## **Chapter 3**

# Development of Epitope-Targeting Protein Catalyzed Capture Agents Against Histidine Rich Protein II

This work described herein is unpublished work that was part of a concerted group effort to develop PCCs against the *unstructured* protein Histidine rich protein II (HRP2), which is a unique biomarker for *Plasmodium falciparum* malaria. These unpublished results (gathered from October 2014 to July 2015) focused on the medicinal chemistry optimization of several hits, and an initial push to develop biligand PCCs. The best ligands (described in JingXin Liang's thesis, **2018**) were characterized for their ability to disrupt the Heme: HRP2 complex *in vitro*, and were found to be potent inhibitors.

# Development of Epitope-Targeting Protein Catalyzed Capture Agents Against Histidine Rich Protein II

## **Section 3.1-Introduction**

Malarial infection is caused by the *Plasmodium* parasite, and is endemic in the developing regions of the world with warm, humid climates including Sub-Saharan Africa, Asia, and Central/South America. The most virulent form of malaria is called *Plasmodium falciparum* (Pf), and accounts for >90% of all malaria related fatalities.<sup>1</sup> The natural product artemisinin is currently the best drug for treating malarial infections;<sup>2</sup> however, resistance to artemisinin has begun to appear in South Asia. The growing resistance of *Plasmodium* parasites to anti-malarial drug treatments including the "gold standard" drug artemisinin underscores the need to develop new anti-malarial medicines ideally with a unique mechanism of action.

Histidine Rich Protein II (HRP2) is an attractive protein target for treating *Plasmodium falciparum* malarial infection. The parasites utilize the protein to sequester Heme, which is a toxic byproduct of hemoglobin catabolism. HRP2 binds Heme and catalyzes its polymerization into the non-toxic crystalline polymer Hemozoin. Chauhan and coworkers determined that HRP2 has two binding modes for Heme that are pH-dependent.<sup>3</sup> Below pH=6, when the histidine (His) side chain is in its acidic form, the carboxylate side chains of aspartate (Asp) and glutamate (Glu) bind Heme, but, above pH=6, binding to the histidine side chain dominates. Chauhan also demonstrated that Heme polymerization is primarily catalyzed by the carboxylate-binding mode, which is consistent with HRP2 sequestration in the acidic food vacuoles of the parasite.<sup>3</sup> Artemisinin's mechanism of action inhibits Hemozoin formation by first covalently

binding to Heme before HRP2 complexes with the Art-Heme moiety.<sup>4–6</sup> The Art-Heme complex cannot undergo polymerization, and consequently Heme builds up and eventually kills the *Plasmodium* parasite. An alternative mechanism to inhibit Hemozoin formation would involve binding to the HRP2 protein and preventing it from complexing with Heme molecules. As this is a novel inhibition pathway this could lead to new therapeutics that treat Pf malarial infection.

While an attractive target, HRP2 presents unusual challenges for developing molecules to bind HRP2 protein. First, the HRP2 sequence primarily consists of repeating sequences of three amino acids: alanine (A), aspartic acid (D), and histidine (H). This results in a very "sticky" polypeptide chain that lacks a defined  $2^{\circ}/3^{\circ}$  structure. Consequently, HRP2 does not have any well-defined grooves or pockets that a small molecule could bind. Secondly, fourteen unique epitopes have been identified in HRP2 samples that appear in varying quantities across different

Table 3.1. The fourteen uniqueepitopes found in HRP2.						
Code	Repeat Sequence					
1	AHHAHHVAD					
2	AHHAHHAAD					
3	AHHAHHAAY					
4	AHA					
5	AHHAAHHASD					
6	AHHATD					
7	AHHAAD					
8	AYY					
9	AHHAAAHHATD					
10	AHHAAAHHATD					
11	AHN					
12	AHAAAHHEAATH					
13	AHHASD					
14	AHHAHHATD					

geographical regions (Table 3.1).<sup>7</sup> The extensive variation in epitope prevalence in Table 3.2 highlights the unusual amount of sequence diversity in HRP2. This makes developing reagents to bind HRP2 in an epitope-targeted manner a challenge as targeting a single epitope could result in ineffective binding in geographic regions where that epitope is either absent or in low abundance.<sup>8</sup>

Region country/island			No. of total and individual repeats													
of origin	No.	Total	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Africa																
Burkino Faso	1	30	1	12	0	1	0	3	8	2	0	2	0	1	0	0
Cameroon	2	32–35	5	11-12	1–2	0	1	4–5	4–8	1	0	1-2	1-2	1	0-1	0-1
Gambia	1	34	2	9	1	0	2	7	9	2	0	1	0	1	0	0
Ghana	2	31-33	1–3	12-14	1–3	0-1	0-1	3-4	6–8	1	0	1–2	0	1	0	0
Liberia	1	33	3	12	1	2	1	3	8	1	0	1	0	1	0	0
Nigeria	1	33	1	9	1	0	2	7	9	2	0	1	0	1	0	0
Sudan	3	28-31	1–5	7-11	1	1	1–2	2–6	5–9	1–2	0	0-2	0	1	0	0
Uganda	1	29	3	13	2	0	0	2	7	1	0	0	0	1	0	0
Zambia	1	31	3	13	2	0	1	3	6	1	0	1	0	1	0	0
Pacific																
Indonesia	2	33	1	9	1	0	2	7	9	2	0	1	0	1	0	0
Malaysia	1	29	3	13	2	0	0	2	7	1	0	0	0	1	0	0
Philippines	21	25–35	1-4	6-15	0–3	0–3	0-2	2–4	1-10	1–2	0-1	0-2	0-1	1	0	0
Papua New Guinea	9	24–38	1–3	7–15	1–2	0-2	1–3	2-7	2–9	1–2	0	1-2	0	1	0	0-1
South America																
Brazil	5	31–33	2-4	9-12	2	0	0-2	2–5	8–10	0-1	0	0–3	0	1	0	0
Ecuador	1	33	5	12	1	0	1	4	7	1	0	1	0	1	0	0
Honduras	1	32	5	12	1	0	1	2	8	1	0	1	0	1	0	0
Peru	1	32	4	13	1	1	0	2	6	1	0	2	0	1	1	0
Southeast Asia																
Cambodia	1	29	3	13	2	0	0	2	7	1	0	0	0	1	0	0
Vietnam	5	30–36	2-6	13–15	1–2	1-2	1	3–4	2-7	1	0	1–2	0	1	0	0
Solomon Islands	7	30–36	2–4	11–16	1–2	0-1	1–2	3–7	4–9	1	0	1–2	0-1	1	0	0-1
Thailand	7	27–36	1–5	10-19	1–3	0–3	0-1	2–4	1–6	0-2	0	0-2	0	1	0-1	0-1
Overall		25–38	1–6	6-19	0–3	0–3	0–3	2–7	1-10	0-2	0-1	0–3	0-2	1	0-1	0-1

 Table 3.2. The prevalence of the different HRP2 epitopes in patient samples from endemic areas.

 Taken from reference 8.

However, PCC agents can be easily raised against a variety of epitopes in a target protein then linked together to form a biligand that can target multiple epitopes. This would mitigate issues with detecting HRP2 as a result of its sequence diversity; also, a multiligand would enhance the binding affinity through cooperativity to enable detection/inhibition of HRP2 at low physiological levels of HRP2 protein. The remainder of this chapter summarizes efforts to develop PCC agents against the HRP2 protein.

## Section 3.2-Methods<sup>9</sup>

## 3.2.1-Preparation of Peptides<sup>10</sup>

The peptides were prepared using standard Fmoc/<sup>7</sup>Bu coupling procedures either by hand or using an automatic synthesizer. Macrocyclization occurred via a coppercatalyzed click reaction before simultaneous resin cleavage and global deprotection of side chains using a 95% trifluoroacetic acid (TFA) cleavage cocktail. Precipitation of the crude peptide into diethyl ether preceded purification using semi-preparative highperformance liquid chromatography (semi-prep HPLC). Fractions containing pure peptide were identified using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and lyophilized (For characterization spectra see **Figures A3B.1-A3B.37**). The resulting powder was reconstituted in dimethyl sulfoxide (DMSO), quantified using Nanodrop, and stored at 4 °C until use.

### 3.2.2-Enzyme-Linked Immunosorbent Assay Analysis of Peptides

The overnight ELISA protocol commenced with immobilizing the biotinylated peptides and blank on a Neutravidin coated 96-well plate before blocking the plate (with 5% milk or 3% BSA) overnight at 4 °C. Protein incubation with a GST-tagged recombinant HRP2 protein also occurred for 7-8 hours at 4 °C before incubation with an anti-GST HRP-conjugated antibody (ab) at RT. The assay was developed for several minutes at RT using a TMB peroxidase development kit before quenching with 1 M H<sub>2</sub>SO<sub>4(aq)</sub> and immediately measuring the absorbance using a Flexstation 3 plate reader. The data was double background corrected before graphing using Graphpad Prism 7.

The all-room temperature ELISA protocol mirrored the overnight ELISA protocol except that the blocking and protein incubation steps occurred at RT for two hours each. The data was double background corrected as before and graphed using Graphpad Prism 7.

## 3.2.3-Linker Library Synthesis

Linker libraries were developed using either the anchor **JXL1** or **DNB**. Linker synthesis occurred by an automated five cycle split and mix protocol using the amino acids pool Fmoc-D-Leu-OH, Fmoc-Gly-OH, Fmoc-D-Pro-OH, Fmoc-protected  $\alpha$ -amino isobutyric acid (Fmoc-Aib-OH), or nothing. The resulting library had a variable linker with between zero and five amino acids. A Boc-protected propargyl click handle was coupled on before global deprotection of all acidic protecting groups. Each library was stored in 0.05% ( $^{W}/_{v}$ ) sodium azide in MQ H<sub>2</sub>O at 4 °C until use.

