligand (19) is achieved. The affinity of the peptide anchor ligand will depend upon the number of amino acids in the peptide, and the three-dimensional structure of the peptide, among other factors. Affinities in the order of 10^{-4}–10^{-6} M are typically achievable.

2.2.5 In Situ Click Screening Procedures for Biligand

Identification of the secondary (2°) ligand and formation of a biligand then can

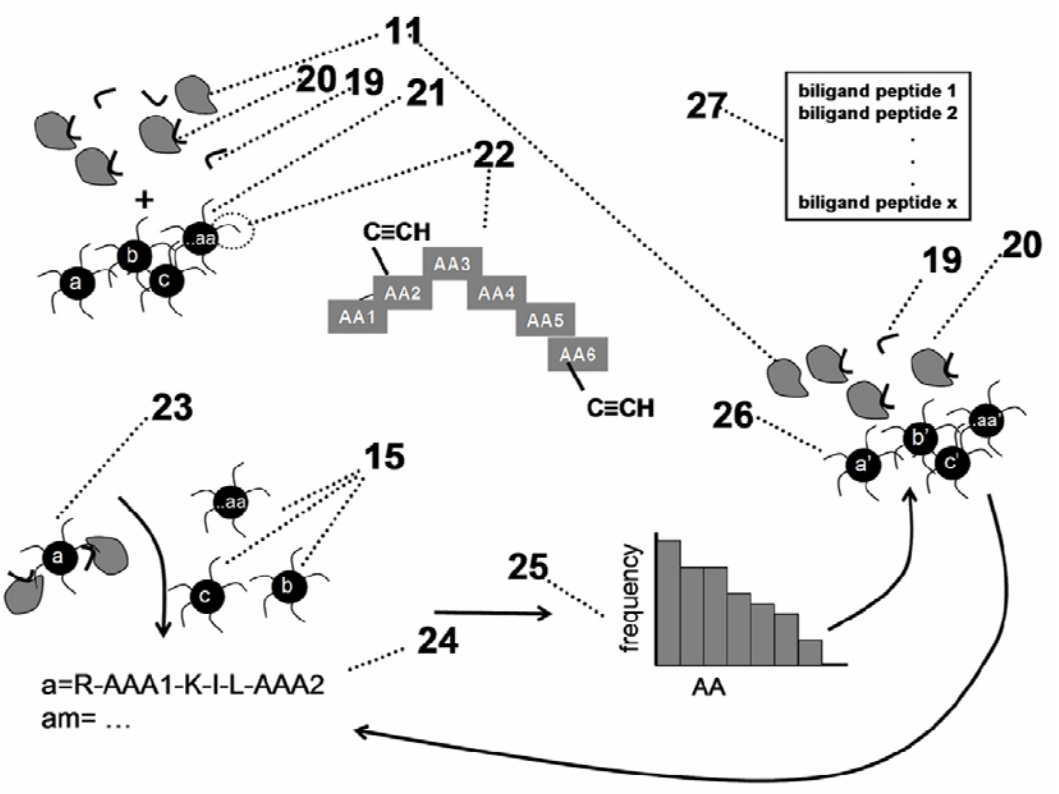
Table 2.2. Screening summary.[a]

Screen	Library	[bCAII-AF647]	Time(h)	% hit beads	Buffer	Other components
An1	**A**	100 nM	1 h	0.02%	PBS	N/A
An2a	**B**	50 nM	1 h	0.09%	PBS	N/A
An2b	**B**	8 nM	24 h	2 hits	PBS	N/A
Bi1	**C**	50 nM	2 h; 37° C (no beads) + 48 h; 37° C	0.007%	PBS + 1% DMSO (v/v)	100 µM of **lklwfk-(D-Pra)**
Bi2a	**D**	50 nM	17 h	0.07%	PBSTBNaN_3	N/A
Bi2b	**D**	10 nM	17 h	0.008%	PBSTBNaN_3	N/A
Tri1	**C**	10 nM	2 h (no beads) +15 h	0.007%	PBSTBNaN_3 + 1% DMSO (v/v)	100 µM of **(D-Pra)-kwlwGl-Tz1-kfwlkl**
Tri2	**E**	10 nM	17 h	0.008%	PBSTBNaN_3	N/A
TriX	**A**	10 nM	17 h	0.007%	PBSTBNaN_3 + 1% DMSO (v/v)	100 µM of **(D-Pra)-kwlwGl-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)-kwlwGl-Tz1-kfwlkl**
Tri4	**G**	0.25 nM	18 h	0.005% -0.01%	PBSTBNaN_3	N/A

[a] All screens were conducted at pH = 7.4 and T = 25 °C, unless otherwise noted.

be performed according to the method schematically illustrated in Scheme 2.3. A typical screen begins with incubation of a library (12) in PBS (pH 7.4) + 0.1% Tween 20 + 0.1% bovine serum albumin (BSA) + 0.05% NaN_3 (PBSTBNaN_3) for 1 h, with shaking, to block non-specific protein binding.[34] One of the anchor ligands (19) from the screening procedures in Section 2.2.4 is added to the protein of interest (11) at a concentration that is dependent upon its binding affinity. It is desirable that this concentration of anchor ligand (19) is at least two orders of magnitude higher than the K_D. All in situ click chemistry screens (Scheme 2.3) started with an initial 2 h pre-incubation of bCAII-Alexa Fluor 647 with the anchor ligand (20), which was followed by addition of the OBOC library of 2° ligands (21) and continuation of the screen (Table 2.2, Screen **Bi1**). This OBOC oligopeptide library is constructed similarly to the candidate library for anchor ligands (12), except that the azide components (22) are replaced by acetylene functionalities. Following in situ screening, beads are washed with 3 × 5 mL PBSTBNaN_3, 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and then 6 × 5 mL PBS (pH 7.4).

As with the screening procedures in Section 2.2.4, the hit beads (23) are identified by their fluorescence and separated from the non-hit beads (15). While the hit beads can contain a certain amount of biligand formed by the protein-catalyzed coupling of bead-bound 2° ligand with anchor ligand, the majority of the peptide on the hit beads (23) is actually 2° ligand that did not participate in the "click" reaction. The protein target and excess anchor ligand are removed from the bead by incubation with 7.5 M GuHCl (pH 2.0) for 1 h, the peptide (24) on the bead is sequenced using standard methods, and a histogram (25) that correlates amino acid frequency vs. amino acid identity is constructed. A second, more focused library (26) that utilizes those most



Scheme 2.3. Selection of biligand by in situ click/OBOC screen. Similarly, triligands (27) may be selected by iteration of this screening method, utilizing a biligand as the anchor unit (19).

commonly identified amino acids may then be prepared and re-screened against the protein (11). Once again, the hit beads are identified via peptide sequencing (24). This second library of 2° ligands can contain slightly longer peptides, and the screening process can involve a lower concentration of the protein (11).

2.2.6 In Situ Click Screening Procedures for Higher-Order Multi-ligands

In situ click screening procedures operate similarly to Scheme 2.3 for identification of higher-order multi-ligands such as the triligand in Figure 2.1. The in situ click/OBOC screen for this triligand (Table 2.2, Screens **Tri1** and **Tri3**) contained an initial 2 h pre-incubation of bCAII-Alexa Fluor 647 with biligand anchor, which was followed by addition of the OBOC library of 3° ligands and continuation of the screen. As a negative control, screen **TriX** was performed with an azide-free OBOC library of 3° ligands.

2.2.7 Validation of In Situ Click/OBOC Multi-ligand Screening Procedures

Binary component screen for in situ biligand. Stock solutions of 2° ligand (azide, Az4-kiwiG, 13.1 mM) and anchor ligand (acetylene, lklwfk-(D-Pra), 2.1 mM) were prepared in DMSO. Stock solutions of bCAII and bovine serum albumin (BSA) were prepared in PBS (pH 7.4). Each reaction contained 394 μM azide, 65 μM alkyne, and 36 μM protein in 100 μL PBS (pH 7.4) + 6% DMSO (v/v). Reactions proceeded for 48 h at 37 °C, followed by 5 days at 25 °C. Reactions were quenched with 100 μL of 7.5 M GuHCl (pH 2.0), and proteins were subsequently removed by centrifugal filtration (Microcon YM-3, Millipore, Billerica, MA).

