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Supporting Information

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The Discriminatory Power of Differential Receptor Arrays is Improved by Prescreening-A Demonstration in the Analysis of Tachykinins and Similar Peptides

Aaron T. Wright, Nicola Y. Edwards, Eric V. Anslyn and John T. McDevitt**

Department of Chemistry and Biochemistry
The University of Texas at Austin
Austin, TX 78712

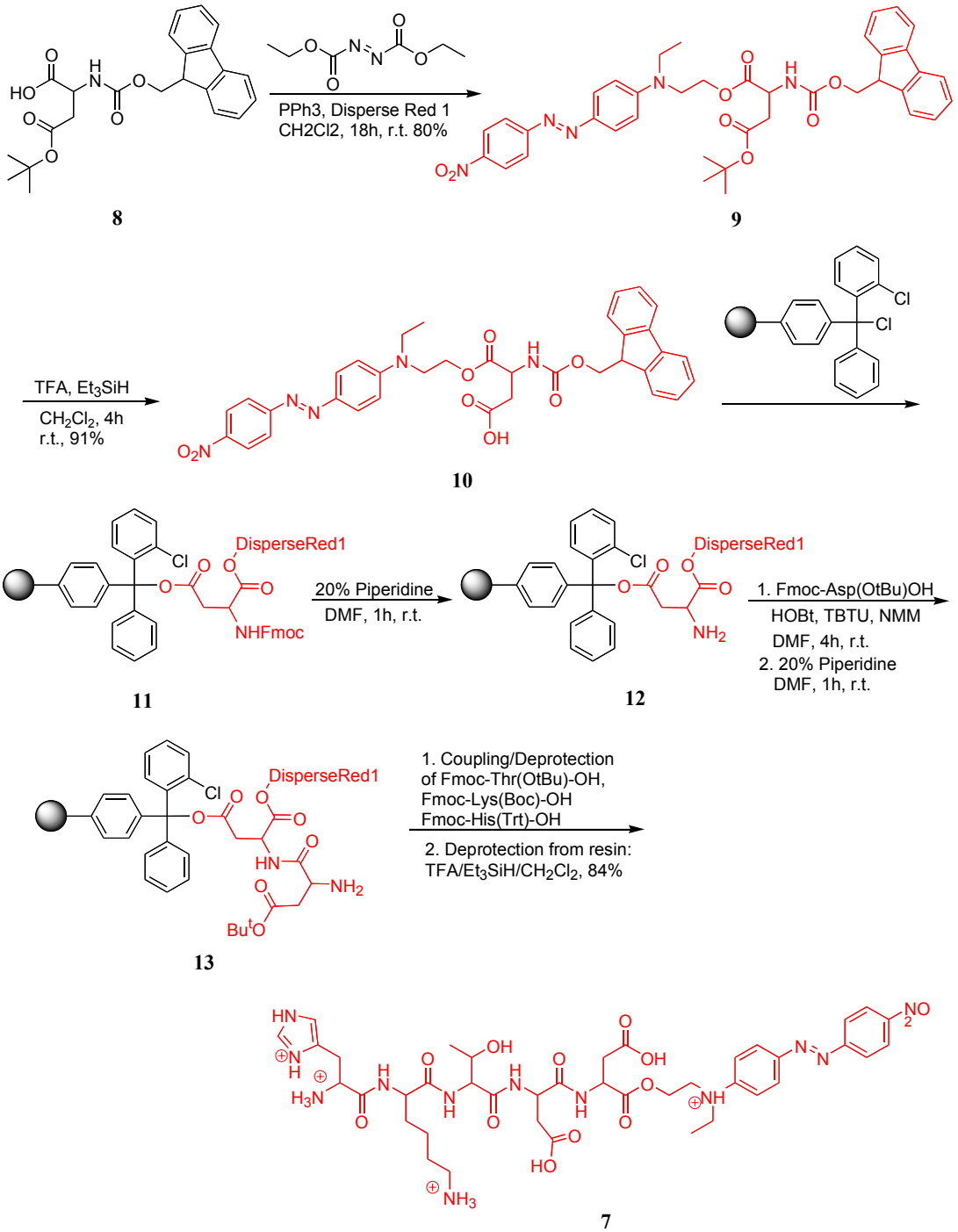
E-mail: anslyn@ccwf.cc.utexas.edu

General. Reactions were run under an atmosphere of argon unless otherwise indicated. Anhydrous solvents were transferred by an oven-dried syringe. Flasks were flame dried under a stream of argon. The chemicals were obtained from Acros Organics, Aldrich, Alfa Aesar, and NovaBiochem and were used without further purification unless otherwise noted. Substance P was purchased from American Peptide Company, Inc., and α-neurokinin was purchased from Biosynthesis Inc. Both of these tachykinins were custom orders. NovaSyn Tentagel Amino resin and 2-chlorotrityl chloride resin were both purchased from NovaBiochem. Methlyene chloride and triethylamine were distilled over calcium hydride. A Varian Gemini 400 MHz NMR was used to obtain ^{1}H and ^{13}C spectra. A Finnigan MAT-VSQ 700 spectrometer was used to obtain low-resolution mass spectra. HPLCs were run on a Gemini Chromasil C18 reverse phase column. The UV-visible absorption measurements were recorded on a Beckman DU640 spectrometer. 96-well plate absorbance spectra were recorded at ambient temperature on a Bio-Tek Synergy HT multi-well plate spectrophotometer. A Costar 96-well flat bottom plate was used for all array experiments. All products were dried for at least 6 hours prior to spectral analysis. Synthesis of solid-supported library **1**,[1] and UV-Vis binding studies between metals and oligopeptides with receptors similar to **1a-1f** have been reported previously.[2]