#### 3.2.4-Linker Library Screen Pre-Clear

For the pre-clear step each library was split into three aliquots and screened individually against the following biotinylated ligands: JXL1 library **DNB**, **N-term**, and **C-term** ligands; DNB library **JXL1**, **N-term**, and **C-term** ligands. After swelling the library in 0.1% BSA binding buffer and blocking overnight with 1% BSA blocking buffer the library was incubated with one of the anchors for one hour at RT. Incubation with a mouse anti-biotin alkaline phosphatase-conjugated antibody preceded stripping with a high salt buffer over one hour and development with the BCIP/NBT development system for thirty minutes. The development reaction was quenched with 6 M hydrochloric acid and all dark purple beads were removed before decolorizing the remaining beads with NMP and stripping with 7.5 M guanidinium chloride (Guan-HCl), pH = 2.0.

#### 3.2.5-Linker Library Product Screens

Each aliquot was swelled in binding buffer and blocked overnight with 1% BSA before incubating with recombinant HRP2 protein and the biotinylated ligand used in the pre-clear stage. Any non-covalently bound material was stripped from the beads using 7.5 M Guan•HCl, pH=2.0 for one hour and incubation with a high salt buffer for an additional hour. Development occurred in the same manner as the pre-clear, and the dark purple beads were picked out and sequenced using Edman degradation.

## Section 3.3-Results and Discussion

#### 3.3.1-Alanine Scan of Sequence Hits

Previous work performed in the Heath group included combinatorial click library

screens of several epitopes that are common in HRP2 samples: the shifted Type 2 epitope AHHAADAHH, the N-term epitope LHETDQAHVDD, the C-term epitope AHHATDAHHAAAHHEAATH, and the Type 6 epitope AHHATD. These screens yielded several dozen first generation hits. Each hit needed to be tested for binding to the full protein before the best binders were optimized to maximize their binding affinity. My research focused on optimizing the structures and identifying a best binder with three hits from the shifted type 2 epitope screen: YYQLL (hL1), YYYNV (hL2), and LGYYY



The initial round of medicinal chemistry optimization involved mapping out the binding contributions of the residues of each peptide with alanine substituted variants. Any alanine variants with dramatically different binding to HRP2 protein would have that position selected for further amino acid substitutions. Each alanine scan set was prepared

(Table A3C.1) and evaluated against the parent ligand in a single point sandwich ELISA (Figure 3.1). The scan for ligand **hL3** revealed that the tyrosine residues in positions 3-5 strongly bind HRP2. Swapping in structurally similar ligands at "intolerant" positions presents a unique opportunity to probe the non-covalent interactions involved in HRP2 binding while potentially improving affinity. Thus, the strongly intolerant residues of **hL3** were also chosen for substitution.

### 3.3.2-Second-Generation Ligand Synthesis and Measurement of HRP2 Binding Affinity

The individual alanine scans yielded between one and four residues for substitution respectively, and the residues to be modified were Glycine (G), Tyrosine (Y), and Glutamine (O). Each residue's characteristics were analyzed to identify appropriate substitutions. For example, Gly lacks a side chain, and consequently does not impart any steric crowding to a peptide sequence; also, Gly makes the macrocyclic peptide ring more conformationally flexible at that position. An increase in HRP2 binding with the addition of a small, but sterically important methyl side chain in the Ala scan suggests that a more rigid ring could lead to an increase in binding affinity. Thus, Val was selected as an amino acid residue substitution for Gly. Tyrosine has an aromatic phenol chain whose hydroxyl group can accept and donate hydrogen bonds. Also, the hydroxy group uses resonance to increase the e<sup>-</sup> density of the aromatic ring overall. Exchanging the –OH group for a fluorine atom could result in stronger H-bonds with the His residues in HRP2. Also, the small size and increased electronegativity of fluorine results in an electrondeficient aromatic ring. A reduction in electron density could increase the strength of anion- $\pi$  interactions with the aspartate residues on HRP2. This effect could be enhanced with additional F on the same aromatic ring so both 4-fluorophenylalanine (<sup>4F</sup>F) and 3.4difluorophenylalanine (<sup>3,4-F</sup>F) were chosen to replace Tyr. Glutamine is the uncharged

amide analogue of the charged amino acid glutamate (Glu). Exchanging an uncharged residue for an analogous charged residue could enhance the binding affinity with HRP2, due to more salt bridge interactions with HRP2. Thus, both Asp and Glu were chosen to replace Gln.

The second-generation ligand sets were prepared (Table A3C.2) and evaluated against the parent ligand as before (Figure 3.2). The Generation II **hL1** single-point ELISA



**Figure 3.2. Testing the second-generation ligands.** The 20 nM HRP2 single point ELISAs comparing the generation two ligands to a) hL1, b) hL2, and c) hL3. In the graphs the bottom dashed lines represent the background signal, and the top dashed lines represent the signal of the parent ligand. D. The binding curve for the best performing second-generation ligand **hL3V2** with an  $EC_{50} = 8.91 \pm 2.5$  nM.

revealed that exchanging the uncharged amide residue for a charged acidic residue did not result in any change in the binding affinity (Figure 3.2A). The His residues ( $pK_a =$ 6.0) are uncharged in the binding buffer (pH = 7.4), so adding a residue that would encourage salt bridge interactions with the acidic form of His was not productive. Glutamine could also have been substituted with arginine (Arg), but since hL1 was the weakest binder of the three ligands studied, further modifications were not pursued. The Generation II hL2 ELISA (Figure 3.2B) did result in enhanced signal with the peptide hL2<sup>f</sup>F2, but as it was not as good a binder as the Y<sup>4F</sup>FYRV (JXL1 ligand) sequence developed by a coworker, this sequence was not subjected to further optimization. The ELISA for the second-generation hL3 ligands revealed that the addition of fluorinated analogues at the Tyr residues did not result in a significant increase of HRP2 binding (Figure 3.2C). This suggests that the cation- $\pi$  interactions between the weakly charged His residues and the Tyr side chains play a strong role in hL3 binding HRP2. Tyr residues appearing in most of the original hits from the shifted Type 2 epitope click library screen further highlighted tyrosine's significance. Surprisingly, the substitution of Gly for Val in ligand **hL3V2** resulted in a dramatic increase in signal, which suggests that ring rigidity played a larger role in HRP2 binding affinity than previously expected. A full binding curve for **hL3V2** was prepared, and it was found to have an EC<sub>50</sub> of  $8.91\pm2.5$ nM (Figure 3.2D). The ligand hL3V2 was the only ligand carried onwards for further optimization.

#### 3.3.3-Further Optimization of Ligand Hit hL3V2

The next round of optimization involved investigating the ideal ring size and closure for the macrocycle. The ligands to date have all been 27-atom macrocycles. A slightly larger macrocyclic ring could result in a binding pocket that better fits the shifted Type 2 HRP2 epitope. A ring-closing metathesis (RCM) ring closure, which gives a ring size of 33 atoms, is an unnatural ring closure that utilizes the non-canonical stapled peptides Fmoc-(S)- $\alpha$ -Me-2'-(7-octenyl)-alanine (Fmoc-Oct-OH) and Fmoc-(R)- $\alpha$ -Me-2'-(4pentenyl)-alanine (Fmoc-Pen-OH).<sup>11,12</sup> A pentameric peptide sequence that is ring closed

with stapled peptides does require adding a Gly residue to one end of the sequence in order to satisfy the *i*, i + 7 or *i*, i + 4 rule.<sup>13</sup> As the RCM closure represents a change in ring flexibility, due to the introduction of quaternary centers and an increase in the hydrophobicity with the stapled peptide side chains, the RCM closure was chosen for ring size/closure optimization. A larger binding pocket could also spontaneously result from the click reaction used to close the macrocycle. The macrocyclic ring closure is the major product of the click reaction, but a side product can result from an *intermolecular* click reaction between two peptide sequences. The resulting ligand would possess two triazole closures for a ring size of 54 atoms (Figure A3B.38). The Type 2 epitope binder YKYYR (**DNB** ligand), which was identified by a colleague, had increased binding affinity for HRP2 in its dimer form.<sup>10</sup> It has been shown in the literature that longer sequences favor intermolecular click reactions as a result of an increase in conformational flexibility.<sup>14</sup> Adding a Gly residue to encourage dimerization also increases the triazole closed macrocyclic ring size up to 30 atoms, which would allow for testing if adding a conformationally flexible residue encouraged dimerization without depressing HRP2 binding affinity; also, the added Gly would provide a triazole analogue to compare to the RCM closed ligands. This comparison would determine whether the change in binding affinity was due to the larger ring size or to the change in macrocyclic ring closing. As a result the following isomers of the ring expanded and RCM-closed ligand variants of hL3V2 were prepared (Table A3C.3).

Although both the heterodimer and homodimer are possible side products of the RCM ring closing, only monomer was observed by MALDI-TOF analysis. Each ligand was isolated in multiple fractions, which results from the Grubbs I catalyst producing a

mixture of E/Z alkene isomers. The different ligand fractions were tested, and the best isomer was **Ak-hL3G1V3** (Figure 3.3). This particular isomer has the stapled peptide with the shorter linker at the C-terminus and places the extra Gly residue away from the Tyr residues. The Tyr residues are heavily involved in binding HRP2, which suggests that optimal binding requires conformational rigidity near the residues that actively bind HRP2.



ELISAS. A. A comparison of the ring-expanded triazole-closed monomer and dimer with the parent ligand. B. A comparison of the RCM ligands with the parent ligand. C. Comparing the best ring-expanded triazole and RCM fractions with the parent ligand. In the graphs the bottom dashed lines represent the background signal, and the top dashed lines represent the signal of the parent ligand.

