Direct Comparison of Linear and Macrocyclic Compound Libraries As A source of Protein Ligands

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ELECTRONIC SUPPPORTING INFORMATION

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Supplementary Material and Methods

All the Fmoc-protected amino acids were purchased from AnaSpec (Fremont, CA). Tentagel resin was purchased from Rapp Polymere GmbH (German). All other reagents were purchased from Sigma-Aldrich or Alfa Aesar, unless otherwise specified. All of the chemical reagents and solvents from commercial sources were used without further purification. 5 mL and 10 mL disposable reaction columns (Intavis AG) were used as reaction vessels for solid phase synthesis. Syntheses of peptoids under microwave conditions were performed in a 1500 W microwave oven (GE model JE 1860BH04) with 10% power. HPLC was carried out on Waters systems equipped with Waters 1525 binary HPLC pumps and a 2487 dual λ absorbance detector, or a 2998 photodiode array detector. The mobile phase comprised of buffer A (H2O containing 20% CH3CN and 0.1% trifluoroacetic acid (TFA)) and buffer B (CH3CN containing 0.1% TFA). Analytical HPLC was conducted using a Vydac C-18 column (5 µm, 250 x 4.6 mm, Alltech, Deerfield, IL) at a flow rate of 1.0 mL/min with UV detection at 220 nm. MS and MS/MS (MALDI-TOF) were performed on a 4800 Proteomics Analyzer (Applied Biosystems) with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. All steps involving water utilized distilled water filtered through a Barnstead Nanopure filtration system (Thermo Scientific).

Library Synthesis

The peptoid library was linked to Tentagel HL-NH2 beads (75 µM diameter, 0.45 mmol/g) by a fixed sequence of amino acid and peptoid residues as shown in the main text. Resin (2.0 g, approximately 10^7 beads) was swelled in DMF for 2 hours prior to use. Amino acids were coupled with diisopropylcarbodiimide (DIC) and ethyl-2-cyano-2-(hydroxyimino) acetate (Oxyma) in DMF without base added to prevent possible racemization (Amino acid: DIC: Oxyma: bead capacity = 4:4:4:1). Methionine was the first amino acid loaded onto the resin. After methionine coupling, Fmoc was removed by 20% piperidine and washed thoroughly in DMF. The rest of the linker was synthesized by standard peptoid sub-monomer synthesis as previously described. A 95:5 mixture of orthogonally protected glutamic acid esters (Fmoc-Glu(OAll)-OH : Fmoc-Glu(OtBu)-OH 95:5, 4 equiv) was used at the last position of the linker region. Fmoc was removed by 20% piperidine and washed thoroughly in DMF. Split and pool synthesis was used to construct the variable region with a theoretical diversity of 50,625 compounds. This was accomplished by incorporating the 15 amines listed in the main text via microwave-assisted sub-monomer peptoid synthesis at four variable positions. Following peptoid synthesis, a fixed region with 0-2 N-methoxyethylamine peptoid units was added to vary the length of different libraries. Allyl esters were deprotected by Pd(0)(PPh3)4 (2 equiv) in 37:2:1 ACN: Morpholine: AcOH M under argon for 2 h at room temperature. All beads were then washed with 0.5% DIEA and 0.5% sodium.
diethyldithiocarbamate in DMF for three times, 5 min each. Cyclization was carried out with PyBOP (4 equiv, 1M) and DIEA (10 equiv, 2.5M) in DMF for 2 hours, and repeated once. Following cyclization, the beads were treated with a mixture of 94% TFA, 2% triisopropylsilane, 2% water, and 2% thioanisole mixture for 2 h to remove side chain protecting groups. After draining the TFA mixture, the beads were washed thoroughly with DCM, then with DMF. For hit re-synthesis, each compound was synthesized following the procedure described above except for the variable region, which was synthesized according to the hit sequence. All re-synthesized hit compounds were purified by HPLC and dried by lyophilization.

**On-bead Screening**

Approximately 500,000 beads, 10 equivalents of theoretical diversity, from each library were combined to form a pool of 3,000,000 beads. Hit compounds were isolated by magnetic screening method as previously described. Specifically, all beads were first thoroughly washed with DMF, then gradually washed with 7:1 DMF:water, 2:1 DMF:water, 1:1 DMF:water, 1:2 DMF:water, 1:7 DMF:water solutions for three times each. To completely remove DMF, all beads were then washed several times with water and then with TBST buffer (50 mM Tris, pH 7.4, 150 mM, NaCl, 0.1% Tween-20) and equilibrated with TBST overnight. Before screening all beads were incubated with Starting Block TBS blocking buffer (Pierce) at room temperature for 4 h. The blocking buffer was filtered out and the beads were incubated with 1:25 (2 ml in 50 ml buffer) streptavidin-coupled Dynabeads M-280 (Invitrogen) in blocking buffer for 2 hours at room temperature. After incubation, hit beads were isolated with a strong magnet. These beads were collected and stripped of bound protein by incubating in 50% acetonitrile/water (3 x 5 mL) for 30 min followed by incubation in acetonitrile for 60 min. Each bead was then isolated into a single well of a 96-well filter plate (Millipore) and washed with water.