A) Synthesis of Colorimetric α-Neurokinin Variant (2). The C-terminal region of tachykinins is conserved, so it was determined that a colorimetric variant of α-neurokinin would be synthesized incorporating key residues of the N-terminus as this is discriminating between the three primary tachykinins. Because neither the complexes of library **6**, or α-neurokinin (**4**), have any inherent color it was necessary to covalently append a dye directly to the tachykinin substrate to identify binding events. Therefore, the colorimetric α-neurokinin variant **7** was developed for screening studies.

Fmoc-Asp(O*t*Bu)-OH (**8**) was coupled to the dye disperse red 1 using diethylazodicarboxylate and triphenylphosphine to give **9** in good yield. The Fmoc/*t*Bu protection scheme on **8** was employed for appropriate coupling to the carboxylic acid and for later incorporation onto solid phase. The *t*Bu group was removed using trifluoroacetic acid (TFA) and triethylsilane as a cation scavenger to give **10**. To complete the synthesis of the colorimetric variant solid phase chemistry was employed. **10** was added to the 2-chlorotrityl chloride resin using N-methylmorpholine (NMM) as base yielding **11**. The Fmoc group was subsequently removed using 20% piperidine in DMF to provide **12**. The second amino acid, Fmoc-Asp(O*t*Bu)-OH, was coupled to the free amine on the resin using HOBt, TBTU, and NMM in DMF. This was followed by deprotection of the Fmoc group to give **13**. The other amino acids, with appropriate side chain protection, were added to the resin and deprotected as with the previous amino acid. The connection between the growing peptide chain and the resin is highly labile when subjected to acid. Therefore, to cleave the colorimetric variant from the resin, and remove the acid-sensitive protecting groups,

TFA and triethylsilane in methylene chloride were added to the resin. Cold ether was added and **7** was collected upon precipitation. The product was dissolved in water and washed repeatedly with ether. The water was removed with lyophilization to give the α-neurokinin colorimetric variant in good yield. The synthesis of **7** is illustrated in Scheme 1.



Scheme 1. Synthesis of the α-neurokinin colorimetric variant **7** using both solution and solid-phase chemistry. Compound **7** was used in subsequent screening studies.

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-succinic acid 4-tert-butyl ester 1-(2-{ethyl-[4-(4-nitro-phenylazo)-phenyl]-amino}-ethyl) ester (9).