Preparing the ring-expanded triazole ligands did lead to the isolation of a significant amount of dimer in addition to the monomeric ligands. As expected, the dimeric ligands outperformed the monomeric ligands, likely as a result of cooperativity (Figure 4.3B). However, dimerizing the ring-expanded ligands did not result in a marked improvement compared to the parent ligand **hL3V2**. The drop in binding affinity, despite having two copies of the HRP2 binding sequence present, could result from the extra Gly residues. Although having the Gly away from the active Tyr residues is preferred, the extra Gly may introduce enough conformational flexibility in the dimer's ring to disfavor HRP2 binding. This suggests that small, conformationally rigid cycles are needed to bind HRP2 selectively. Directly comparing the best RCM ligand to the best ring-expanded triazole did show an increase in binding affinity for the RCM peptide, but overall both ligands behaved worse than the parent ligand **hL3V2** (Figure 3.3C). This reinforced that, for targeting the shifted type 2 epitope, the original macrocycle ring size and ring closure was optimal.

### 3.3.4-Developing PCC Agent Biligands

Although the capture agents developed to bind select HRP2 epitopes have EC<sub>50</sub> values in the 10-20 nM range, the best antibodies against HRP2 have much lower K<sub>d</sub> values: 1 nM (C1-13 epitope, IgG antibody) and 160 pM (PTL3 epitope, IgM antibody).<sup>15</sup> The difference in binding affinity between the antibodies and the much smaller capture agents can be explained by cooperativity. Improving the EC<sub>50</sub> of the PCC agents further would require linking multiple copies of the cyclic peptides together. One such method would be to prepare a biligand composed of two monomeric PCC agents coupled through a spacer than can either be a flexible PEG<sub>n</sub> molecule or a rigid polypeptide linker. Thus, work was undergone collaboratively to identify the optimal amino acid spacer for linking together the best Type 2, shifted Type 2, N-term, and the C-terminal epitope targeting monoligands. Two linker libraries were prepared with the ligands **JXL1** (EC<sub>50</sub> = 538 pM) and **DNB** (type 2 epitope, EC<sub>50</sub> = 218±44 nM) as anchors that were coupled to linkers that were 0-5 amino acids in length. The amino acid pool for linker library split and mix synthesis was: glycine (G), D-proline (p), D-leucine (l), 2-amino isobutyric acid (Aib), or nothing. These linker libraries were individually screened against the following biotinylated ligands: **DNB**, **JXL1**, **N-term** (EC<sub>50</sub> = 4 nM), and **C-term** (Figure 3.4).



The linker screens generated a total of 25 hits (Table A3C.4). Two of the hits (**DNBvCtermH6** and **DNBvCtermH8**) were evaluated in a double-point ELISA against the monoligands and were found to outperform the anchors. The **DNBCtermH8** biligand was found to have an  $EC_{50} = 540$  pM, which represents a forty-fold increase over the  $EC_{50}$  value of the original DNB anchor.

At this stage the project leader wrapped up the HRP2 protein study by examining ligands' ability to prevent complexation between free Heme and HRP2 protein. The functional study revealed that the **N-term**, **DNB**, and **JXL1** ligands all inhibited the complexation, but that the **JXL1-PEG-DNB** biligand inhibited Heme: HRP2 complex formation even better than chloroquine, which is a commercially available malaria

prophylactic. The results of this functional study are summarized both in a recent publication under review<sup>16</sup> and in JingXin Liang's dissertation.

## **Section 3.4-Conclusion**

In closing, this report disclosed efforts to optimize the library screen hits **hL1**, **hL2**, and **hL3** to arrive at the ligand **hL3V2**. Further studies on changing macrocyclic ring closure and macrocyclic ring size revealed that low-conformational flexibility and a smaller ring size are optimal for binding the shifted Type 2 epitope AHHAADAHH. The best ligands discovered during the group effort to test and optimize all of the HRP2 epitope screen hits were tested for inhibition of the Heme: HRP2 complex and found to inhibit at a rate comparable to or better than chloroquine, a common anti-malarial.

## **Section 3.5-References**

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# Appendix 3A

# Materials and Experimental Procedures

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## Materials

Unless otherwise stated all chemicals were used as received.

## <u>Peptide Synthesis</u>

The 9-Fluorenylmethoxycarbonyl (Fmoc) and t-butyl carboxy (Boc) protected amino acids were purchased from Anaspec, AAPTec, Bachem, ChemPep, and Sigma-Aldrich. The Fmoc-protected stapled amino acids Fmoc-(S)-2-(7-octenyl) Ala-OH and Fmoc-(R)-2-(pentenyl) Ala-OH used to prepare the RCM-closed peptides were purchased from Sigma Aldrich. Biotin NovaTag<sup>™</sup> resin was obtained from EMD Chemicals, Inc. and used for synthesis of biotinylated peptides. The Seiber amide resin used for the synthesis of protected peptide fragments and the Rink Amide MHBA Resin used for the preparation of C-term amide peptides were purchased from AAPPTec. The peptide onebead-one compound (OBOC) library was prepared on Tentagel Resin purchased from RAPP Polymere. The Fmoc-protected propionic acid polyethylene glycol (PEG<sub>n</sub>) linkers were purchased from ChemPep Inc. The L-Ascorbic Acid and copper (I) iodide (CuI) used for click reactions were purchased from Sigma Aldrich. The N-methyl pyrrolidine (NMP), 1-[Bis (dimethylamino) methylene]-1H-1, 2, 3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), N, N'-dimethyl formamide (DMF), and N, N'diisopropylethylamine (DIPEA) used in peptide synthesis were bought from EMD Chemicals, Inc., ChemPep, and Sigma-Aldrich respectively. The piperidine used for the removal of Fmoc protecting groups was purchased from Sigma-Aldrich. The Hydrazine (60 (%  $(^{v}/_{v})$  in H<sub>2</sub>O) and 2-azido acetic acid used for the deprotection of 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-ethyl (Dde) protecting groups and formation of Az4' from groups purchased Sigma Aldrich. The **Benzylidene-bis** were

(tricyclohexylphosphine)-dichlororuthenium (Grubbs I) catalyst and 1,2-dichloroethane (DCE) used for the preparation of stapled peptides were purchased from Fischer Scientific.

### **Peptide Isolation and Purification**

The trifluoroacetic acid (TFA), and triethylsilane (TESH) used for the cleavage of peptides from resin and global deprotection of side chains were purchased from Sigma-Aldrich. The diethyl ether used to precipitate crude peptide was purchased from JT Baker. The Omnisolv grade acetonitrile (MeCN) used for peptide purification was purchased from EMD Millipore. Unless otherwise stated, peptide preparation was performed using a Titan 357 Automatic Peptide Synthesizer (AAPPTec, Louisville, KY). Mass analysis was performed using a Voyager De-Pro matrix assisted laser desorption ionization time-offlight mass spectrometer (MALDI-TOF MS) (Applied Biosystems, California). The crude peptides were dissolved in either DMSO (Sigma Aldrich) or (1:1) MeCN/doubly distilled water (MQ  $H_2O$ ) w/ 0.1% TFA before purification by a gradient of 0% to 50% acetonitrile in MQ H<sub>2</sub>O with 0.01% ( $^{V}$ ) TFA using a RP-HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector) using a C18 reversed phase semipreparative column (Phenomenex Luna 10  $\mu$ m, 250  $\times$  10 mm). The concentration of peptides was determined using a Nanodrop 2000 Spectrophotomer (ThermoFischer Scientific Inc., Massachusetts).

## ELISA Analysis of Peptides

The bovine serum albumin (BSA) and nonfat milk powder used in ELISAs was purchased from Sigma-Aldrich and Best Value respectively. The Glutathione Stransferase (GST)-labeled recombinant *P. falciparum* antigen HRP2 protein that was used in the ELISAs was purchased from CTK Biotech Inc. The anti-GST HRP-conjugated antibody (ab58626) was purchased from Abcam. The ELISAs were run on either 96-well clear Pierce Neutravidin Plates (#15129) or Pierce Neutravidin Coated Plates (#15127) purchased from ThermoFischer Scientific. The tris (hydroxymethyl) aminomethane hydrochloride salt (Tris•HCl), sodium chloride, and magnesium chloride pentahydrate (MgCl<sub>2</sub>•5H<sub>2</sub>O) used to prepare the Tris buffer were purchased from Sigma Aldrich. The surfactant polysorbate 20 (Tween20) was purchased from Sigma Aldrich. The TMB Microwell Peroxidase Substrate System B (#50-76-00) that was used to develop ELISAs was purchased from KPL. The sulfuric acid (H<sub>2</sub>SO<sub>4(aq)</sub>) used to quench the enzymatic amplification reaction in the ELISAs was purchased from JT Baker. The 96-well ELISA plates were read using a Flexstation 3 plate reader (Molecular Devices LLC, Sunnyvale, CA).

### Linker Library Screens

The recombinant, unlabeled HRP2 protein used for the linker screens was a generous gift from the Program for Appropriate Technology in Health (PATH). The mouse antibiotin-alkaline phosphatase conjugated antibody (ab) (#A6561) pre-clear and the subsequent product screens were purchased from Sigma Aldrich. The combined 5bromo-4-chloro-3-indoyl phosphate (BCIP)/ nitro blue tetrazolium (NBT) (#S3771) used to develop hits during the library screens was purchased from Promega. The concentrated hydrochloric acid used to quench the BCIP/NBT development was purchased from Sigma Aldrich. The methanol (MeOH) used to rinse the library beads was purchased from EMD Millipore. The guanidinium chloride (Guan•HCl) used to strip library beads was purchased from Sigma Aldrich. Sigma Aldrich. Sequencing of bead hits occurred via Edman degradation sequencing on a Procise Protein Sequencer (Applied Biosystems, California).