**Fluorescent Polarization Assay**

For single-bead labelling with fluorescein azide, 130 µl of a 1:1 mixture of t-BuOH and water was first added to each well. A fluorescein azide (structure shown above) methanol solution (0.3 mM) was dispensed to each well, 10 µl per well.
10 µl of freshly prepared water solution of CuSO₄ (0.3 mM) and ascorbic acid (0.6 mM) was then dispensed to each well, and the whole plate was sealed and left on shaker for 24 hours. Final concentrations were: fluorescein azide (20 µM), CuSO₄ (20 µM), ascorbic acid (40 µM). The beads were then thoroughly washed with water and then CH₃CN. Compound was cleaved from the bead by incubating each bead in 20 µL of a 50 mg/ml solution of cyanogen bromide (CNBr) dissolved in AcOH:CH₃CN:water (5:4:1) overnight at room temperature. The next day, the CNBr solution was evaporated by vacuum centrifugation. After drying under vacuum, the cleaved compounds were dissolved in 60% acetonitrile/water, the final concentration of all the compounds were normalized to 100 nM by UV absorption at 495nm (ε₄₉₅ = 78,000 M⁻¹cm⁻¹). Serial dilution of the streptavidin (homo-tetramer) in PBS buffer was dispensed in 384-well plate, 10 µl each, and then the dissolved compounds in PBS were added in each well. The whole plate was then mounted on EnVision plate reader (PerkinElmer), and FP data was taken as direct read outs of each well in mP using 450 excitation and 515 nm emission filters. Fitting of the saturation curves to obtain Kᵩ values was accomplished using Prism (GraphPad Software, Inc.) with a one-site specific saturation binding model. For re-synthesized compounds, after purification, each compound was dissolved in PBS buffer to obtain a 10 nM solution. Serial dilutions of streptavidin were prepared in a 96-well plate, 50 µl per well. 50 µl of compound solution was added to each well and Kᵩ values were obtained as described above.

All 450 hit identified from screening were measured using this method. The raw Kᵩ values are listed in Table S3. Sequences were obtained by tandem mass spectroscopy.

To determine if the identified compounds bind to streptavidin at the same binding site of biotin, we performed a biotin competition assay. Re-synthesized KYGG-1171 and KYGG-1181 were measured by FP at the presence of 0 µM, 320 µM and 1 mM biotin respectively. Kᵩ values were obtained from fitting FP data as described above. As shown in Figure S3, no significant change was observed, suggesting that KYGG-1171 and KYGG-1181 do not bind to the biotin-binding site.

Hit characterization

For hit sequence identification, all solution was first evaporated by vacuum centrifugation and the dry compound in each well was dissolved in 20 µL of a 75:25 mixture of water:CH₃CN containing 0.1% TFA. 0.6 µL from each well was co-spotted with a 10 mg/mL solution of CHCA dissolved in 50% acetonitrile in water containing 0.1% TFA. The spot was dried, and the mass spectra and tandem mass spectra of these compounds were collected using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems). For quality control purpose and to determine if sequence from different libraries could be differentiated, 20 beads
were isolated manually from each library. All 120 beads are combined sequenced as a pool. The results are listed in Table S2.
Figure S1. Optimization of cyclization efficiency of peptoids with various ring sizes. Besides the desired cyclic product, dimer and uncyclized linear peptoids are identified as major by-products.
Figure S2. Encoding with mixture of glutamic acid esters. A) HPLC shows a 95:5 mixture of Fmoc-Glu(OAll)-OH and Fmoc-Glu(OtBu)-OH coupled after peptoid provides 95:5 products after cleavage. B) HPLC shows clear trace of cyclic and linear compounds cleaved from the single bead after on-bead cyclization. (See main text Figure 1 for reaction) C) Typical MS spectrum of cleaved product from single bead. [M+1]⁺ = cyclic compound, [M+18+1]⁺ = linear encoding tail, [M+23]⁺ = cyclic compound + Na peak. D) MS/MS spectrum shows that sequence of peptoid can be easily obtained from linear encoding tail cleaved from single bead.
Figure S3. K\textsubscript{D} of KYG-1171 and KYG-1181 at the presence of biotin. A) Re-synthesized KYG-1171 titrated with streptavidin with no biotin (K\textsubscript{D}=10.71 µM), 320 µM biotin (1:1 to streptavidin monomer, K\textsubscript{D}=14.83 µM), 1 mM biotin (K\textsubscript{D}=14.05 µM). B) Re-synthesized KYG-1181 titrated with streptavidin with no biotin (K\textsubscript{D}=12.51 µM), 320 µM biotin (1:1 to streptavidin monomer, K\textsubscript{D}=13.53 µM), 1 mM biotin (K\textsubscript{D}=14.54 µM).
Figure S4. Comparison of $K_D$ obtained from single-bead FP assay and re-synthesized compound. A) Single-bead FP of each of the 8 repeats of KYG-1181. Compound #1-#8 $K_D$=1.1, 8.9, 2.0, 1.9, 0.4, 2.0, 6.1, 10.0 µM respectively. B) Re-synthesized KYG-1181 measured by FP. $K_D$=8.8 µM.
Table S1. Optimization of macrocyclization condition. Reaction as shown in Figure S1.
Percentage of linear, dimer and cyclized products are determined by HPLC peak areas.