The formation of in situ biligands was identified by MALDI-MS. Control experiments were conducted (1) in the absence of bCAII, and (2) replacing bCAII with BSA, to verify that the click reaction between the azide and alkyne is specific to the bCAII protein target. A third control, performed in the absence of protein, represents the slow thermally driven reaction between solutions of azide and alkyne.

On-bead biligand screen. Synthesis of Library D was achieved on bead via the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC),[43–45] as described in Section 2.2.9. Screens **Bi2a** and **Bi2b** (Table 2.2) were conducted using Library D following the general OBOC screening protocol described in Section 2.2.4. After initial blocking with PBSTBNaN_3 for 1 h, 10 nM to 50 nM bCAII-Alexa647 in PBSTBNaN_3 was incubated with the library for 17 h at 25 °C, with shaking. The screened beads were washed with 3 × 5 mL PBSTBNaN_3, then 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and finally 6 × 5 mL PBS (pH 7.4). The beads were imaged for fluorescence, and the hits were selected by micropipette. After washing the hits to remove bound protein [7.5 M GuHCl (pH 2.0)], their sequences were determined by Edman degradation.

On-bead triligand screen. Synthesis of Libraries E and G was achieved on bead via the CuAAC, as described in Section 2.2.9. Screens **Tri2** and **Tri4** (Table 2.2) were conducted following the general OBOC screening protocol described in Section 2.2.4, using <10 nM bCAII-Alexa647 and fluorescent detection of hits.

2.2.8 Bulk Peptide Synthesis

Bulk synthesis of hit peptide sequences was performed on either Fmoc-Rink amide MBHA (50 μm, 0.67 mmol/g, AnaSpec) or Biotin-PEG-NovaTag resin (0.48 mmol/g; Novabiochem), on a typical resin scale of 0.2 g per sequence. Crude peptides

were precipitated with ether, and then purified to >95% by HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector, Fullerton, CA) on a C_{18} reversed-phase semi-preparative column (Phenomenex Luna 10 μm, 250 × 10 mm). The pure peptides were used for affinity measurements, in situ click/OBOC screens, and binding assays. Hit peptide sequences were also re-synthesized on TentaGel S-NH_2 on a similar resin scale, and used for on-bead binding assays.

Installation of polyethylene glycol linkers $(EG)_n$ was achieved by Fmoc-NH-$(PEG)_5$-COOH (22 atoms) (Novabiochem) via SPPS with standard HATU/DIEA coupling. N-terminal biotin labeling of certain sequences was achieved via SPPS with standard HATU/DIEA coupling and overnight reaction.

It should be noted that the protein-templated in situ click reaction may yield product regioisomers that are either *anti* (1,4), *syn* (1,5), or a mixture of the two geometries. Although we have not yet determined which regioisomers of the in situ click products were formed, the authentic multi-ligands synthesized by CuAAC to test affinity and specificity were definitely the 1,4-triazole (see Chapter 3).

All anchor ligands, biligands, and triligands were prepared in bulk by solid-phase synthesis, purified by HPLC, and analyzed by mass spectrometry prior to further study. Their characterization is as follows:

lklwfk-(D-Pra) (Figure 2.2A). MALDI-MS of the purified 1° ligand gave peaks at *m/z* 928.7 for $[M + H]^+$ and 950.7 for $[M + Na]^+$.

Az4-kiwiG (Figure 2.2B). ESI-MS of the purified 2° ligand gave peaks at *m/z* 385.2 for $[M + 2H]^{2+}$ and 769.5 for $[M + H]^+$.

lklwfk-Tz1-kiwiG (Figure 2.2C). MALDI-MS of the purified biligand gave a peak at *m/z* 1808.4 for $[M + H]^+$.

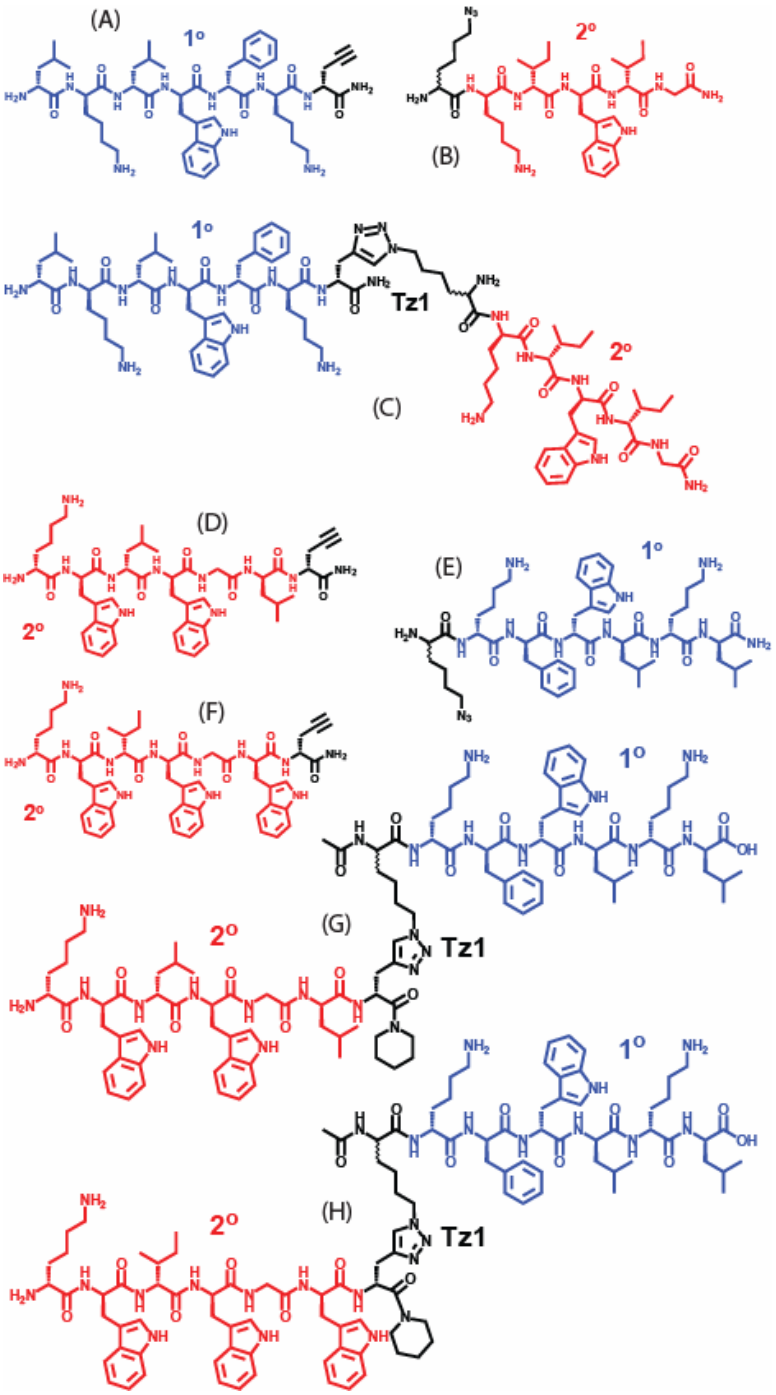


Figure 2.2. Structures of representative 1° ligands **(A, E)**, 2° ligands **(B, D, F)**, and biligands **(C, G, H)** which were isolated as moderate affinity binders of bCAII.

Az4-kfwlkl (Figure 2.2E). ESI-MS of the purified 1° ligand gave peaks at *m/z* 329.9 for $[M + 3H]^{3+}$, 494.3 for $[M + 2H]^{2+}$, and 987.6 for $[M + H]^{+}$.

kwlwGl-(D-Pra) (Figure 2.2D). MALDI-MS of the purified 2° ligand gave peaks at *m/z* 897.0 for $[M + H]^{+}$, 919.0 for $[M + Na]^{+}$, and 935.0 for $[M + K]^{+}$.

kwiwGw-(D-Pra) (Figure 2.2F). MALDI-MS of the purified 2° ligand gave peaks at *m/z* 970.1 for $[M + H]^{+}$ and 992.1 for $[M + Na]^{+}$.

kwlwGl-Tz1-kfwlkl (Figure 2.2G). MALDI-MS of the purified biligand gave a peak at *m/z* 1993.6 for $[M + H]^{+}$.

kwiwGw-Tz1-kfwlkl (Figure 2.2H). MALDI-MS of the purified biligand gave peaks at *m/z* 2066.9 for $[M + H]^{+}$ and 2088.7 for $[M + Na]^{+}$.