## Methods

## General Procedures for the Preparation of Ligands

## **Preparation of Resin for Coupling**

Novatag Biotin Resin (0.44  $^{\text{mmol amine}/g}$ , 300 mg, 0.132 mmol) was placed into a 8mL solid-phase peptide synthesis (SPPS) tube fitted with a stopcock and swelled in 4mL of NMP for a minimum of two hours.

## Hand Deprotection of Fmoc Protecting Group

The resin was incubated with 4mL portions of Piperidine (20% ( $^{v}/_{v}$ ) in NMP) for 2 min, 5 min, and 25 min before rinsing with NMP (5x).

## **Peptide Hand Couplings**

The appropriate Fmoc-protected amino acid (2.00-4.00 equiv.) was added directly to the SPPS tube with HATU (2.00-4.00 equiv.). After solubilizing the coupling reagents in NMP, the solution was charged with DIPEA (6.00-12.0 equiv.) and allowed to react on a peptide shaker at room temperature (35 min  $\rightarrow$  overnight) before washing with NMP (5x).

#### **Titan Synthesis**

Automated synthesis of the 5-mer and alkyne click handle was conducted on the Titan 357 Automatic Peptide Synthesizer. Wells were charged with 50mg each of Novatag resin following Fmoc-Az4-OH coupling. Titan synthesis prepared the peptides in a linear fashion: Fmoc-NH-Pra- $X_1X_2X_3X_4X_5$ -Az4-PEG<sub>5</sub>-Resin.

#### Click Reaction

The click reaction solution for each ligand (50mg, 0.0220 mmol) was prepared as a solution in 3mL of Piperidine (20% ( $^{v}/_{v}$ ) in NMP): CuI (8.4 mg, 0.0440 mmol) and L-ascorbic acid (19.4 mg, 0.110 mmol). The solution was added to the resin and allowed to cyclize overnight with shaking at room temperature. A solution of click wash was prepared immediately before use: DIPEA (5% ( $^{v}/_{v}$ )) and sodium diethyl dithiocarbonate (5% ( $^{w}/_{v}$ )) in NMP. After draining the SPPS tube the resin was rinsed with click wash (3x), and incubated with click wash for ten minutes before rinsing with NMP (5x).

#### **Ring-Closing Metathesis of Macrocyclic Peptide Ring**

The resin (200 mg, 0.0880 mmol) was rinsed with DCM (5x) and left to dry for a minimum of one hour before reswelling in 1,2-DCE (4 mL) for one hour. An oven-dried 2-neck round-bottom flask (RBF) under  $Ar_{(g)}$  was charged with the resin solution and degassed for thirty minutes. Grubbs I catalyst (50 mg) was added quickly and the reaction was left to stir until the solution went from purple to red-brown (about six hours). The solution was placed into an SPPS tube, drained, charged with fresh Grubbs I catalyst (50 mg) in 1,2-DCE (4 mL), and returned to the RBF under  $Ar_{(g)}$  for another six hours. The resin was collected into an SPPS tube and rinsed with DCM until the washes were clear and colorless before adding 3 mL click wash for five-minute washes until the drained liquid came back colorless. The resin was Fmoc-deprotected (3 x 20 minutes) prior to cleavage from resin. The first coupling reaction after the RCM was run overnight to prevent incomplete formation of the amide bond due to Grubbs I catalyst interference.

#### MTT Deprotection

Prior to an MTT deprotection the resin was washed with DCM (3x). Deprotection occurred with washes of 2% TFA in DCM (5 mL, 9 times) until the drained solvent went

from bright yellow to colorless. The resin was washed with DCM (5x) then NMP (5x), and reswelled in NMP for at least thirty minutes before further manipulation.

## **Dde Deprotection and Formation of Az4' Group**

The Dde group was removed by incubating with Hydrazine (2 % ( $^{v}/_{v}$ ) in DMF) (5 mL x 10 minutes) three times before rinsing with DMF (5x). The resin was then reacted with 2-azido acetic acid using the standard hand coupling procedure for a minimum of two hours (**with regular venting for the first thirty minutes of shaking**) and worked up by rinsing with NMP (5x).

#### Seiber Amide Resin Deprotection to Isolate Fully Protected Peptides

The resin (3.00 g, 2.01 mmol) was rinsed with DCM (5x), and dried under vacuum for a minimum of one hour. The resin was then washed with TFA (1% ( $^{v}/_{v}$ ) in DCM) (25 mL x 1 minute once and 10 mL x 1 minute, 10 times) draining into a RBF containing 5mL of DIPEA to neutralize the solution. An additional 1 mL of DIPEA was added to the flask before isolating the crude protein by rotary evaporation and purifying by prep-scale HPLC, identification of pure protected peptides using electrospray ionization mass spectrometry (ESI-MS), and lyophilization of desired fractions.

#### Resin Cleavage, Global Side-Chain Deprotection, and Purification of Crude Peptide

The resin was rinsed with DCM (5x) and dried under vacuum for a minimum of one hour. A 20 mL scintillation vial was charged with a stir-bar, resin and cleavage solution (95% TFA, 2.5% TESH, 2.5% MQ H<sub>2</sub>O) before stirring at room temperature for 2 hours. The solution was then filtered into 40 mL of cold ether, vortexed briefly, and stored at 4 °C overnight. The precipitated protein was centrifuged into a pellet (4500 RPM, 10 minutes @ 4 °C) prior to decantation of the supernatant. After dissolving the crude peptide in DMSO purification occurred by a gradient of 0% to 50% acetonitrile in water with 0.01% ( $^{v}/_{v}$ ) TFA using an 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), identification of pure peptide using MALDI-TOF mass spectrometry, and lyophilization of desired fractions.

## Linker Library Synthesis

The peptide anchors DNB and JXL1 were synthesized on Tentagel resin and cyclized before a split and mix coupling of the linker library on Titan occurred. Each library was split into five wells and couple with Fmoc-D-Pro-OH, Fmoc-Gly-OH, Fmoc-D-Leu-OH, Fmoc-2-aminoisobutyric acid (Fmoc-Aib-OH), or nothing. Recombination of the resin preceded washing and Fmoc deprotection before repeating for an additional four cycles to get a linker library with a variable 0 to 5 amino acids linker length. A final coupling with Boc-Pra-OH preceded global deprotection of side chains with cleavage cocktail (95% TFA, 2.5% TESH, 2.5% H<sub>2</sub>O) and storage under 0.05% sodium azide in water at 4 °C.

**Overnight ELISA Procedure** 

Binding Buffer: TBS + 0.05% Tween20 + 0.1% BSA

Blocking Buffer: TBS + 5-7% milk + 0.05% Tween20

All washing steps were 200<sup>µL</sup>/<sub>well</sub>

All incubation steps were  $100^{\mu L}/_{well}$  except for the blocking step, which was  $200^{\mu L}/_{well}$ Prior to incubating with the blank and ligands the 96 well plate was washed with binding buffer (3 x 5 minutes). The wells were then loaded with 100  $\mu$ L of either 0.5% ( $^{v}/_{v}$ ) DMSO/ Biotin-PEG<sub>5</sub> linker (2  $\mu$ M in binding buffer) or biotinylated ligand (2  $\mu$ M in binding buffer), and the plate was incubated at RT for two hours. The washing step was repeated before adding blocking solution and the plate was incubated for 12-14 hours in the cold room. After washing the plate in the cold room, each well was charged with either binding buffer or HRP2 protein (20-40 nM in binding buffer), and the plate was left to incubate in the cold room for 7-8 hours. Unbound protein was washed away at room temperature before each well was charged with the anti-GST HRP-conjugated antibody (2000:1 dilution in binding buffer) before incubating at RT for one hour. The plate was then washed with binding buffer (3 x 5 minutes) then with TBS buffer (1 x 5 minutes) before adding developing solution (1:1 TMB Peroxidase Substrate: Peroxidase Substrate Solution B) and agitating until a blue color began to appear. Approximately five minutes after the initial appearance of color the reaction was quenched with 100  $\mu$ L of 1M H<sub>2</sub>SO<sub>4(aq)</sub> at which point the color changed from blue to golden yellow. The absorbance was measured immediately at  $\lambda = 450$ nM, background corrected, and subsequently plotted in Graphpad.

A similar procedure to the above was utilized to prepare the LVYYY binding curve with the exceptions that the ligand was immobilized at a 200 nM concentration, and the plate was blocked with 3% BSA.

The cocktail ELISA experiments were run using a similar experimental protocol with the exception that the blank used was Biotin-Gly-NHAc and the plate was blocked with 3% BSA.

*Room-Temperature ELISA Procedure*<sup>1</sup>

Binding Buffer: PBS + 0.05% Tween20 Blocking Buffer: PBS + 2% BSA+ 0.05% Tween20 All washing steps were 200<sup>μL</sup>/<sub>well</sub>

## All incubation steps were $100^{\mu L}/_{well}$ except for the blocking step, which was $200^{\mu L}/_{well}$

Prior to incubating with the biotinylated blank and ligands the 96 well plate was washed with binding buffer (3 x 5minutes). The wells were then loaded with 100 mL of either Biotin-PEG<sub>5</sub>-NH<sub>2</sub> (blank) (2  $\mu$ M in binding buffer) or biotinylated ligand (2  $\mu$ M in binding buffer) and the plate incubated with shaking at RT for two hours. The washing step was repeated before adding blocking solution and incubating with shaking for two hours at room temperature. After washing the plate, each well was charged with either binding buffer or a HRP2 protein solution (1-5 nM in binding buffer) and the plate was left to incubate at room temperature for two hours. Unbound protein was washed away at room temperature before each well was charged with the anti-GST HRP Antibody (2000:1 dilution in binding buffer), and the plate was incubated at RT for one hour. The plate was then washed with binding buffer  $(3 \times 5 \text{ minutes})$  and PBS  $(1 \times 5 \text{ minutes})$ before adding 100  $\mu$ L of the developing solution (1:1 TMB Peroxidase Substrate: Peroxidase Substrate Solution B) and agitated until a blue color began to appear. Approximately five to ten minutes after the initial appearance of color the reaction was quenched with 100  $\mu$ L of 1 M H<sub>2</sub>SO<sub>4(aq)</sub> at which point the color changes from blue to golden yellow. The absorbance was measured immediately at  $\lambda = 450$  nM, and subsequently plotted in Graphpad.