| Condition       | Linear | Dimer | Cyclized |
|-----------------|--------|-------|----------|
| Trimer          | PyBOP, HOBt, DIPEA, 1 h X 2 | 76%   | 11%      | 13%      |
| Trimer          | PyAOP, HOAt, DIPEA, 1 h X 2 | 73%   | 5%       | 22%      |
| Trimer          | PyBOP, HOBt, DIPEA, 2 h X 4 | 52%   | 21%      | 27%      |
| Tetramer        | PyBOP, HOBt, DIPEA, 1 h X 2 | 24%   | 1%       | 75%      |
| Tetramer        | PyBOP, HOBt, DIPEA, 2 h X 2 | 11%   | 1%       | 88%      |
| Pentamer        | PyBOP, HOBt, DIPEA, 1 h X 2 | 10%   | 5%       | 85%      |
| Hexamer         | PyBOP, HOBt, DIPEA, 1 h X 2 | 7%    | 8%       | 85%      |
| Heptamer        | PyBOP, HOBt, DIPEA, 1 h X 2 | 8%    | 6%       | 86%      |
| Octamer         | PyBOP, HOBt, DIPEA, 1 h X 2 | 5%    | 13%      | 82%      |
Table S2. Sequences of 120 quality control beads. 20 beads from each library. Letter codes are listed in main text Figure 1. The source of each sequence is listed in the last column.

| #     | Sequence | lib | #     | Sequence | lib | #     | Sequence | lib |
|-------|----------|-----|-------|----------|-----|-------|----------|-----|
| QC-1-01 | V C Y S | L2  | QC-1-41 | I K S L | L3  | QC-1-81 | W W I P | L3  |
| QC-1-02 | W L T C | C2  | QC-1-42 | W Q M F | L2  | QC-1-82 | P P Q S | C3  |
| QC-1-03 | I M L K | L2  | QC-1-43 | C P I I | C3  | QC-1-83 | P A P F | C3  |
| QC-1-04 | W Q V F | L3  | QC-1-44 | T M K L | C2  | QC-1-84 | A K M S | L3  |
| QC-1-05 | Q V L F | L3  | QC-1-45 | V Y Q K | C2  | QC-1-85 | T H M L | L2  |
| QC-1-06 | I W K Y | L2  | QC-1-46 | L A S M | L2  | QC-1-86 | C K S I | C2  |
| QC-1-07 | V Y M L | L1  | QC-1-47 | L S C M | C1  | QC-1-87 | K H F Y | C3  |
| QC-1-08 | C C F T | L3  | QC-1-48 | L W V W | C1  | QC-1-88 | V P P W | C2  |
| QC-1-09 | H K S A | C2  | QC-1-49 | Y Y A I | L1  | QC-1-89 | K Y Y K | C2  |
| QC-1-10 | F A C I | L2  | QC-1-50 | H P M Y | C1  | QC-1-90 | M P Q W | C1  |
| QC-1-11 | F T Y P | C3  | QC-1-51 | A H W V | L1  | QC-1-91 | A M F I | L1  |
| QC-1-12 | C P F P | L3  | QC-1-52 | M I Q P | C1  | QC-1-92 | K M V K | C2  |
| QC-1-13 | I W Y Q | C2  | QC-1-53 | H Y T I | L2  | QC-1-93 | I V Y C | L3  |
| QC-1-14 | T F Y A | C3  | QC-1-54 | K Y Y P | L2  | QC-1-94 | H I Q T | L1  |
| QC-1-15 | Q M A H | C2  | QC-1-55 | I L M P | C3  | QC-1-95 | W P S L | L2  |
| QC-1-16 | W Y P H | C3  | QC-1-56 | W L Y L | C1  | QC-1-96 | H L F S | L2  |
| QC-1-17 | Y K Y K | L2  | QC-1-57 | F L P A | L3  | QC-1-97 | H F S V | C3  |
| QC-1-18 | F Q S K | C1  | QC-1-58 | Y A I C | L1  | QC-1-98 | P C P I | L2  |
| QC-1-19 | M P V A | L1  | QC-1-59 | Q C F I | C1  | QC-1-99 | H S P A | L3  |
| QC-1-20 | A L W P | C1  | QC-1-60 | C M L I | C3  | QC-1-100 | M I L I | L2  |
| QC-1-21 | T Q Y Y | C1  | QC-1-61 | K L Y H | L1  | QC-1-101 | A W H M | C2  |
| QC-1-22 | S P A S | L3  | QC-1-62 | P M L W | C1  | QC-1-102 | L S A H | C2  |
| QC-1-23 | H W M W | L3  | QC-1-63 | K I S Q | L2  | QC-1-103 | M C P M | L3  |
| QC-1-24 | V S S P | L1  | QC-1-64 | T F C P | L2  | QC-1-104 | A F Q C | L2  |
| QC-1-25 | W W M F | L2  | QC-1-65 | W V S I | L2  | QC-1-105 | A F A M | C2  |
| QC-1-26 | S C Y F | L3  | QC-1-66 | F I T A | C1  | QC-1-106 | I V L V | L1  |
| QC-1-27 | Q Q W P | L2  | QC-1-67 | V Q H T | L3  | QC-1-107 | S H Y Y | C3  |
| QC-1-28 | S A W K | C3  | QC-1-68 | M T Y Y | L3  | QC-1-108 | Y A F P | C2  |
| QC-1-29 | L Y F C | C3  | QC-1-69 | S L L W | C1  | QC-1-109 | F M M V | L3  |
| QC-1-30 | H C A Q | L1  | QC-1-70 | Q Q F M | L2  | QC-1-110 | P T Y I | L2  |
| QC-1-31 | A K T H | C2  | QC-1-71 | I K H C | L2  | QC-1-111 | P F P A | L1  |
| QC-1-32 | Q T M V | L3  | QC-1-72 | P T P W | L2  | QC-1-112 | C A L V | C3  |
| QC-1-33 | M K Y K | C1  | QC-1-73 | M H T Y | C2  |
| QC-1-34 | M Y M M | C3  | QC-1-74 | F A W I | L2  |
| QC-1-35 | H Q T Q | L2  | QC-1-75 | H L F Q | C3  |
| QC-1-36 | I V H V | C3  | QC-1-76 | L Y C K | L3  |
| QC-1-37 | F A V P | C1  | QC-1-77 | H H Y W | L1  |
| QC-1-38 | H L Q S | L1  | QC-1-78 | P T W Y | C3  |
| QC-1-39 | W I Q W | C2  | QC-1-79 | K F T F | L3  |
| QC-1-40 | F F F F | C3  | QC-1-80 | L Y I T | C1  |
### Table S3. a) Single-bead FP of all hits from Library C1.