(D-Pra)-kwlwGl-Tz1-kfwlkl (Figure 2.3A). ESI-MS of the purified biligand anchor gave peaks at *m/z* 711.1 for $[M + 3H]^{3+}$ and 1066.1 for $[M + 2H]^{2+}$.

(D-Pra)-k(Boc)w(Boc)lw(Boc)Gl-Tz1-k(Boc)fw(Boc)lk(Boc)l (Figure 2.3B). ESI-MS of the biligand anchor as the fully protected peptide gave peaks at *m/z* 1365.3 for $[M + 2H]^{2+}$ and 2731.6 for $[M + H]^{+}$.

Biotin-$(EG)_5$-(D-Pra)-kwlwGl-Tz1-kfwlkl (Figure 2.3C). MALDI-MS of the purified biotinylated biligand anchor gave peaks at *m/z* 1325.9 for $[M + 2H]^{2+}$ (minor) and 2649.9 for $[M + H]^{+}$ (major).

Az4-nlivfr (Figure 2.4A). MALDI-MS of the purified 3° ligand gave a peak at *m/z* 914.5 for $[M + H]^{+}$.

Az4-nlivfr-$(EG)_3$-Biotin (Figure 2.4B). MALDI-MS of the purified biotinylated 3° ligand gave a peak at *m/z* 1343.8 for $[M + H]^{+}$.

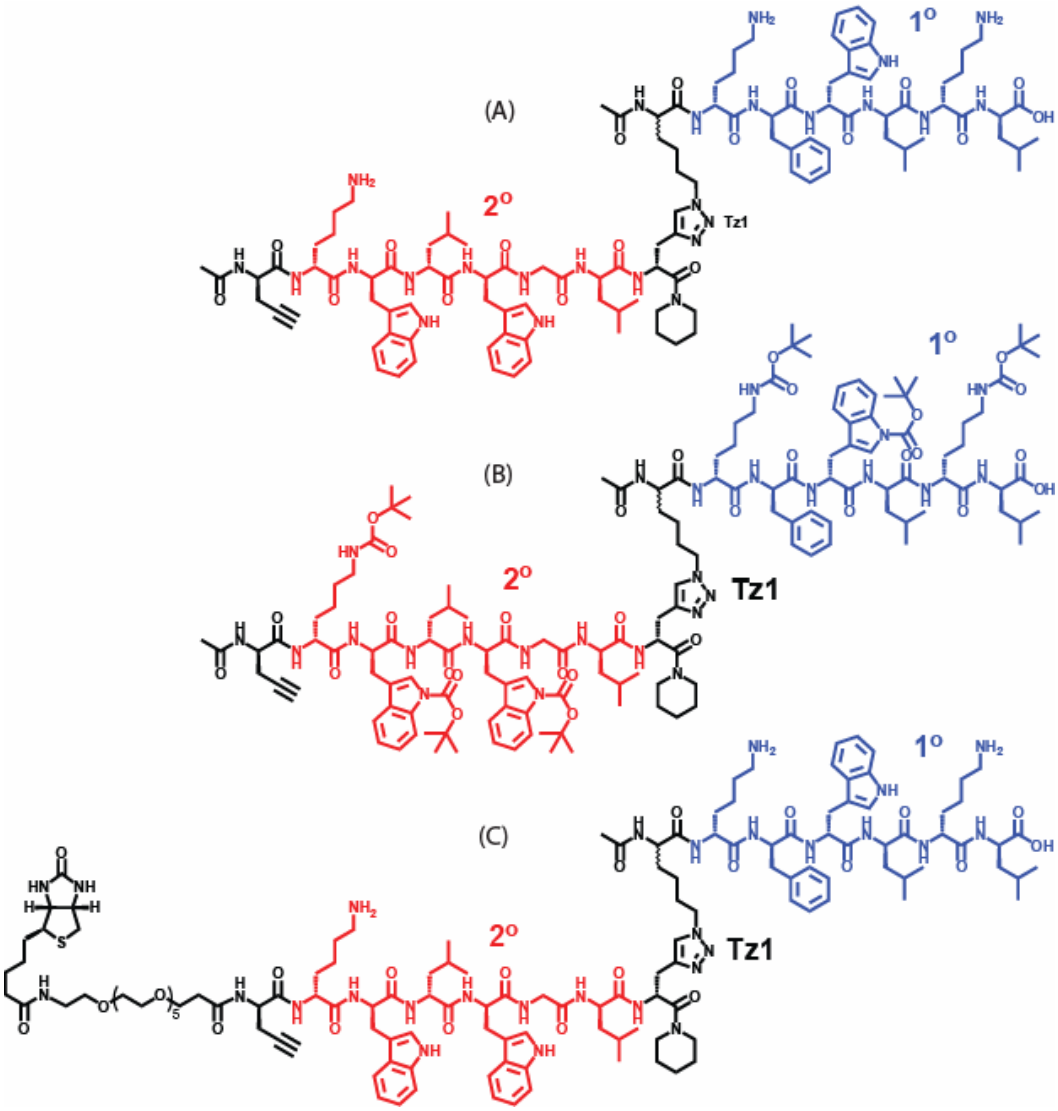


Figure 2.3. **(A)** Biligand anchor, employed for in situ click/OBOC screens. **(B)** Fully protected biligand anchor, used in bulk triligand synthesis. **(C)** Biotinylated biligand anchor, used in specificity experiments and assays for detecting on-bead, protein-templated multi-ligand.