### Linker Library Screens

The procedures described herein are a modification of the procedures given in **reference 10** of the main text.

## Linker Library Pre-Clear

Each anchor library was split into three aliquots and pre-cleared separately. Each aliquot was rinsed with MeOH (5x), MQ H<sub>2</sub>O (10x), and swelled in 500  $\mu$ L of binding buffer (0.1% BSA, 0.1% Tween 20 in TBS) @ RT for three hours. The library aliquots were then mixed with 500 µL blocking buffer (1% BSA, 0.1% Tween 20 in TBS) and left to shake at 4 °C overnight. After draining and rinsing with 500  $\mu$ L binding buffer the library was incubated for four hours @ RT with 500 µL of binding buffer containing 5 µM of one of the biotinylated ligands: JXL library DNB, C-term, or N-term; DNB Library JXL1, C-term, or N-term. The beads were then rinsed with binding buffer (3 x 500  $\mu$ L) before incubation with 500  $\mu$ L binding buffer containing a 10,000:1 dilution of the mouse anti-biotin conj. alkaline phosphatase antibody for one hour @ RT. Rinses with binding buffer (3 x 500 µL), TBS (1 x 500 µL) preceded incubations with a high salt (25 mM Tris•HCl, 10 mM MgCl<sub>2</sub>, 700 mM NaCl, pH = 7.4) TBS buffer @ RT (500  $\mu$ L, 3 x 20 minutes). The beads were then rinsed with BCIP Buffer (3 x 500  $\mu$ L) and dried under vacuum for eight minutes before transferring to a petri dish. The beads were developed with 13  $\mu$ L of BCIP (50 <sup>mg</sup>/<sub>mL</sub> in 70% DMF) in 10mL of developing buffer (100 mM Tris•HCl, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, pH=xx) and incubated with the library beads in a 20cm petri dish for ten minutes before adding 26 µL of NBT (50 mg/mL in 70% DMF) and incubating for an additional thirty minutes. The development was quenched with 100  $\mu$ L of 6 M HCl<sub>(aq)</sub> and the dark purple beads that represent promiscuous binders to the biotinylated ligand were removed using a 10  $\mu$ L micropipette. Each individual library aliquot was prepared for a subsequent product screen by rinsing with NMP (5x), decolorizing with NMP overnight, rinsing with MeOH (5x), MQ H<sub>2</sub>O (10x), and stripping with 7.5 M Gua • HCl, pH = 2 for thirty minutes @ RT.

The aliquots were screened individually against the ligand with which they had been pre-cleared. Each library aliquot was swelled in binding buffer for four hours @ RT before rinsing with binding buffer (10 x 500 µL) and incubating with 500 µL blocking buffer overnight at 4 °C. The beads were then incubated with 100 nM rHRP2 and  $5\mu$ M of one of the following: N-term, C-term, JXL1, or DNB ligand in 500 µL binding buffer @ RT for six hours. After a quick rinse with binding buffer (500  $\mu$ L), the beads were stripped with 500 µL of 7.5M Guan HCl, pH=2.0 @ RT for one hour. A second overnight block with 500 µL of blocking buffer @ 4 °C preceded incubation with a 10,000:1 dilution of mouse anti-biotin-alkaline phosphatase conj. Ab in 500  $\mu$ L binding buffer for one hour @ RT. The beads were washed with binding buffer (3 x 500 µL), TBS, pH = 7.4 (3 x 500 µL) before incubating with high salt buffer (4 x 15 minutes). Finally they were rinsed with TBS (3 x 500  $\mu$ L) and BCIP Buffer (pH = 9, 3 x 500  $\mu$ L), and dried under vacuum for one minute before development in a 20 cm petri dish with 26 µL BCIP, 13 µL NBT in 15 mL of BCIP buffer for thirty minutes. The development reaction was quenched with 100  $\mu$ L 6 M HCl<sub>(aq)</sub>, and the dark purple beads were picked with a 10 µL micropipette. The beads were placed individually into Spinex tubes and washed with 250  $\mu$ L 7.5 M Guan•HCl, pH = 2.0, MQ H<sub>2</sub>O (5 x 250  $\mu$ L), and stored in NaN<sub>3</sub> (0.05 %  $(^{m}/_{v})$ ) in MQ H<sub>2</sub>O at 4 °C until ready to be sequenced.

## References

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# Appendix 3B

# **Supplemental Figures**

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in the click reaction.	164



**Figure A3B.1. Characterization spectra for ligand hL1A1.** Ligand hL1A1:  $C_{65}H_{106}N_{16}O_{17}S$ . (Top) The HPLC trace for ligand **hL1A1**. The arrows correspond to the fractions with the correct mass. (Middle) The MALDI-TOF spectra for the first fraction. The peak labeled "i" corresponds to the [M+H] mass, expected [M+H]= 1416.677, and the peak labeled "i" corresponds to the [M+Na] mass, expected [M+Na]= 1438.762. (Bottom) The MALDI-TOF spectra for the second fraction. The peak labeled "i" corresponds to the [M+H] mass, and the peak labeled "ii" corresponds to the [M+Na] mass.



Figure A36.2. Representative characterization spectra for ligand nL1A2. Ligand nL1A2  $C_{65}H_{106}N_{16}O_{17}S$ . (Top) The HPLC trace for ligand hL1A2. The indicated regions correspond to fractions 1-4 that had the correct mass. (Middle and Bottom) The MALDI spectra for fractions 1 and 2 respectively. The peaks labeled "i" correspond to the [M+H] mass, expected [M+H] = 1416.677, and the peaks labeled "ii" correspond to the [M+Na] mass, expected [M+Na] = 1438.762.



 $C_{69}H_{107}N_{15}O_{17}S$ . (Top) The HPLC trace for ligand **hL1A3**. The indicated regions correspond to fractions 1-3 that had the correct mass. (Middle and Bottom) The MALDI spectra for fractions 1 and 2 respectively. The peaks labeled "i" correspond to the [M+H] mass, expected [M+H] = 1450.777, and the peaks labeled "ii" correspond to the [M+Na] mass, expected [M+Na] = 1472.759.



**Figure A3B.4.** Characterization spectra for ligand hL1A4. Ligand hL1A4  $C_{68}H_{104}N_{16}O_{18}S$ . (Top) The HPLC trace for ligand hL1A4. The indicated regions correspond to the two fractions that had the correct mass. (Middle and Bottom) The MALDI spectra for fractions 1 and 2 respectively. The peaks labeled "i" correspond to the [M+H] peak, expected [M+H] = 1465.751, and the peaks labeled "ii" correspond to the [M+Na] peak, expected [M+Na] = 1487.733.



Figure A3B.5. Representative characterization spectra for ligand hL1A5. Ligand hL1A5:  $C_{68}H_{104}N_{16}O_{18}S$ . (Top) The HPLC trace for ligand hL1A5. The indicated regions correspond to the four fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The peaks labeled "i" correspond to the [M+H] peak, expected [M+H] = 1465.751, the peaks labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1487.733, and the peak labeled "ii" corresponds to the [M+K] peak, expected [M+K] = 1503.707. (Bottom) The MALDI spectra for fraction 2. The indicated peak corresponds to the [M+Na] peak.



**Figure A3B.6. Characterization spectra for ligand hL2.** Ligand **hL2**  $C_{72}H_{104}N_{16}O_{19}S$ . (Top) The HPLC trace for ligand **hL2**. The indicated regions correspond to the two fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The indicated peak corresponds to the [M+H] peak, expected [M+H] = 1530.765. (Bottom) The MALDI spectra for fraction 2. The peak labeled "i" corresponds to the [M+H] peak, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na]= 1552.747.



Figure A3B.7. Representative characterization spectra for ligand hL2A1. Ligand hL2A1  $C_{66}H_{100}N_{16}O_{18}S$ . (Top) The HPLC trace for ligand hL2A1. The indicated regions correspond to the three fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1437.720, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na]= 1459.702. (Bottom) The MALDI spectra for fraction 2. The indicated peak corresponds to the [M+Na] peak.






**Figure A3B.10. Characterization spectra for ligand hL2A4.** Ligand **hL2A4:**  $C_{71}H_{103}N_{15}O_{18}S$ . (Top) The HPLC trace for ligand **hL2A4.** The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The peaks labeled "i" corresponds to the [M+H] peak, expected [M+H]= 1486.740, and the peaks labeled "i" correspond to the [M+Na] peak, expected [M+Na]= 1508.722.



The HPLC trace for ligand **hL2A5**. The indicated regions correspond to the fraction that had the correct mass. (Bottom) The MALDI spectra for fraction 1. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H]= 1501.715, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na]= 1523.697.



**Figure A3B.12. Representative characterization spectra for ligand hL3A1.** Ligand **hL3A1:**  $C_{68}H_{97}N_{15}O_{18}S$ . (Top) The HPLC trace for ligand **hL3A1**. The indicated regions correspond to the three fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The peaks labeled "i" correspond to the [M+H] peak, expected [M+H] = 1444.693, the peaks labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1466.675, and the peak labeled "ii" corresponds to the [M+K] peak, expected [M+K] = 1482.649. (Bottom) The MALDI spectra for fraction 2. The peak labeled "i" corresponds to the [M+H] peak, and the peak labeled "ii" corresponds to the [M+Na] peak.