| #    | Sequence | Kd | #    | Sequence | Kd | #    | Sequence | Kd |
|------|----------|----|------|----------|----|------|----------|----|
| KYG 111-01 | Q W Y C | 197 | KYG 1141 | A W W K | 60 | KYG 1164 | L M H T | 32 |
| KYG 111-02 | H W L S | 151 | KYG 1141 | A W W K | 41 | KYG 1164 | L M H T | 40 |
| KYG 111-03 | Y W W P | 174 | KYG 1142 | A Y F K | 46 | KYG 1164 | L M H T | 27 |
| KYG 111-04 | T V H K | 51  | KYG 1142 | A Y F K | 39 | KYG 1171 | I W W Y | 21 |
| KYG 111-05 | H K I Y | 25  | KYG 1142 | A Y F K | 59 | KYG 1171 | I W W Y | 2.6 |
| KYG 111-06 | I W H V | 108 | KYG 1142 | A Y F K | 46 | KYG 1171 | I W W Y | 5.0 |
| KYG 111-07 | F W V P | 125 | KYG 1143 | T V Y V | 46 | KYG 1171 | I W W Y | 3.0 |
| KYG 111-08 | P K I F | 51  | KYG 1143 | T V Y V | 39 | KYG 1171 | I W W Y | 4.0 |
| KYG 111-09 | A H K Q | 85  | KYG 1143 | T V Y V | 34 | KYG 1171 | I W W Y | 7.0 |
| KYG 111-10 | L L Y K | 108 | KYG 1143 | T V Y V | 62 | KYG 1171 | I W W Y | 8.0 |
| KYG 111-11 | A W S K | 48  | KYG 1144 | W I P C | 26 | KYG 1172 | T P W A | 7.1 |
| KYG 111-12 | L V I Y | 46  | KYG 1144 | W I P C | 49 | KYG 1172 | T P W A | 9.0 |
| KYG 111-13 | M F K Y | 147 | KYG 1144 | W I P C | 32 | KYG 1172 | T P W A | 7.0 |
| KYG 111-14 | L I L W | 45  | KYG 1144 | W I P C | 35 | KYG 1172 | T P W A | 10.0 |
| KYG 111-15 | F T K C | 61  | KYG 1151 | L I H L | 57 | KYG 1172 | T P W A | 7.0 |
| KYG 111-16 | H Q F A | 138 | KYG 1151 | L I H L | 22 | KYG 1172 | T P W A | 11 |
| KYG 111-17 | L T K F | 79  | KYG 1151 | L I H L | 56 | KYG 1172 | T P W A | 3.0 |
| KYG 111-18 | W A L A | 141 | KYG 1151 | L I H L | 48 | KYG 1173 | T P W L | 21 |
| KYG 111-19 | T Y Q W | 48  | KYG 1151 | L I H L | 31 | KYG 1173 | T P W L | 23 |
| KYG 112-01 | Q W T F | 139 | KYG 1152 | L Y W S | 26 | KYG 1173 | T P W L | 2.0 |
| KYG 112-02 | Q W T F | 123 | KYG 1152 | L Y W S | 57 | KYG 1173 | T P W L | 2.0 |
| KYG 112-03 | Q M W H | 84  | KYG 1152 | L Y W S | 38 | KYG 1173 | T P W L | 1.0 |
| KYG 112-04 | Q M W H | 58  | KYG 1152 | L Y W S | 53 | KYG 1173 | T P W L | 31 |
| KYG 112-05 | L W L A | 103 | KYG 1152 | L Y W S | 42 | KYG 1173 | T P W L | 2.0 |
| KYG 112-06 | L W L A | 61  | KYG 1153 | K F S I | 49 | KYG 1181 | L W W W | 1.0 |
| KYG 112-07 | S A Q I | 107 | KYG 1153 | K F S I | 59 | KYG 1181 | L W W W | 9.0 |
| KYG 112-08 | S A Q I | 73  | KYG 1153 | K F S I | 23 | KYG 1181 | L W W W | 2.0 |
| KYG 112-09 | L K L L | 85  | KYG 1153 | K F S I | 56 | KYG 1181 | L W W W | 2.0 |
| KYG 112-10 | L K L L | 108 | KYG 1153 | K F S I | 39 | KYG 1181 | L W W W | 0.4 |
| KYG 112-11 | K C W Y | 148 | KYG 1161 | H Y T W | 42 | KYG 1181 | L W W W | 2.0 |
| KYG 112-12 | K C W Y | 119 | KYG 1161 | H Y T W | 28 | KYG 1181 | L W W W | 6.0 |
| KYG 112-13 | A Y W V | 114 | KYG 1161 | H Y T W | 46 | KYG 1181 | L W W W | 10.0 |
| KYG 112-14 | A Y W V | 148 | KYG 1161 | H Y T W | 49 |
| KYG 112-15 | L F L A | 67  | KYG 1161 | H Y T W | 27 |
| KYG 112-16 | L F L A | 60  | KYG 1161 | H Y T W | 43 |
| KYG 112-17 | L F L A | 50  | KYG 1162 | F F W S | 32 |
| KYG 112-18 | M H M W | 87  | KYG 1162 | F F W S | 31 |
| KYG 112-19 | M H M W | 59  | KYG 1162 | F F W S | 17 |
| KYG 112-20 | M H M W | 45  | KYG 1162 | F F W S | 32 |
| KYG 112-21 | W W W Q | 91  | KYG 1162 | F F W S | 41 |
| KYG 112-22 | W W W Q | 84  | KYG 1162 | F F W S | 18 |
| KYG 112-23 | W W W Q | 99  | KYG 1163 | L Y H T | 33 |
| KYG 112-24 | V C T L | 80  | KYG 1163 | L Y H T | 47 |
| KYG 112-25 | V C T L | 69  | KYG 1163 | L Y H T | 42 |
| KYG 112-26 | V C T L | 49  | KYG 1163 | L Y H T | 42 |
| KYG 112-27 | S P Y Y | 97  | KYG 1163 | L Y H T | 23 |
| KYG 112-28 | S P Y Y | 51  | KYG 1163 | L Y H T | 45 |
| KYG 112-29 | S P Y Y | 43  | KYG 1164 | L M H T | 37 |
| KYG 112-30 | A W W K | 39  | KYG 1164 | L M H T | 49 |
| KYG 112-31 | A W W K | 48  | KYG 1164 | L M H T | 24 |