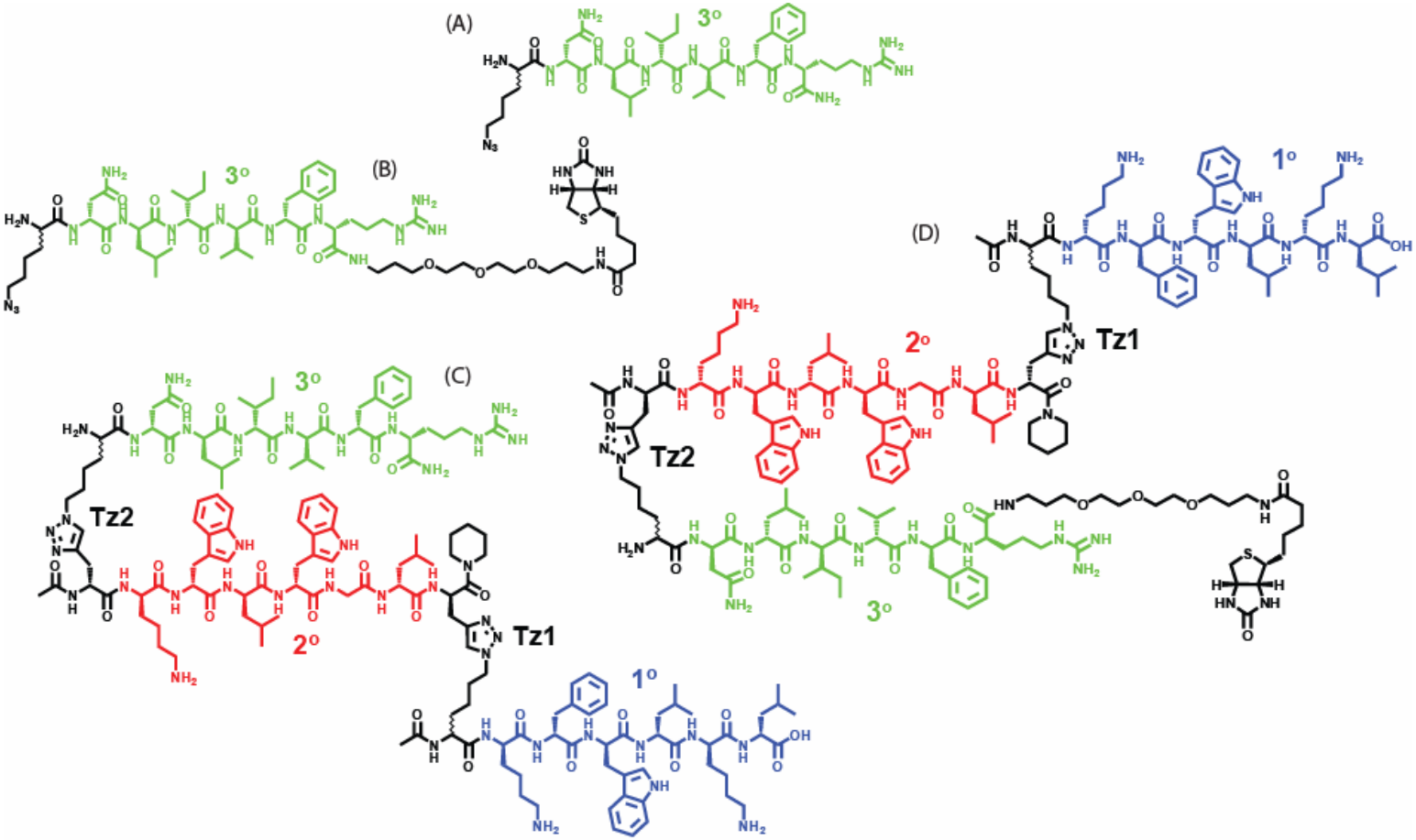


Figure 2.4. **(A, B)** Tertiary (3°) ligands. **(C, D)** Triligand capture agent, where the 1° ligand is colored in blue, the 2° ligand in red, and the 3° ligand in light green. The connections between the ligands are formed by 1,2,3-triazoles (Tz1 and Tz2).

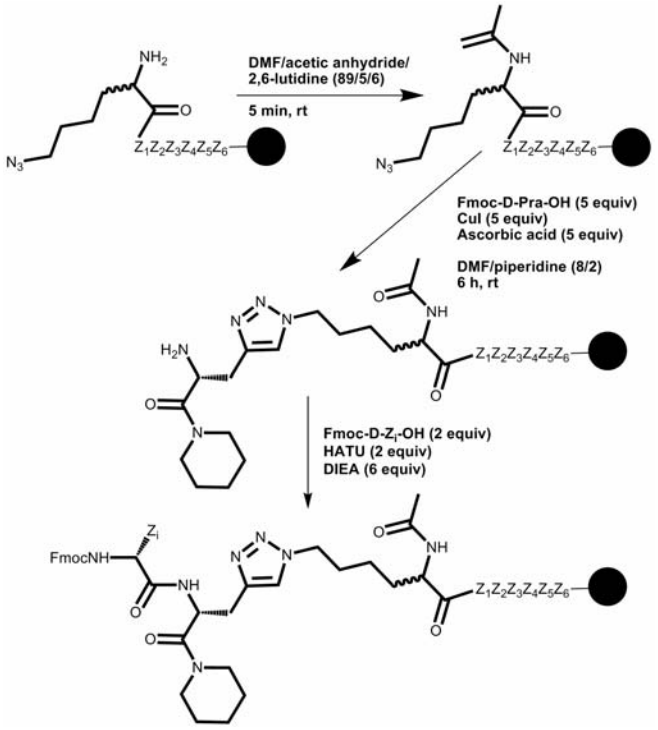
rfviln-Tz2-kwlwGl-Tz1-kfwlkl (Figure 2.4C). MALDI-MS of the purified triligand gave peaks at *m/z* 1522.9 for $[M + 2H]^{2+}$ (minor) and 3045.7 for $[M + H]^{+}$ (major).

rfviln-Tz2-kwlwGl-Tz1-kfwlkl-(EG)$_3$-Biotin (Figure 2.4D). MALDI-MS of the purified biotinylated triligand gave peaks at *m/z* 1737.5 for $[M + 2H]^{2+}$ (minor) and 3472.0 for $[M + H]^{+}$ (major).

2.2.9 On-Bead Biligand and Triligand Synthesis

For preparing Libraries D, E, and G (Table 2.1), as well as for bulk synthesis of biligand and triligand candidates, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)[43–45] was carried out on bead, with 4 general steps: (1) anchor ligand synthesis, (2) acetylation, (3) click reaction, and (4) addition of 2° ligand sequence. Scheme 2.4 illustrates the acetylation and click reactions for a 6-mer peptide (Z = any amino acid). The fully protected TentaGel S-NH_2 bead-bound anchor ligand (0.420 g, 0.13 mmol) was capped by a solution of acetic anhydride (1 mmol) in 2,6-lutidine and DMF.[46] The acetylated peptide was reacted with Fmoc-D-Pra-OH (0.218 g, 0.65 mmol) in the presence of CuI (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 h.[47] The resin was washed with 5 × 5 mL $Et_2NCSSNa•3H_2O$ (sodium diethyldithiocarbamate trihydrate, 1% w/v), containing 1% DIEA (v/v) in DMF to remove the coordinated copper from click reaction.[48]

The biligand anchor (D-Pra)-kwlwGl-Tz1-kfwlkl was synthesized on 2-chlorotrityl chloride (1.6 mmol/g) resin (Anaspec, San Jose, CA) using Scheme 2.4. The biligand anchor was released either as the fully deprotected peptide by cleavage with



Scheme 2.4. Acetylation and click reactions for a 6-mer peptide (Z = any amino acid) by solid-phase synthesis. Peptide synthesis may continue via the Fmoc-protected primary amine of Z_i to generate a linear multi-ligand capture agent.

95:5 TFA:water (+ 2 mol equiv TES per side chain protecting group), or as the fully protected peptide by cleavage with 99:1 DCM:TFA.[49] To facilitate the on-bead click reaction, it is noted that the 1° ligand was synthesized here as Az4-kfwlkl (displaying N-terminal Az_n modification), and to this sequence was coupled D-Pra and the 2° ligand to produce the linear biligand.

Triligands were synthesized by click reaction between the fully protected biligand anchor (D-Pra)-kwlwGl-Tz1-kfwlkl (0.274 g, 0.1 mmol, >95% HPLC) and bead-bound 3° ligand Az4-nlivfr (0.1 g, 0.03 mmol) using CuI (0.021 g, 0.1 mmol) and L-ascorbic acid (0.020 g, 0.1 mmol) in DMF/piperidine (8/2).

2.3 RESULTS AND DISCUSSION

2.3.1 Screening for Anchor (1°) Ligand against bCAII

The anchor (1°) ligand was selected from a two-generation screen (**An1, An2a, An2b**) as summarized in Table 2.2. For the first screen (**An1**), following Edman sequencing of hits, a histogram correlating the (position-independent) frequency of amino acid occurrence vs. amino acid identity (Figure 2.5A) suggested the importance of basic/charged (k, r) and aromatic residues (y, f, w) for an 1° ligand for bCAII. A second, more focused library (Library B) of ~10^5 D-peptide compounds was constructed from the most commonly occurring amino acids, as identified from screen **An1**, but expanded into a 6-mer peptide, and screened under 50 nM bCAII (**An2a**) and 8 nM bCAII (**An2b**) conditions. Figure 2.5B illustrates the results of these second-generation 1° ligand screens. The more stringent screen (**An2b**) yielded two hits, hlyflr and lklwfk. From these two candidates, one peptide (lklwfk) was arbitrarily chosen as the starting point for