Figure A3B.13. Representative characterization spectra for ligand hL3A2. Ligand hL3A2:  $C_{72}H_{105}N_{15}O_{18}S_r$ . (Top) The HPLC trace for ligand hL3A2. The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 2 and fraction 3 respectively. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1500.756 and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1522.738.



Figure A3B.14. Characterization spectra for ligand hL3A3. Ligand hL3A3:  $C_{65}H_{99}N_{15}O_{17}S$ . (Top) The HPLC trace for ligand hL3A3. The indicated regions correspond to the two fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1394.714, the peak labeled "ii" corresponds to the [M+K] peak, expected [M+K] = 1432.670. (Bottom) The MALDI spectra for fraction 2. The peak labeled "i" corresponds to the [M+H] peak, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1416.696.



Figure A3B.15. Characterization spectra for ligand hL3A4. Ligand hL3A4:  $C_{65}H_{99}N_{15}O_{17}S$ . (Top) The HPLC trace for ligand hL3A4. The indicated regions correspond to the two fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The peak labeled "i" corresponds to the [M+H] mass, expected [M+H] = 1394.714, and the peak labeled "ii" corresponds to the [M+Na] mass, expected [M+Na] = 1416.696. (Bottom) The MALDI spectra for fraction 2. The indicated peak corresponds to the [M+H] mass.



**Figure A3B. 16. Characterization spectra for ligand hL3A5.** Ligand **hL3A5**:  $C_{65}H_{99}N_{15}O_{17}S$ . (Top) The HPLC trace for ligand **hL3A5**. The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1394.714, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1416.696.



**Figure A3B.17. Characterization spectra for ligand hL1D3.** Ligand **hL1D3:**  $C_{70}H_{107}N_{15}O_{19}S$ . (Top) The HPLC trace for ligand **hL1D3.** The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The peaks labeled "i" correspond to the [M+H] peak, expected [M+H] = 1494.767, the peaks labeled "ii" correspond to the [M+Na] peak, expected [M+Na] = 1516.749, and the peaks labeled "ii" correspond to the [M+K] = 1532.723.







**Figure A3B.19. Characterization spectra for ligand hL2fF1.** Ligand **hL2fF1**:  $C_{72}H_{103}FN_{16}O_{18}S$ . (Top) The HPLC trace for ligand **hL2fF1**. The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H]= 1531.742, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1553.724.



Figure A3B.20. Representative characterization spectra for ligand hL2f\*F1. Ligand hL2f\*F1  $C_{72}H_{102}F_2N_{16}O_{18}S$ . (Top) The HPLC trace for ligand hL2f\*F1. The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 2 and fraction 3 respectively. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H]= 1549.733, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na]= 1571.714.



Figure A3B.21. Characterization spectra for ligand hL2fF2. Ligand hL2fF2:  $C_{72}H_{103}FN_{16}O_{18}S$ . (Top) The HPLC trace for ligand hL2fF2. The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The peaks labeled "i" correspond to the [M+H] peak, expected [M+H] = 1531.742, and the peaks labeled "ii" correspond to the [M+Na] peak, expected [M+Na] = 1553.724.



**Figure A3B.22.** Representative characterization spectra for ligand hL2f\*F2. Ligand hL2f\*F2:  $C_{72}H_{102}F_2N_{16}O_{18}S$ . (Top) The HPLC trace for ligand hL2f\*F2. The indicated regions correspond to the two fractions that had the correct mass. (Middle) The MALDI spectra for fraction 2. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1549.733, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1571.714. (Bottom) The MALDI spectra for fraction 3. The indicated peak corresponds to the [M+Na] peak.





**Figure A3B.24. Characterization spectra for ligand hL3fF3.** Ligand **hL3fF3**:  $C_{71}H_{102}FN_{15}O_{17}S$ . (Top) The HPLC trace for ligand **hL3fF3**. The indicated regions correspond to the two fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The peak labeled "i" corresponds to the [M+Na] peak, expected [M+Na] = 1510.718, and the peak labeled "ii" corresponds to the [M+K] peak, expected [M+K] = 1526.692. (Bottom) The MALDI spectra for fraction 2. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1488.736, and the peak labeled "ii" corresponds to the [M+Na] peak.





Figure A3B.26. Characterization spectra for ligand hL3fF4. Ligand hL3fF4:  $C_{71}H_{102}FN_{15}O_{17}S$ . (Top) The HPLC trace for ligand hL3fF4. The indicated regions correspond to the two fractions that had the correct mass. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The peaks labeled "i" correspond to the [M+H] peak, expected [M+H] = 1488.736, and the peaks labeled "ii" correspond to the [M+Na] peak, expected [M+Na] = 1510.718.



Figure A3B.27. Characterization spectra for hL3f\*F4. Ligand hL3f\*F4:  $C_{72}H_{102}F_2N_{16}O_{18}S$ . (Top) The HPLC trace for ligand hL3f\*F4. The indicated regions correspond to the two fractions that had the correct mass. (Middle) The MALDI spectra for fraction 1. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1506.727, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1528.709. (Bottom) The MALDI spectra for fraction 2. The indicated peak corresponds to the [M+Na] peak.



(Top) The HPLC trace for ligand **hL3fF5**. The indicated regions correspond to the fraction that had the correct mass. (Bottom) The MALDI spectra for fraction 1. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1488.736, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1510.718.





**hL3G1V3**:  $C_{82}H_{125}N_{13}O_{19}S$ . (Top) The HPLC trace for ligand **Ak-hL3G1V3**. The indicated peaks i-iii correspond to the three fractions that had the correct mass. The peak labeled "iv" corresponds to oxidized Grubbs I catalyst. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The indicated peaks correspond to the [M+Na] peak, expected [M+Na] = 1650.883.



Figure A3B.31. Characterization spectra for ligand Ak-hL3V2G6. Ligand Ak-hL3V2G6:  $C_{82}H_{125}N_{13}O_{19}S$ . (Top) The HPLC trace for ligand Ak-hL3V2G6. The indicated peaks i-ii correspond to the two fractions that had the correct mass. The peak labeled "iii" corresponds to oxidized Grubbs I catalyst. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The indicated peaks correspond to the [M+Na] peak, expected [M+Na] = 1650.883.



Figure A3B.32. Representative characterization spectra for ligand Ak'-hL3GIV3. Ligand Ak'-hL3GIV3:  $C_{82}H_{125}N_{13}O_{19}S$ . (Top) The HPLC trace for ligand Ak'-hL3GIV3. The indicated peaks i-iii correspond to the three fractions that had the correct mass. The peak labeled "iv" corresponds to oxidized Grubbs I catalyst. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The indicated peaks correspond to the [M+Na] peak, expected [M+Na] = 1650.883.



Figure A3B.33. Characterization spectra for ligand Ak'-hL3V2G6. Ligand Ak'-hL3V2G6:  $C_{82}H_{125}N_{13}O_{19}S$ . (Top) The HPLC trace for ligand Ak'-hL3V2G6. The indicated peaks i-ii correspond to the two fractions that had the correct mass. The peak labeled "iii" corresponds to oxidized Grubbs I catalyst. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The indicated peaks correspond to the [M+Na] peak, expected [M+Na] = 1650.883.



**Figure A3B.34. MALDI spectra for ligand Tz-hL3G1V3.** Ligand **Tz-hL3G1V3**:  $C_{76}H_{112}N_{16}O_{19}S$  and  $C_{152}H_{224}N_{32}O_{38}S_2$  (dimer). (Top) The MALDI trace for fraction 1, which is mostly monomer. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1585.809, and the peak labeled "ii" corresponds to the [M+Na] peak, expected [M+Na] = 1607.791. (Middle) The MALDI spectra for fraction 2, which is mostly monomer. The indicated peak corresponds to the [M+Na] peak. (Bottom) The MALDI spectra for fraction 3, which is the dimeric form of ligand **Tz-hL3G1V3**. The indicated peaks correspond to the [D+Na] peak, expected [D+Na] = 3191.441.



**Figure A3B.35. Characterization spectra for ligand Tz-hL3V2G6.** Ligand **Tz-hL3V2G6**:  $C_{76}H_{112}N_{16}O_{19}S$  and  $C_{152}H_{224}N_{32}O_{38}S_2$  (dimer). (Top) The HPLC trace for ligand **Tz-hL3V2G6**. The indicated peaks correspond to the two fractions with the correct mass. (Middle) The MALDI trace for fraction 1, which is all monomer. The peak labeled "i" corresponds to the [M+H] peak, expected [M+H] = 1585.809, and the peak labeled "i" corresponds to the [M+Na] peak, expected [M+Na] = 1607.791. (Bottom) The MALDI spectra for fraction 3, which is the dimeric form of ligand **Tz-hL3V2G6**. The indicated peak corresponds to the [D+Na] peak, expected [D+Na] = 3192.592.





Figure A3B.37. Characterization spectra for ligand DNBCtermH8. Ligand DNBCtermH8:  $C_{150}H_{226}N_{44}O_{32}S$ . (Top) The HPLC trace for ligand DNBCtermH8. The indicated peaks i-ii correspond to the two fractions that had the correct mass. The peak labeled "iii" corresponds to oxidized Grubbs I catalyst. (Middle, Bottom) The MALDI spectra for fraction 1 and fraction 2 respectively. The indicated peaks correspond to the [M+H] peak, expected [M+H] = 3188.721.