Continued on next page.
Table S3. b) Single-bead FP of all hits from Library C2.

| #    | Sequence | Kd | #    | Sequence | Kd | #    | Sequence | Kd |
|------|----------|----|------|----------|----|------|----------|----|
| KYG 121-01 | C H T H | 96 | KYG 1232 | I Q L W | 73 | KYG 1252 | W C C W | 31 |
| KYG 121-02 | F C M I | 68 | KYG 1232 | I Q L W | 54 | KYG 1252 | W C C W | 26 |
| KYG 121-03 | A V W M | 129 | KYG 1232 | I Q L W | 57 | KYG 1252 | W C C W | 42 |
| KYG 121-04 | M M K W | 90 | KYG 1233 | T F V V | 93 | KYG 1261 | P V A I | 29 |
| KYG 121-05 | S F V A | 55 | KYG 1233 | T F V V | 43 | KYG 1261 | P V A I | 1.0 |
| KYG 121-06 | S I F K | 193 | KYG 1233 | T F V V | 76 | KYG 1261 | P V A I | 27 |
| KYG 121-07 | T L P I | 75 | KYG 1234 | Q Q W Q | 34 | KYG 1261 | P V A I | 13 |
| KYG 121-08 | T L W H | 39 | KYG 1234 | Q Q W Q | 61 | KYG 1261 | P V A I | 2.2 |
| KYG 121-09 | P S W H | 182 | KYG 1234 | Q Q W Q | 90 | KYG 1261 | P V A I | 33 |
| KYG 121-10 | L K V V | 112 | KYG 1235 | L Q P A | 51 |
| KYG 121-11 | C K W M | 182 | KYG 1235 | L Q P A | 38 |
| KYG 121-12 | L K F M | 66 | KYG 1235 | L Q P A | 34 |
| KYG 121-13 | S H K F | 148 | KYG 1241 | I H W C | 30 |
| KYG 121-14 | M S F L | 98 | KYG 1241 | I H W C | 25 |
| KYG 121-15 | M K S I | 61 | KYG 1241 | I H W C | 23 |
| KYG 121-16 | T T C W | 145 | KYG 1241 | I H W C | 25 |
| KYG 1221 | Q A H W | 70 | KYG 1242 | L Y W F | 58 |
| KYG 1221 | Q A H W | 71 | KYG 1242 | L Y W F | 68 |
| KYG 1222 | L Y K W | 79 | KYG 1242 | L Y W F | 45 |
| KYG 1222 | L Y K W | 149 | KYG 1242 | L Y W F | 45 |
| KYG 1223 | P W W L | 69 | KYG 1243 | A H I L | 32 |
| KYG 1223 | P W W L | 116 | KYG 1243 | A H I L | 70 |
| KYG 1224 | I Q C F | 60 | KYG 1243 | A H I L | 43 |
| KYG 1224 | I Q C F | 71 | KYG 1243 | A H I L | 64 |
| KYG 1225 | L W I P | 99 | KYG 1244 | H V C I | 57 |
| KYG 1225 | L W I P | 129 | KYG 1244 | H V C I | 62 |
| KYG 1226 | W F L C | 69 | KYG 1244 | H V C I | 43 |
| KYG 1226 | W F L C | 79 | KYG 1244 | H V C I | 33 |
| KYG 1227 | W H M L | 103 | KYG 1251 | C K L M | 45 |
| KYG 1227 | W H M L | 103 | KYG 1251 | C K L M | 19 |
| KYG 1228 | H W W I | 111 | KYG 1251 | C K L M | 45 |
| KYG 1228 | H W W I | 88 | KYG 1251 | C K L M | 37 |
| KYG 1231 | W Q W F | 93 | KYG 1251 | C K L M | 51 |