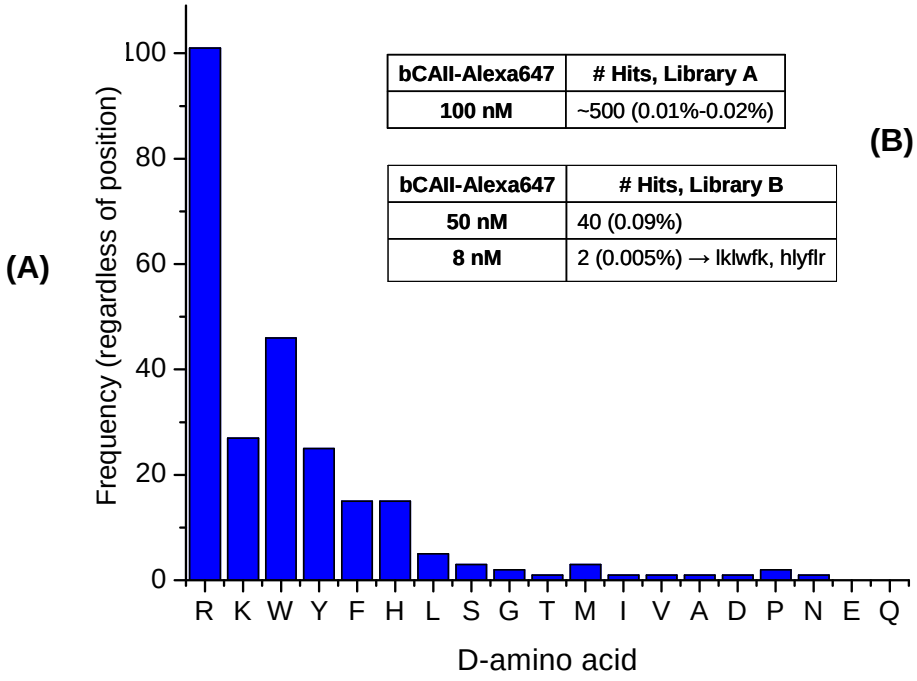


Figure 2.5. Results of selecting a primary or anchor ligand of bCAII. **(A)** Diagram plotting frequency vs. D-amino acid for 51 hit sequences isolated from screening Library A (first-generation anchor ligand screen). **(B)** Hit rates for Library A and B (second-generation anchor ligand) screens, leading to the selection of two anchor ligands (lklwfk and hlyflr).

a 1° ligand for use in multi-ligand screens. A complete list of 1° ligand hit sequences from OBOC selections can be found in Appendix B.

The peptide lklwfk was then functionalized with either an azide ($-N_3$) or acetylene (-C≡C-H) terminus, fluoresceinated, and produced in bulk quantities for affinity measurements by fluorescence polarization. Chapter 3 will describe that one such 1° ligand lklwfk-(D-Pra) displays an equilibrium dissociation constant of $K_D \approx$ 500 μM for its interaction with bCAII. This value is an estimate, since weak affinities are hard to quantify. Surface plasmon resonance (SPR) was also employed to measure the affinity of bCAII for Az4-kfwlkl and lklwfk-(D-Pra) as 1° ligands, and a similarly low affinity was recorded (at least >10 μM, see Chapter 3).

2.3.2 Identification of Secondary (2°) Ligands: Biligand Screens

A biligand is constructed of a 2° ligand that is covalently attached, via a 1,2,3-triazole linkage, to the 1° ligand. As illustrated by Figure 2.6, secondary (2°) ligands were identified by two complementary approaches: (1) in situ click/OBOC biligand screens; (2) on-bead biligand screens. In the first approach (Figure 2.6A), the protein acts as a catalyst for the in situ click assembly of the biligand on bead. During this screen, the 1° ligand and protein coexist in solution, while the cognate library of 2° ligands is on bead. In the second approach (Figure 2.6B), the 1° ligand is covalently coupled to the on-bead library of 2° ligands via the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). Such a library of pre-assembled biligands is screened against the protein target to discover 2° ligand candidates. The protein target is not a catalyst in this approach; this screen was used as a validation tool for comparison against the in situ click/OBOC screens.

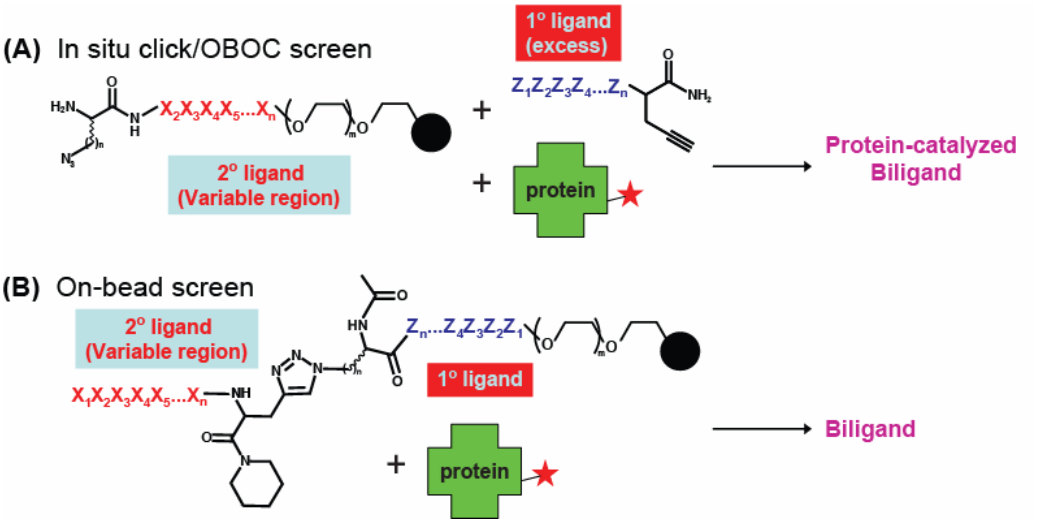
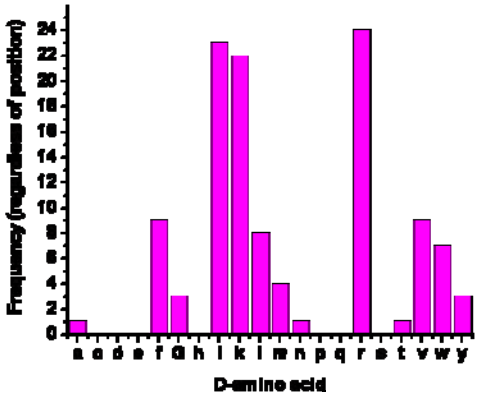


Figure 2.6. A schematic illustrating the two types of biligand screen. **(A)** In situ screen for a secondary (2°) ligand. **(B)** The on-bead screen for a secondary (2°) ligand was utilized as confirmation that the in situ screen was performing its designed function.

In situ click/OBOC biligand screen. Based on the protein-catalyzed in situ click reactions reported by the Sharpless group[17–21] only those 2° ligands that bind with bCAII *and* are in close proximity with the 1° ligand, *and* are in the correct orientation, will react to form the 1,2,3-triazole product. Figure 2.7A illustrates the result of the first-generation in situ biligand screen **Bi1** against bCAII, which utilized 100 μM lklwfk-(D-Pra) as the 1° ligand and a comprehensive azide-modified Library C. From histogram and raw analysis of hits, a 2° ligand Az4-kiwiG emerged as the best candidate, since its inherent motif was repeated several times. Figure 2.7B shows an abbreviated list of the hit sequences isolated from screening Library C against 50 nM bCAII-Alexa647 (**Bi1**). A complete list of biligand hit sequences from the in situ click/OBOC screens can be found in Appendix B.

The very high sequence homology observed here was not witnessed for the 1° ligand screens, but is characteristic of all of the in situ biligand and triligand (see Section 2.3.3) screens discussed in this thesis. Note also that all of the peptides in Figure 2.7B contain at least one azide group, although, statistically, over one-third of the OBOC library does not contain azide groups at positions 1 or 7. The high sequence homology, coupled with the persistence of azide groups in the selected 2° ligands, provides strong circumstantial evidence that the in situ click/OBOC screen worked to produce a biligand.