## Appendix 3C

## Supplemental Tables

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hL1	hL2	hL3
AYQLL (hL1a1)	AYYNV (hL2a1)	AGYYY (hL3a1)
YAQLL (hL1a2)	YAYNV (hL2a2)	LAYYY (hL3a2)
YYALL (hL1a3)	YYANV (hL2a3)	LGAYY (hL3a3)
YYQAL (hL1a4)	YYYAV (hL2a4)	LGYAY (hL3a4)
YYQLA (hL1a5)	YYYNA (hL2a5)	LGYYA (hL3a5)

hL1	hL2	]	hL3
YYELL (hL1E3)	fFYYNV (hL2fF1)	LVYYY (hL3V2)	LG <sup>3,4-f</sup> FYY (hL3 <sup>f*</sup> F3)
YYDLL (hL1D3)	Y <sup>f</sup> FYNV (hL2 <sup>f</sup> F2)	LG <sup>f</sup> FYY (hL3 <sup>f</sup> F3)	LGY <sup>3,4-f</sup> FY (hL3 <sup>f*</sup> F4)
	<sup>3,4-f</sup> FYYNV (hL2 <sup>f*</sup> F1)	LGY <sup>f</sup> FY (hL3 <sup>f</sup> F3)	LGYY <sup>3,4-f</sup> F (hL3 <sup>f*</sup> F5)
	Y <sup>3,4-f</sup> FYNV (hL2 <sup>f*</sup> F2)	LGYY <sup>f</sup> F (hL3 <sup>f</sup> F5)	

Triazole Closure	RCM (	Closure
GLVYYY (Tz-hL3G1V3)	OctGLVYYYPen (Ak-hL3G1V3)	PenGLVYYYOct (Ak'-hL3G1V3)
LVYYYG (Tz-hL3V2G6)	OctLVYYYGPen (Ak-hL3V2G6)	PenLVYYYGOct (Ak'-hL3V2G6)

Table A	3C.4.	Linker hit	s from	JXL1	vs. N-	term l	inker	scree
	Hit	Click	<b>X</b> <sub>1</sub>	$\mathbf{X}_2$	<b>X</b> <sub>3</sub>	X <sub>4</sub>	<b>X</b> 5	
	1	L-Pra	р	1	G	G		
	2	L-Pra	р	1	Aib	Aib		
	3	L-Pra	Aib	1	G	G		
	4	-	1	Aib	Aib	G	1	
	5	L-Pra	р	1	G	р	1	
	6	L-Pra	l	р	1			
	7	L-Pra	1	1	Aib			

e A3	C.5. L	inker hit	s from	DNB	vs. C-	term li	inker	SC
	Hit	Click	<b>X</b> <sub>1</sub>	$X_2$	<b>X</b> <sub>3</sub>	<b>X</b> 4	<b>X</b> 5	
	1	L-Pra	G	l	G	l	G	
	2	L-Pra	р	l	Aib	Aib		
	3	L-Pra	l	р	l	G		
	4	L-Pra	l	G	l	р		
	5	L-Pra	р	Aib	G	G		
	6	L-Pra	р	l	G			
	7	L-Pra	1	l	Aib	Aib		
	8	L-Pra	р	G	l	l		
								•

 Table A3C.5. Linker hits from DNB vs. C-term linker screen.

Hit	Click	X <sub>1</sub>	$X_2$	<b>X</b> <sub>3</sub>	X4	<b>X</b> 5	
1	L-Pra	1	G	1	G	1	
2	L-Pra	G	Aib	Aib	G		
3	-	1	1	G	1		
4	L-Pra	р	Aib	G	Aib		
5	L-Pra	р	1	Aib			
6	L-Pra	р	G	р	Aib	Aib	
7	L-Pra	1	G	G			
	Hit 1 2 3 4 5 6 7	HitClick1L-Pra2L-Pra3-4L-Pra5L-Pra6L-Pra7L-Pra	Hit         Click         X1           1         L-Pra         1           2         L-Pra         G           3         -         1           4         L-Pra         p           5         L-Pra         p           6         L-Pra         p           7         L-Pra         1	Hit         Click         X1         X2           1         L-Pra         1         G           2         L-Pra         G         Aib           3         -         1         1           4         L-Pra         p         Aib           5         L-Pra         p         1           6         L-Pra         p         G           7         L-Pra         1         G	HitClickX1X2X31L-Pra1G12L-PraGAibAib3-11G4L-PrapAibG5L-Prap1Aib6L-PrapGp7L-Pra1GG	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$\begin{array}{c c c c c c c c c c c c c c c c c c c $

Hit	Click	<b>X</b> <sub>1</sub>	$\mathbf{X}_2$	<b>X</b> <sub>3</sub>	$X_4$	<b>X</b> 5
1	L-Pra	р	l			
2	L-Pra	G	р	Aib	Aib	
3	L-Pra	1	р	1	Aib	Aib

Table A3C.8. Linker hits from JXL1 vs. C-term linker screen.								
	Hit	Click	X <sub>1</sub>	$\mathbf{X}_2$	<b>X</b> <sub>3</sub>	$X_4$	<b>X</b> 5	
	1	L-Pra	Aib	G	G	1	Aib	
	2	L-Pra	1	G	Aib	G	1	
	3	L-Pra	1	1			1	
	4	L-Pra	G	р	l	1	G	

Structure	Chemical Formula	HPLC Ret. Time (min)	Exp. mass (amu)	Obs. mass (amu)
	C71H110N16O18S	*	[M+H]= 1506.790	*
$ \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \\ \end{array} \\ \end{array} \\ \\ \end{array} \\ \\ \end{array} \\ \end{array} $	C65H106N16O17S	46.5-47 (f1); 47-48.5 (f2)	[M+H]= 1416.677; [M+Na]= 1438.762	1414.613; 1436.605 (f1) 1436.962 (f2)
HO = HO = HI = HO = HO	$C_{65}H_{106}N_{16}O_{17}S$	32.5- 33.5 (f1); 33.5- 34.5 (f2); 34.5- 35.5 (f3); 35.5-36 (f4)	[M+H]= 1416.677; [M+Na]= 1438.762	1415.510; 1437.511 (f1) 1415.678; 1437.650 (f2) 1415.836; 1437.838 (f3) 1415.975; 1437.974 (f4)
HO = O = O = O = O = O = O = O = O = O =	C69H107N15O17S	46.5-48 (f1); 48-48.5 (f2); 48.5-50 (f3)	[M+H]= 1450.777; [M+Na]= 1472.759	1450.047 (f1) 1450.283; 1472.253 (f2); 1450.467; 1472.442 (f3)
HO =	$C_{68}H_{104}N_{16}O_{18}S$	41:30- 44 (f1); 44-46.5 (f2)	[M+H]= 1465.751; [M+Na]= 1487.733; [M+K]= 1503.707	1464.937; 1502.864 (f1) 1465.173; 1487.151 (f2)
$H_{O} = H_{O} = H_{O$	C <sub>68</sub> H <sub>104</sub> N <sub>16</sub> O <sub>18</sub> S	40-43 (f1); 43-45 (f2); 45-46.5 (f3); 46.5-49 (f4)	[M+H]= 1465.751; [M+Na]= 1487.733; [M+K]= 1503.707	1465.417; 1487.401; 1503.373 (f1) 1488.548 (f2) 1465.313; (f3) 1466.837; 1487.835 (f4)

Table A3C.9. Ligand Characterization Data.<sup>1</sup>

Structure	Chemical Formula	HPLC Ret. Time (min)	Exp. mass (amu)	Obs. mass (amu)
HO = O = O = O = O = O = O = O = O = O =	$C_{72}H_{104}N_{16}O_{19}S$	37-40 (f1); 40-44 (f2)	[M+H]= 1530.765	1529.177 (f1) 1529.348; 1551.317 (f2)
$ \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \\ \end{array} \\ \\ \\ \end{array} \\ \\ \end{array} \\ \\ \\ \\ \end{array} \\ \\ \\ \end{array} \\ \\ \\ \end{array} \\ \\ \\ \\ \end{array} \\ \\ \\ \\ \end{array} \\ \\ \\ \\ \\ \end{array} \\ \\ \\ \\ \\ \end{array} \\ \\ \\ \\ \\ \\ \\ \end{array} \\$	C <sub>66</sub> H <sub>100</sub> N <sub>16</sub> O <sub>18</sub> S	3.5-5 (f1); 11.5- 12.5 (f2); 12.5- 13.5 (f3)	[M+H]= 1437.720 [M+Na]= 1459.702	1437.655; 1459.863 (f1) 1459.262 (f2) 1459.433 (f3)
HO = O = O = O = O = O = O = O = O = O =	C66H100N16O18S	6.5-7.5	[M+H]= 1437.720 [M+Na]= 1459.702	1458.747
$HO_{O} = \begin{pmatrix} 0 & H & 0 & 0 \\ H & 0 & H & 0 \\ HO_{O} = \begin{pmatrix} 0 & H & 0 & 0 \\ H & 0 & H & 0 \\ H & 0 & H & 0 \\ H & H & H & H \\ H & H & H & H \\ H & H &$	$C_{66}H_{100}N_{16}O_{18}S$	30.5-31 (f1); 31-33.5 (f2)	[M+H]= 1437.720 [M+Na]= 1459.702	1436.878; 1458.864 (f1) 1437.200; 1458.190 (f2)
HO = O = O = O = O = O = O = O = O = O =	C71H103N15O18S	42-42.5 (f1); 42.5-44 (f2)	[M+H]= 1486.740 [M+Na]= 1508.722	1487.214; 1508.178 (f1) 1486.406; 1508.364 (f2)
$H_{O} = \begin{pmatrix} 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 &$	C <sub>70</sub> H <sub>100</sub> N <sub>16</sub> O <sub>19</sub> S	39-41.5	[M+H]= 1501.715 [M+Na]= 1523.697	1501.307; 1523.288
Structure	Chemical Formula	HPLC Ret. Time (min)	Exp. mass (amu)	Obs. mass (amu)
--	--	---	--	---
$\begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	C <sub>71</sub> H <sub>103</sub> N <sub>15</sub> O <sub>18</sub> S	*	[M+H]= 1485.733	*
$\begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	C <sub>68</sub> H <sub>97</sub> N <sub>15</sub> O <sub>18</sub> S	35-38.5 (f1); 38.5- 40.5 (f2); 40.5- 42.5 (f3)	[M+H]= 1444.693 [M+Na]= 1466.675 [M+K]= 1482.649	1444.662; 1466.648 1482.232 (f1) 1443.219; 1465.181 (f2) 1443.628; 1465.609 (f3)
$ \begin{array}{c} \begin{array}{c} & & & \\ & & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\$	C72H105N15O18S	39-42 (f1); 42-43 (f2); 43-44.5 (f3)	[M+H]= 1500.756 [M+Na]= 1522.738 [M+K]= 1538.712	1499.292; 1537.254 (f1) 1499.644; 1521.604 (f2) 1499.894; 1521.845 (f3)
$\begin{array}{c} & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\$	C <sub>65</sub> H <sub>99</sub> N <sub>15</sub> O <sub>17</sub> S	36.5-40 (f1); 40-42.5 (f2)	[M+H]= 1394.714 [M+Na]= 1416.696 [M+K]= 1432.670	1394.717; 1432.688 (f1) 1394.798; 1416.772 (f2)
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	C65H99N15O17S	40-42.5 (f1); 42.5-45 (f2)	[M+H]= 1394.714 [M+Na]= 1416.696	1393.923; 1415.884 (f1) 1394.117 (f2)
$ \begin{array}{c} \begin{array}{c} & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & &$	C <sub>65</sub> H <sub>99</sub> N <sub>15</sub> O <sub>17</sub> S	39.5-41 (f1); 41-42 (f2)	[M+H]= 1394.714 [M+Na]= 1416.696	1393.788; 1415.760 (f1) 1394.019; 1416.017 (f2)
HO =	C70H107N15O19S	45.5- 48.5 (f1); 51-55.3 (f2)	[M+H]= 1494.767 [M+Na]= 1516.749 [M+K]= 1532.723 [M- H <sub>2</sub> O+H]= 1476.756	1494.693; 1516.670; 1532.633 (f1) 1476.902 (f2)