| KYG 1231 | W Q W F | 38 | KYG 1252 | W C C W | 22 |
| KYG 1231 | W Q W F | 88 | KYG 1252 | W C C W | 45 |

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Table S3. c) Single-bead FP of all hits from Library C3.

| #     | Sequence | Kd | #     | Sequence | Kd  |
|-------|----------|----|-------|----------|-----|
| KYG 131-01 | I H F P | 76 | KYG 1333 | I F P P | 100 |
| KYG 131-02 | P V P F | 92 | KYG 1334 | L F A Q | 45  |
| KYG 131-03 | T M C M | 86 | KYG 1334 | L F A Q | 92  |
| KYG 131-04 | F I A K | 190 | KYG 1334 | L F A Q | 51  |
| KYG 131-05 | T Q I W | 82 | KYG 1341 | L H H L | 30  |
| KYG 131-06 | F C M Y | 129 | KYG 1341 | L H H L | 32  |
| KYG 131-07 | Y K V V | 200 | KYG 1341 | L H H L | 30  |
| KYG 131-08 | Y L V K | 43 | KYG 1341 | L H H L | 44  |
| KYG 131-09 | I T A H | 193 | KYG 1342 | A Q W A | 60  |
| KYG 131-10 | H H H M | 33 | KYG 1342 | A Q W A | 67  |
| KYG 131-11 | F S M A | 169 | KYG 1342 | A Q W A | 55  |
| KYG 1321 | V W C F | 108 | KYG 1342 | A Q W A | 62  |
| KYG 1321 | V W C F | 110 | KYG 1343 | W Y Y A | 46  |
| KYG 1322 | L P M K | 80 | KYG 1343 | W Y Y A | 66  |
| KYG 1322 | L P M K | 120 | KYG 1343 | W Y Y A | 73  |
| KYG 1323 | H M H W | 112 | KYG 1343 | W Y Y A | 40  |
| KYG 1323 | H M H W | 105 | KYG 1351 | L C C K | 1.0 |
| KYG 1324 | C H M L | 54 | KYG 1351 | L C C K | 8.0 |
| KYG 1324 | C H M L | 83 | KYG 1351 | L C C K | 7.5 |
| KYG 1325 | W F A L | 76 | KYG 1351 | L C C K | 9.9 |
| KYG 1325 | W F A L | 79 | KYG 1351 | L C C K | 10.0 |
| KYG 1326 | L H L T | 105 | KYG 1352 | H W V W | 70  |
| KYG 1326 | L H L T | 52 | KYG 1352 | H W V W | 84  |
| KYG 1327 | P F M V | 76 | KYG 1352 | H W V W | 69  |
| KYG 1327 | P F M V | 128 | KYG 1352 | H W V W | 76  |
| KYG 1328 | I Q C L | 93 | KYG 1352 | H W V W | 78  |
| KYG 1328 | I Q C L | 82 | KYG 1361 | V H W P | 41  |
| KYG 1331 | I L M V | 63 | KYG 1361 | V H W P | 20  |
| KYG 1331 | I L M V | 93 | KYG 1361 | V H W P | 31  |
| KYG 1331 | I L M V | 46 | KYG 1361 | V H W P | 1.7 |
| KYG 1332 | Q P Y L | 83 | KYG 1361 | V H W P | 31  |
| KYG 1332 | Q P Y L | 39 | KYG 1361 | V H W P | 33  |
| KYG 1332 | Q P Y L | 65 | KYG 1361 | V H W P | 33  |
| KYG 1333 | I F P P | 79 | KYG 1361 | V H W P | 33  |
| KYG 1333 | I F P P | 39 |KYG 1361 | V H W P | 33  |