On-bead biligand screen. On-bead biligand screens (**Bi2a** and **Bi2b**) were carried out utilizing a focused CuAAC biligand library (Library D) that was prepared based on the sequencing results from screen **Bi1**. All 2° ligand sequences obtained by screens **Bi2a** and **Bi2b** (Table 2.2) also display striking sequence homology. Several sequences were repeated more than once, including kwlwGl and kwiwGw. A residue-



(A)

	Az_n	x_2	x_3	x_4	x_5	x_6	Az_n
hit1	Az4	k	i	w	i	G	
hit2	Az8	r	l	w	v	G	Az4
hit3	Az8	r	r	r	k	r	Az8
hit4	Az4	l	l	v	i	k	Az4
hit5	Az4	m	i	l	i	k	
hit6	Az8	i	i	i	m	r	Az4
hit7	Az8	i	i	i	w	r	Az8
hit8	Az4	n	v	i	i	f	
hit9	Az4	i	f	l	v	k	Az8
hit10	Az4	k	i	w	i	G	Az8
hit11	Az4	r	r	k	f	r	Az8
hit12	Az4	r	v	w	l	r	Az8
hit13	Az8	k	y	r	r	r	Az4
hit14	Az8	r	r	k	v	w	Az4
hit15	Az4	i	f	l	v	k	Az8
hit16		k	r	k	r	f	Az4
hit17	Az8	k	i	w	i	k	

(B)

Figure 2.7. Identification of a 2° ligand by in situ click/OBOC screening against bCAII. **(A)** Diagram illustrating frequency (y-axis) of D-amino acids (x-axis) for 2° ligand candidates of a biligand isolated from screening Library C in the presence of the 1° ligand lklwfk-(D-Pra) and bCAII (screen **Bi1**). **(B)** Abbreviated list of the 2° ligand sequences isolated from the screen of Figure 2.7A.

by-residue histogram analysis (Figure 2.8) of all 2° ligand hits illustrates a strong preference for only one particular amino acid at each residue position—1 (k), 2 (w), 4 (w), and 5 (G)—in the 2° ligand component of the biligand capture agent. The distribution of D-amino acids illustrated in Figure 2.8, based on the analysis of 37 biligand hit beads, suggests this consensus sequence k-w-x_3-w-G (where x_3 = hydrophobic amino acid). A complete list of biligand hit sequences from the on-bead biligand screens can be found in Appendix B.

Several methods were employed to characterize the properties of biligand candidates. First, homology derived from the 2° ligand sequences from both the in situ click/OBOC and on-bead screens provided clues. Second, the three candidate biligands—kwlwGl-Tz1-kfwlkl, kwiwGw-Tz1-kfwlkl, and lklwfk-Tz1-kiwiG—were synthesized in bulk, and their binding affinities for bCAII were measured by SPR. Chapter 3 will describe that an equilibrium dissociation constant of $K_D \approx 3$ μM (bCAII) was determined for the best-binding biligand kwlwGl-Tz1-kfwlkl. This value is two orders of magnitude greater than the affinity for the 1° ligand alone, meeting our goal of affinity enhancement.

Binary component screen for in situ biligand. Finally, the in situ click/OBOC biligand screening method can be validated by a binary component screen. The 1° ligand lklwfk-(D-Pra) and 2° ligand Az4-kiwiG were combined in solution in the presence of protein target. The bCAII-catalyzed assembly of biligand is typically monitored by analytical methods such as LC/MS.[17–21] Here, MALDI-MS was used to monitor the extent of this reaction over several days (Figure 2.9). The bCAII-catalyzed biligand product is marked with an arrow. While it was encouraging that the

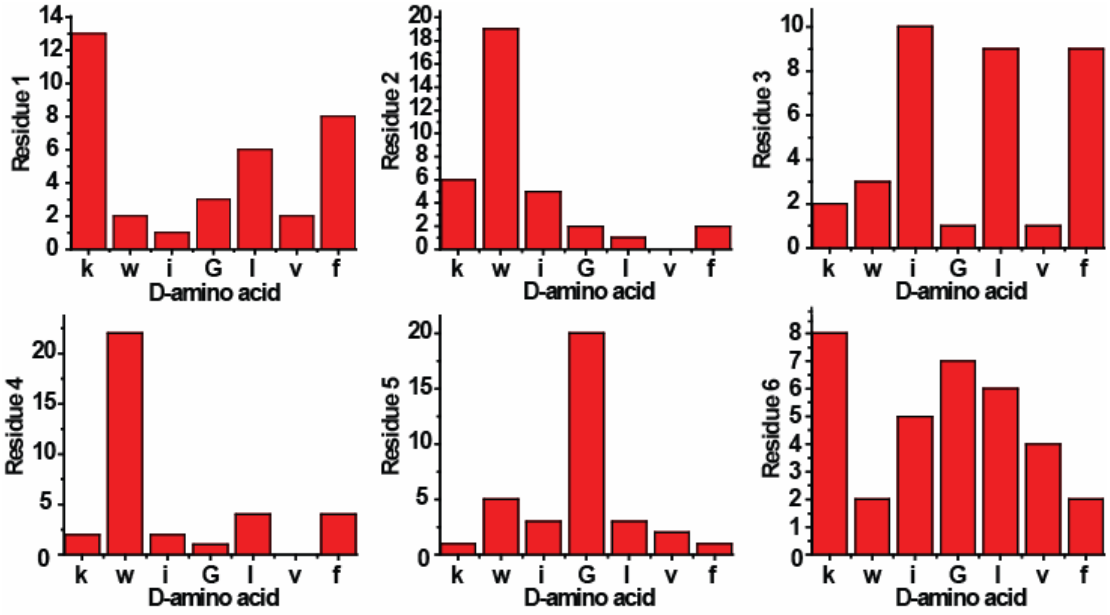


Figure 2.8. Distribution of D-amino acids found in positions 1 to 6 based on the analysis of 37 biligand hit beads from screens **Bi2a** and **Bi2b**.

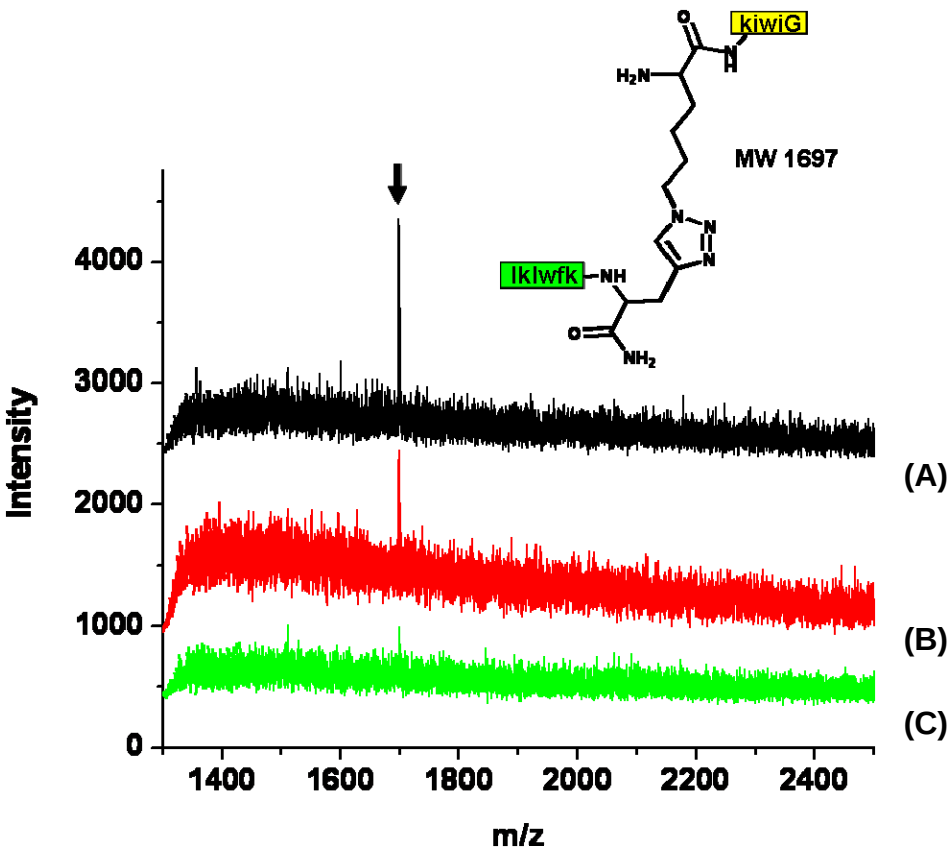


Figure 2.9. Binary component in situ click chemistry screen of 1° ligand lklwfk-(D-Pra) and 2° ligand Az4-kiwiG, illustrating bCAII-catalyzed formation of biligand (marked by arrow). **(A)** Bovine carbonic anhydrase II (bCAII). **(B)** Bovine serum albumin (BSA) control. **(C)** Buffer-only (no protein) control.

background reactions (BSA, no protein) were less, the MALDI-MS result did not provide quantitative measurement of the signal-to-noise ratio and overall yield for the bCAII-catalyzed reaction. Methods to quantitatively assess these were developed at the triligand level and are discussed in detail in Chapter 4.