Structure	Chemical Formula	HPLC Ret. Time (min)	Exp. mass (amu)	Obs. mass (amu)
$HO_{O} = \begin{pmatrix} 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 &$	C71H109N15O19S	45-47.5	[M+H]= 1509.787	1507.978
$ \begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & \\ & & & \\ & & & & \\ & & & & \\ & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ $	C72H103FN16O18S	42-43 (f1); 43-45 (f2)	[M+H]= 1531.742 [M+Na]= 1553.724	1530.483; 1552.463 (f1) 1530.693; 1552.664 (f2)
$ \begin{array}{c} & & & & & \\ & & & & & \\ & & & & & \\ & & & & \\ & & & & \\ & & & $	$C_{72}H_{102}F_2N_{16}O_{18}S$	40-42 (f1); 42-43.5 (f2); 43.5- 44.5 (f3)	[M+H]= 1549.733 [M+Na]= 1571.714	1564.202 (f1) 1548.644; 1570.611 (f2) 1548.868; 1570.840 (f3)
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	C72H103FN16O18S	43.5- 44.5 (f1); 44.5- 45.5 (f2)	[M+H]= 1531.742 [M+Na]= 1553.724	1530.534; 1552.511 (f1) 1530.800; 1552.775 (f2)
HO = (HO) = (H	$C_{72}H_{102}F_2N_{16}O_{18}S$	41.5- 44.5 (f1); 44.5- 45.5 (f2); 45.5- 46.5 (f3)	[M+H]= 1549.733 [M+Na]= 1571.714	1564.199 (f1) 1548.644; 1570.611 (f2) 1576.669 (f3)
	C <sub>74</sub> H <sub>109</sub> N <sub>15</sub> O <sub>18</sub> S	43-45	[M+H]= 1528.787 [M+Na]= 1550.718	1527.260; 1550.217

Structure	Chemical Formula	HPLC Ret. Time (min)	Exp. mass (amu)	Obs. mass (amu)
$\begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	C <sub>71</sub> H <sub>102</sub> FN <sub>15</sub> O <sub>17</sub> S	42-45 (f1); 45-46.5 (f2)	[M+H]= 1488.736 [M+Na]= 1510.718 [M+K]= 1526.692	1524.900 (f1) 1487.261; 1509.233 (f2)
$ \begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	$C_{71}H_{101}F_2N_{15}O_{17}S$	46-47.5	[M+H]= 1506.727 [M+Na]= 1528.709	1506.250; 1527.212
$ \begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	C71H102FN15O17S	44-45.5 (f1); 45.5-47 (f2)	[M+H]= 1488.736 [M+Na]= 1510.718 [M+K]= 1526.692	1487.286; 1509.237 (f1) 1487.513; 1509.467 (f2)
$ \begin{array}{c} & & & \\ & & & $	C71H101F2N15O17S	46-47.5 (f1); 47.5- 49.5 (f2)	[M+H]= 1506.727 [M+Na]= 1528.709	1505.647; 1527.611 (f1) 1505.927 (f2)
$ \begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	C71H102FN15O17S	46-47.5	[M+H]= 1488.736 [M+Na]= 1510.718	1489.199; 1509.170
$ \begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	$C_{71}H_{101}F_2N_{15}O_{17}S$	42-46 (f1); 46-49.5 (f2)	[M+H]= 1506.727 [M+K]= 1544.683	1544.018 (f1) 1544.363 (f2)
$\begin{array}{c} & \underbrace{Me}_{H_{2}}^{Me} \\ & \underbrace{H}_{2}^{NH} \\ & \underbrace{H}_{2}^{NH} \\ & \underbrace{H}_{1}^{NH} \\ & \underbrace{H}_{1}^{N$	C <sub>82</sub> H <sub>125</sub> N <sub>13</sub> O <sub>19</sub> S	49-50 (f1); 50-50.5 (f2); 50.5-51 (f3)	[M+H]= 1628.901 [M+Na]= 1650.883	1649.923 (f1) 1648.839 (f2) 1649.211 (f3)
$\begin{array}{c} & & & & & & \\ & & & & & & \\ & & & & & $	C <sub>82</sub> H <sub>125</sub> N <sub>13</sub> O <sub>19</sub> S	48.5- 49.5 (f1); 50-50.5 (f2); 50.5-51 (f3)	[M+H]= 1628.901 [M+Na]= 1650.883	1648.836 (f1) 1649.201 (f2) 1649.211 (f3)

Structure	Chemical Formula	HPLC Ret. Time (min)	Exp. mass (amu)	Obs. mass (amu)
$\begin{array}{c c} & & & & & & & & & & & & & & & & & & &$	C <sub>82</sub> H <sub>125</sub> N <sub>13</sub> O <sub>19</sub> S	48.5- 49.5 (f1); 50-51 (f2)	[M+H]= 1628.901 [M+Na]= 1650.883	1651.204 (f1) 1650.208 (f2)
$\begin{array}{c c} & & & & & & & & & & & & & & & & & & &$	C <sub>82</sub> H <sub>125</sub> N <sub>13</sub> O <sub>19</sub> S	34.5-35 (f1); 35-36.5 (f2)	[M+H]= 1628.901 [M+Na]= 1650.883	1650.626 (f1) 1649.784 (f2)
$ \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \\ \\ \end{array} \end{array} \end{array} \\ \begin{array}{c} \\ \\ \end{array} \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} $	C <sub>76</sub> H <sub>112</sub> N <sub>16</sub> O <sub>19</sub> S	42-44	[M+H]= 1585.809 [M+Na]= 1607.791	1584.127; 1606.108
$\begin{array}{c} \begin{array}{c} H_{2}N \\ $	C76H112N16O19S	17-18 (f1); 18-18.5 (f2)	[M+H]= 1585.809 [M+Na]= 1607.791	1584.743; 1606.728 (f1) 1606.855 (f2)
$\begin{array}{c} H_{0} \\ H_{0} \\$	$C_{152}H_{224}N_{32}O_{36}S_2$	44-46	[M+Na]= 3192.592	3190.337
$\begin{array}{c} \begin{array}{c} & & & & & & & & & & & & & & & & & & &$	$C_{152}H_{224}N_{32}O_{38}S_2$	19-20	[M+Na]= 3192.252	3191.441

Structure	Chemical Formula	HPLC Ret. Time (min)	Exp. mass (amu)	Obs. mass (amu)
$H_{2N} \xrightarrow{Me}_{HN} \underbrace{Cterm}_{HN} \xrightarrow{H}_{HN} \underbrace{H_{2N}}_{HN} \xrightarrow{H}_{HN} \underbrace{Cterm}_{HN} \xrightarrow{H}_{HN} \underbrace{H_{2N}}_{HN} \underbrace{H_{2N}}_{HN} \xrightarrow{H}_{HN} \underbrace{H_{2N}}_{HN} \underbrace{H_{2N}}_{HN$	C76H120N24O17S	36.5-38 (f1); 38-39 (f2)	[M+H]= 1673.906 [M- N <sub>2</sub> +H]= 1647.916	1645.33; 1671.71 (f1) 1672.42 (f2)
	$C_{150}H_{226}N_{44}O_{32}S$	44-45.5 (f1); 45.5- 46.5 (f2)	[M+H]= 3188.721	3187.95 (f1) 3190.21 (f2)

\*Spectra lost due to computer failure

## References

(1) Expected masses were calculated using the mass calculator at the following: http://www.lfd.uci.edu/~gohlke/molmass/?q=C152H224N32O38S2Na.