Continued on next page.
Table S3. d) Single-bead FP of all hits from Library L1.

| #       | Sequence | Kd | #       | Sequence | Kd |
|---------|----------|----|---------|----------|----|
| KYG 211-01 | Y H I F | 189 | KYG 2151 | A W K W | 44 |
| KYG 211-02 | K H H M | 41  | KYG 2151 | A W K W | 42 |
| KYG 211-03 | Q K P Y | 133 | KYG 2151 | A W K W | 12 |
| KYG 211-04 | P L Q P | 159 |         |          |    |
| KYG 211-05 | Y S F W | 126 |         |          |    |
| KYG 211-06 | Q I T Q | 146 |         |          |    |
| KYG 211-07 | Q V P P | 111 |         |          |    |
| KYG 211-08 | M L Q I | 115 |         |          |    |
| KYG 211-09 | H T S V | 154 |         |          |    |
| KYG 211-10 | P H C T | 108 |         |          |    |
| KYG 211-11 | A F K L | 83  |         |          |    |
| KYG 2121   | A C Q K | 122 |         |          |    |
| KYG 2121   | A C Q K | 83  |         |          |    |
| KYG 2122   | L H W I | 133 |         |          |    |
| KYG 2123   | W W W L | 150 |         |          |    |
| KYG 2123   | W W W L | 118 |         |          |    |
| KYG 2124   | L W Y I | 149 |         |          |    |
| KYG 2124   | L W Y I | 144 |         |          |    |
| KYG 2131   | L H Q Y | 86  |         |          |    |
| KYG 2131   | L H Q Y | 77  |         |          |    |
| KYG 2131   | L H Q Y | 80  |         |          |    |
| KYG 2132   | L H H K | 73  |         |          |    |
| KYG 2132   | L H H K | 82  |         |          |    |
| KYG 2132   | L H H K | 98  |         |          |    |
| KYG 2141   | L K Y L | 64  |         |          |    |
| KYG 2141   | L K Y L | 47  |         |          |    |
| KYG 2141   | L K Y L | 68  |         |          |    |
| KYG 2141   | L K Y L | 85  |         |          |    |
| KYG 2142   | W K W W | 63  |         |          |    |
| KYG 2142   | W K W W | 56  |         |          |    |
| KYG 2142   | W K W W | 55  |         |          |    |
| KYG 2142   | W K W W | 58  |         |          |    |
| KYG 2151   | A W K W | 12  |         |          |    |
| KYG 2151   | A W K W | 22  |         |          |    |

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Table S3. e) Single-bead FP of all hits from Library L2.

|   | Sequence | Kd  |   | Sequence | Kd  |
|---|----------|-----|---|----------|-----|
| KYG 221-01 | L Y A P | 90 | KYG 2242 | A K F F | 27 |
| KYG 221-02 | S I P Q | 115 | KYG 2242 | A K F F | 94 |
| KYG 221-03 | W V T C | 52 | KYG 2242 | A K F F | 49 |
| KYG 221-04 | Q P H C | 149 | KYG 2242 | A K F F | 56 |
| KYG 221-05 | Q T C Y | 95 | KYG 2243 | P W Q Q | 92 |
| KYG 221-06 | T W K C | 111 | KYG 2243 | P W Q Q | 30 |
| KYG 221-07 | K S A I | 159 | KYG 2243 | P W Q Q | 34 |
| KYG 221-08 | K F A K | 40 | KYG 2243 | P W Q Q | 41 |
| KYG 221-09 | Q P T I | 47 | KYG 2251 | W P Q W | 58 |
| KYG 221-10 | F F F K | 135 | KYG 2251 | W P Q W | 64 |
| KYG 2221 | I T Q C | 72 | KYG 2251 | W P Q W | 33 |
| KYG 2221 | I T Q C | 79 | KYG 2251 | W P Q W | 33 |
| KYG 2222 | L L H L | 65 | KYG 2251 | W P Q W | 60 |
| KYG 2222 | L L H L | 94 | KYG 2252 | Q A Y W | 95 |
| KYG 2223 | I L Y L | 76 | KYG 2252 | Q A Y W | 76 |
| KYG 2223 | I L Y L | 52 | KYG 2252 | Q A Y W | 78 |
| KYG 2231 | I H K Q | 67 | KYG 2252 | Q A Y W | 82 |
| KYG 2231 | I H K Q | 40 | KYG 2252 | Q A Y W | 87 |
| KYG 2231 | I H K Q | 73 | KYG 2253 | C V L A | 59 |
| KYG 2232 | L C K K | 47 | KYG 2253 | C V L A | 66 |
| KYG 2232 | L C K K | 89 | KYG 2253 | C V L A | 62 |
| KYG 2232 | L C K K | 54 | KYG 2253 | C V L A | 73 |
| KYG 2233 | Q Y L C | 41 | KYG 2253 | C V L A | 56 |
| KYG 2233 | Q Y L C | 26 | KYG 2253 | T Y W L | 22 |
| KYG 2233 | Q Y L C | 99 | KYG 2261 | T Y W L | 1.1 |
| KYG 2234 | M K H A | 56 | KYG 2261 | T Y W L | 31 |
| KYG 2234 | M K H A | 82 | KYG 2261 | T Y W L | 29 |
| KYG 2234 | M K H A | 80 | KYG 2261 | T Y W L | 14 |
| KYG 2235 | W T Q L | 34 | KYG 2261 | T Y W L | 6.3 |
| KYG 2235 | W T Q L | 89 |
| KYG 2235 | W T Q L | 34 |
| KYG 2241 | V H W T | 57 |
| KYG 2241 | V H W T | 73 |
| KYG 2241 | V H W T | 89 |
| KYG 2241 | V H W T | 68 |