2.3.3 Identification of Tertiary (3°) Ligands: Triligand Screens

Once a biligand is identified, that biligand can serve as the new anchor ligand, as illustrated in Figure 2.1, and *the same OBOC library* may be employed to identify a triligand. This process may be repeated with the same OBOC library until a multi-ligand with the desired affinity and specificity is reached. With the biligand (D-Pra)-kwlwGl-Tz1-kfwlkl serving as the anchor ligand, the Figure 2.1 in situ click/OBOC screen was repeated with Library C (Table 2.1) to identify a triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl (Figure 2.4C). It is crucial to note that the comprehensive Library C was applied again here, demonstrating the versatility of this type of general library.

For the case of the triligand screens, a histogram charting the position-dependent frequency of amino acids observed in the hit beads was generated. The consensus tertiary (3°) ligand was Az4-nlivfr (Figure 2.4A). Figure 2.10 shows position-dependent histograms for the first-generation in situ click/OBOC screens, for peptides (a) with and (c) without an azide-containing amino acid, to generate a triligand. For the in situ screen (**Tri1**, Figure 2.10A), one-third of the beads had no azide at the x_1 or x_7 positions, but interestingly, all hit beads contained an azide. On the other hand, the first- and second-generation on-bead CuAAC library screens (**Tri2** and **Tri4**, Figure 2.10B), where the 3° ligand variable region was coupled via CuAAC (Tz2) to the biligand, yielded independent validation of the in situ result. The final, consensus triligand sequence is

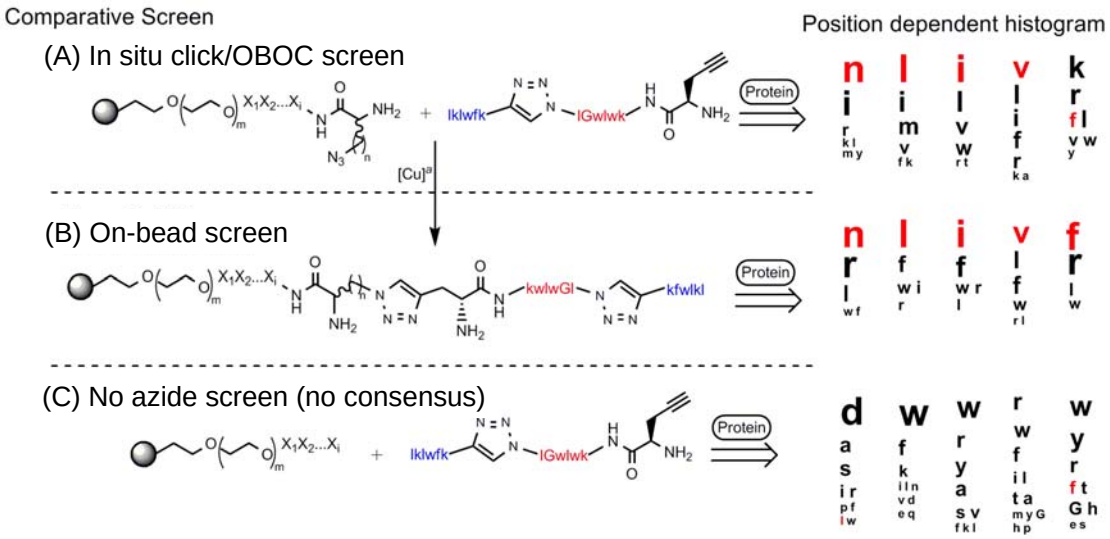


Figure 2.10. Method to validate protein-templated formation of a multi-ligand capture agent. Position-dependent histograms are illustrated for the first-generation in situ click/OBOC screens, for tertiary ligands **(A)** with and **(C)** without an azide-containing amino acid, to generate a triligand. First- and second-generation on-bead CuAAC library screens **(B)** independently confirmed the in situ result. The final consensus triligand sequence is indicated in red. Sample size: *in situ* = 25 hits; *in situ no azide* = 24 hits; *CuAAC library* = 21 hits.

[a] CuAAC conditions: Fully protected (D-Pra)-kwlwGl-Tz1-kfwlkl (0.274 g, 0.1 mmol, >98% HPLC), 0.03 mmol Library C, CuI (0.021 g, 0.1 mmol), and L-ascorbic acid (0.020 g, 0.1 mmol) were stirred in DMF/piperidine (8/2) overnight at 25 °C.

indicated by red font. Both this on-bead triligand screen, and the in situ click/OBOC screen, yielded *the same* consensus sequence and confirmed the equivalence of the two types of screens.

In the absence of azide (Figure 2.10C), the in situ triligand screens yielded completely different, and much less homologous, hit sequences. This phenomenon resulted from the prevention of triligand capture agent formation by click chemistry (control screen **TriX**). This screen illustrates the importance of the azide and acetylene functional groups, and their specific interaction on the surface of the target to produce a multi-ligand capture agent.

The consensus 3° ligand obtained by second-generation in situ screen **Tri3** resembles almost exactly the 3° ligand isolated by the first-generation screen (**Tri1**). Such sequence homology is unique to the in situ screens, which display target-guided selection. A complete list of triligand hit sequences from the in situ click/OBOC screens and on-bead triligand screens can be found in Appendix B.

The interaction between bCAII and triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl (Figure 2.4C) was measured by SPR. Chapter 3 will describe that equilibrium dissociation constants of $K_D \approx 45$ nM (hCAII) and $K_D \approx 64$ nM (bCAII) were determined, and represent a fifty-fold affinity enhancement from the protein/biligand interaction.

2.4 CONCLUSIONS

It was our goal to develop a high-affinity protein capture agent with high affinity and specificity through the iterative conjugation of modest affinity peptides using in situ click chemistry. An affinity enhancement due to in situ click conjugation was apparent at each screening level. Even for a weakly binding anchor ligand ($K_D \approx 500$ μM), the

hits from biligand screens displayed high sequence homologies and affinities ($K_D \approx 3$ to 10 μM). Both types of biligand screens, in situ and on-bead, demonstrated this effect, suggesting that although the mechanism of the selection is different, the hits identified are essentially equivalent.

At the triligand level, a similar concept was explored. When the peptide ligand became approximately larger than a 15-mer, the OBOC library size was practically limited to <5 million sequences, and the in situ screen (**Tri1**) became the only way to sample increasing diversity and length. Based on analysis of sequence homology, we discovered that the final triligand capture agent reflected in situ assembly, as the on-bead CuAAC triligand library (Table 2.2, Library E) was not comprehensive.

The final triligand capture agent (Figure 2.4C) was demonstrated to bind to bCAII and hCAII with affinities of $K_D \approx 64$ nM and $K_D \approx 45$ nM, respectively, and in Chapter 3, we will provide evidence that it is a specific binder for the enzyme.