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Table S3. f) Single-bead FP of all hits from Library L3.

| #       | Sequence | Kd | #       | Sequence | Kd |
|---------|----------|----|---------|----------|----|
| KYG 231-01 | Y W V L | 155 | KYG 2341 | Y Y I W | 75 |
| KYG 231-02 | H V Y P | 103 | KYG 2341 | Y Y I W | 35 |
| KYG 231-03 | K L M Y | 162 | KYG 2341 | Y Y I W | 51 |
| KYG 231-04 | M I L V | 165 | KYG 2341 | Y Y I W | 36 |
| KYG 231-05 | L H K S | 175 | KYG 2342 | Q T Q W | 36 |
| KYG 231-06 | L F F L | 90  | KYG 2342 | Q T Q W | 66 |
| KYG 231-07 | K T K W | 56  | KYG 2342 | Q T Q W | 52 |
| KYG 231-08 | C I F H | 42  | KYG 2342 | Q T Q W | 56 |
| KYG 231-09 | F P V T | 26  | KYG 2343 | V M Y P | 68 |
| KYG 231-10 | K V C Q | 162 | KYG 2343 | V M Y P | 74 |
| KYG 231-11 | I M H I | 78  | KYG 2343 | V M Y P | 69 |
| KYG 2321 | L F W L | 28  | KYG 2343 | V M Y P | 45 |
| KYG 2321 | L F W L | 63  | KYG 2351 | L C K K | 29 |
| KYG 2322 | I V Q H | 50  | KYG 2351 | L C K K | 47 |
| KYG 2322 | I V Q H | 31  | KYG 2351 | L C K K | 29 |
| KYG 2323 | V F P I | 77  | KYG 2351 | L C K K | 33 |
| KYG 2323 | V F P I | 92  | KYG 2351 | L C K K | 22 |
| KYG 2324 | W W W C | 62  | KYG 2352 | L W H L | 42 |
| KYG 2324 | W W W C | 77  | KYG 2352 | L W H L | 27 |
| KYG 2325 | H I W C | 73  | KYG 2352 | L W H L | 23 |
| KYG 2325 | H I W C | 35  | KYG 2352 | L W H L | 49 |
| KYG 2326 | L W S Q | 81  | KYG 2352 | L W H L | 46 |
| KYG 2326 | L W S Q | 66  | KYG 2361 | S L I W | 25 |
| KYG 2331 | C L W A | 35  | KYG 2361 | S L I W | 22 |
| KYG 2331 | C L W A | 86  | KYG 2361 | S L I W | 23 |
| KYG 2331 | C L W A | 55  | KYG 2361 | S L I W | 40 |
| KYG 2332 | P L C C | 77  | KYG 2361 | S L I W | 6.6 |
| KYG 2332 | P L C C | 65  | KYG 2361 | S L I W | 41 |
| KYG 2332 | P L C C | 92  | KYG 2371 | I H Q F | 23 |
| KYG 2333 | L C S A | 99  | KYG 2371 | I H Q F | 8.7 |
| KYG 2333 | L C S A | 66  | KYG 2371 | I H Q F | 6.3 |
| KYG 2333 | L C S A | 91  | KYG 2371 | I H Q F | 2.0 |
| KYG 2334 | C M L K | 35  | KYG 2371 | I H Q F | 5.6 |
| KYG 2334 | C M L K | 66  | KYG 2371 | I H Q F | 8.0 |
| KYG 2334 | C M L K | 57  | KYG 2371 | I H Q F | 10.0 |

Table S3. Sequences and raw Kd (µM) values for all 450 hits identified. Sequences were obtained by tandem mass spectroscopy. Letter code see main text Figure 1. All aromatic residues are colored in blue and the linear aliphatic residues without heteroatom are colored red. Side chains with non-aromatic cyclic structures are colored in green, heteroatom-containing aliphatic chains are colored in purple.
Reference

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