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2.6 REFERENCES

1. Borrebaeck, C. A. K. *Immunol. Today* **2000**, *21*, 379–382.
2. Kodadek, T.; Reddy, M. M.; Olivos, H. J.; Bachhawat-Sikder, K.; Alluri, P. G. *Acc. Chem. Res.* **2004**, *37*, 711–718.
3. Blow, N. *Nature* **2007**, *447*, 741–744.
4. (a) Fan, R.; Vermesh, O.; Srivastava, A.; Yen, B. K. H.; Qin, L.; Ahmad, H.; Kwong, G. A.; Liu, C.-C.; Gould, J.; Hood, L.; Heath, J. R. *Nat. Biotechnol.* **2008**, *26*, 1373–1378. (b) Hood, L.; Heath, J. R.; Phelps, M. E.; Lin, B. *Science* **2004**, *306*, 640–643. (c) Phelan, M. L.; Nock, S. *Proteomics* **2003**, *3*, 2123–2134.
5. Smith, G. P.; Petrenko, V. A. *Chem. Rev.* **1997**, *97*, 391–410.
6. Cox, J. C.; Hayhurst, A.; Hesselberth, J.; Bayer, T. S.; Georgiou, G.; Ellington, A. D. *Nucleic Acids Res.* **2002**, *30*, e108–e108.
7. McCauley, T. G.; Hamaguchi, N.; Stanton, M. *Anal. Biochem*. **2003**, *319*, 244–250.
8. Lee, J. F.; Hesselberth, J. R.; Meyers, L. A.; Ellington, A. D. *Nucleic Acids Res.* **2004**, *32*, D95–D100.
9. Gold, L.; Polisky, B.; Uhlenbeck, O.; Yarus, M. *Annu. Rev. Biochem.* **1995**, *64*, 763–797.
10. Burmeister, P. E.; Lewis, S. D.; Silva, R. F.; Preiss, J. R.; Horwitz, L. R.; Pendergrast, P. S.; McCauley, T. G.; Kurz, J. C.; Epstein, D. M.; Wilson, C.; Keefe, A. D. *Chem. Biol.* **2005**, *12*, 25–33.
11. Proske, D.; Blank, M.; Buhmann, R.; Resch, A. *Appl. Microbiol. Biotechnol.* **2005**, *69*, 367–374.
12. Lam, K. S.; Lebl, M.; Krchňák, V. *Chem. Rev.* **1997**, *97*, 411–448.

13. Furka, A.; Sebestyén, F.; Asgedom, M.; Dibó, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487–493.

14. Geysen, H. M.; Mason, T. J. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 397–404.

15. Alluri, P. G.; Reddy, M. M.; Bachhawat-Sikder, K.; Olivos, H. J.; Kodadek, T. *J. Am. Chem. Soc.* **2003**, *125*, 13995–14004.

16. Reddy, M. M.; Bachhawat-Sikder, K.; Kodadek, T. *Chem. Biol.* **2004**, *11*, 1127–1137.

17. Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radić, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2002**, *41*, 1053–1057.

18. Manetsch, R.; Krasiński, A.; Radić, Z.; Raushel, J.; Taylor, P.; Sharpless, K. B.; Kolb, H. C. *J. Am. Chem. Soc.* **2004**, *126*, 12809–12818.

19. Bourne, Y.; Kolb, H. C.; Radić, Z.; Sharpless, K. B.; Taylor, P.; Marchot, P. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 1449–1454.

20. Mocharla, V. P.; Colasson, B.; Lee, L. V.; Röper, S.; Sharpless, K. B.; Wong, C.-H.; Kolb, H. C. *Angew. Chem. Int. Ed.* **2005**, *44*, 116–120.

21. Whiting, M.; Muldoon, J.; Lin, Y.-C.; Silverman, S. M.; Lindstrom, W.; Olson, A. J.; Kolb, H. C.; Finn, M. G.; Sharpless, K. B.; Elder, J. H.; Fokin, V. V. *Angew. Chem. Int. Ed.* **2006**, *45*, 1435–1439.

22. Kehoe, J. W.; Maly, D. J.; Verdugo, D. E.; Armstrong, J. I.; Cook, B. N.; Ouyang, Y.-B.; Moore, K. L.; Ellman, J. A.; Bertozzi, C. R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 329–332.

23. Ramstrom, O.; Lehn, J. M. *Nature Rev. Drug Discov.* **2002**, *1*, 26–36.

24. Poulin-Kerstien, A. T.; Dervan, P. B. *J. Am. Chem. Soc.* **2003**, *125*, 15811–15821.

25. Jain, A.; Huang, S. G.; Whitesides, G. M. *J. Am. Chem. Soc.* **1994**, *116*, 5057–5062.

26. Mammen, M.; Choi, S. K.; Whitesides, G. M. *Angew. Chem. Int. Ed.* **1998**, *37*, 2755–2794.

27. Kodadek, T.; Reddy, M. M.; Olivos, H. J.; Bachhawat-Sikder, K.; Alluri, P. G. *Acc. Chem. Res.* **2004**, *37*, 711–718.

28. Parkkila, S.; Rajaniemi, H.; Parkkila, A.-K.; Kivelä, J.; Waheed, A.; Pastoreková, S.; Pastorek, J.; Sly, W. S. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2220–2224.

29. Yoshiura, K.; Nakaoka, T.; Nishishita, T.; Sato, K.; Yamamoto, A.; Shimada, S.; Saida, T.; Kawakami, Y.; Takahashi, T. A.; Fukuda, H.; Imajoh-Ohmi, S.; Oyaizu, N.; Yamashita, N. *Clin. Cancer Res*. **2005**, *11*, 8201–8207.

30. Haapasalo, J.; Nordfors, K.; Järvelä, S.; Bragge, H.; Rantala, I.; Parkkila, A.-K.; Haapasalo, S. *Neuro-Oncol*. **2007**, *9*, 308–313.

31. Krishnamurthy, V. M.; Kaufman, G. K.; Urbach, A. R.; Gitlin, I.; Gudiksen, K. L.; Weibel, D. B.; Whitesides, G. M. *Chem. Rev.* **2008**, *108*, 946–1051.

32. Jude, K. M.; Banerjee, A. L.; Haldar, M. K.; Manokaran, S.; Roy, B.; Mallik, S.; Srivastava, D. K. Christianson, D. W. *J. Am. Chem. Soc.* **2006**, *128*, 3011–3018.

33. Melkko, S.; Scheuermann, J.; Dumelin, C. E.; Neri, D. *Nat. Biotechnol.* **2004**, *22*, 568–574.

34. Wilkinson, B. L.; Bornaghi, L. F.; Houston, T. A.; Innocenti, A.; Supuran, C. T.; Poulsen, S.-A. *J. Med. Chem.* **2006**, *49*, 6539–6548.

35. Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 6354–6364.

36. van Hest, J. C. M.; Kiick, K. L.; Tirrell, D. A. *J. Am. Chem. Soc.* **2000**, *122*, 1282–1288.

37. Lee, H.-S.; Park, J.-S.; Kim, B. M.; Gellman, S. H. *J. Org. Chem.* **2003**, 68, 1575–1578.

38. (a) I. Coin, M. Beyermann, M. Bienert, *Nat. Protocols* **2007**, *2*, 3247–3256. (b) Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161–214.

39. Carpino, L. A.; El-Faham, A.; Minor, C. A.; Albericio, F. *J. Chem. Soc., Chem. Commun*. **1994**, 201–203.

40. Dixon, S. M.; Li, P.; Liu, R.; Wolosker, H.; Lam, K. S.; Kurth, M. J.; Toney, M. D. *J. Med. Chem.* **2006**, *49*, 2388–2397.

41. (a) Edman, P. *Acta Chem. Scand.* **1950**, *4*, 283–293. (b) Laursen, R. A. *Eur. J. Biochem.* **1971**, *20*, 89–102. (c) Niall, H. D. *Methods Enzymol.* **1973**, *27*, 942–1010.

42. Lee, S. S.; Lim, J.; Cha, J.; Yeo, S. Y.; Tan, S.; Agnew, H. D.; Heath, J. R. *Anal. Chem.* **2010**, *82*, 672–679.

43. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.

44. Tornøe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057–3064.

45. Tornøe, C. W.; Meldal, M. "Peptidotriazoles: Copper(I)-catalyzed 1,3-dipolar cycloadditions on solid-phase" in *Peptides: The Wave of the Future* (Lebl, M.; Houghten, R. A., editors), Kluwer, Dordrecht, 2001, p. 263.

46. Atherton, E.; Sheppard, R. C. in *Solid Phase Peptide Synthesis—A Practical Approach*, Oxford University Press, USA, 1989, p. 136.

47. Zhang, Z.; Fan, E. *Tetrahedron Lett.* **2006**, *47*, 665–669.

48. Weterings, J. J.; Khan, S.; van der Heden, G. J.; Drijfhout, J. W.; Melief, C. J. M.; Overkleeft, H. S.; van der Burg, O. H.; Ossendorp, F.; van der Marel, G. A.; Filippov, D. V. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3258–3261.

49. García-Martín, F.; Bayó-Puxan, N.; Cruz, L. J.; Bohling, J. C.; Albericio, F. *QSAR Comb. Sci.* **2007**, *26*, 1027–1035.