To an argon purged dry flask was added Fmoc-Asp(O*t*Bu)-OH (0.562 g, 1.37 mmol), disperse red 1 (0.515 g, 1.64 mmol), triphenylphosphine (0.358 g, 1.37 mmol), and methylene chloride (20 mL). This solution mixed at room temperature for 30 min at which point diethylazodicarboxylate (0.216 mL, 1.37 mmol) was added dropwise to the reaction mixture. The reaction was left to stir overnight (16 h) at room temperature. The solvent was removed *in vacuo*, and the dried residue was redissolved in methylene chloride (2 mL) and purified on a SiO_2 column (eluent: progressed from 20% ethyl acetate to 30% ethyl acetate in hexanes). The product was collected as a red solid (0.77 g, 0.992 mmol, 80%). ^{1}H NMR ($CDCl_3$): 8.28 (d, 2H), 7.87 (d, 2H), 7.85 (d, 2H), 7.73 (d, 2H), 7.57 (d, 2H), 7.37 (t, 2H), 7.28 (t, 2H), 6.75 (d, 2H), 5.80 (d, NH), 4.55 (t, 1H), 4.41 (t, 1H), 4.34 (d, 2H), 4.21 (t, 2H), 3.66 (t, 2H), 3.49 (q, 2H), 2.92 (d, 2H), 1.46 (s, 9H), 1.22 (t, 3H). ^{13}C NMR ($CDCl_3$): 170.6, 169.4, 156.5, 155.8, 150.9, 147.2, 143.7, 143.5, 141.1, 127.6, 126.9, 126.1, 125, 124.9, 124.5, 122.5, 119.9, 111.2, 67.1, 61.6, 60.2, 56.9, 50.8, 48.4, 41.1, 38.0, 27.7, 12.1. MS (CI^+) *m/z* 708 $[M]^+$.

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-succinic acid 1-(2-{ethyl-[4-(4-nitro-phenylazo)-phenyl]-amino}-ethyl) ester (10).

9 (0.70 g, 0.992 mmol) was added to a dry flask and dissolved in methylene chloride (8 mL). Trifluoroacetic acid (1.1 mL, 14.9 mmol) and triethylsilane (0.48 mL, 2.98 mmol) were then slowly dripped into the solution. The reaction was stirred for 4 h at room temperature. The solvent was removed and the product was redissolved in methylene chloride (1.5 mL) and purified on a SiO_2 column (eluent: 1:1 ethyl acetate:hexanes). The product was collected as a red solid (0.638 g, 0.979 mmol, 91%) from the column. ^{1}H NMR ($CDCl_3$): 8.28 (d, 2H), 7.87 (d, 2H), 7.85 (d, 2H), 7.73 (d, 2H), 7.57 (d, 2H), 7.37 (t, 2H), 7.28 (t, 2H), 6.75 (d, 2H), 5.80 (d, NH), 4.55 (t, 1H), 4.41 (t, 1H), 4.34 (d, 2H), 4.21 (t, 2H), 3.66 (t, 2H), 3.49 (q, 2H), 2.92 (d, 2H), 1.22 (t, 3H). ^{13}C NMR ($CDCl_3$): 170.6, 169.4, 156.5, 155.8, 150.9, 147.2, 143.7, 143.5, 141.1, 127.6, 126.9, 126.1, 125, 124.9, 124.5, 122.5, 119.9, 111.2, 67.1, 61.6, 60.2, 56.9, 50.8, 48.4, 41.1, 38.0, 12.1. MS (CI^+) *m/z* 652 $[M]^+$.

[11].

10 (0.266 g, 0.408 mmol) was dissolved in methylene chloride (9 mL) in a dried flask. N-methylmorpholine (0.15 mL, 1.36 mmol) was added to the flask followed immediately by the 2-chlorotrityl chloride resin (1.4 mmol/g, 0.244 g, 0.34 mmol). The mixture stirred for 1 h at room temperature. At this point a solution of methylene chloride/methanol/*N,N*-diisopropylethyl amine (17:5:2, 8 mL) was added to quench unreacted resin, and the solution stirred for 10 min. The solvents were filtered from

the resin, and the resin was washed with DMF, methylene chloride (2x), methanol (2x), and DMF once again. MS (CI^+) *m/z* 652 $[M]^+$.

[12].

A solution of 20% piperidine in DMF (5 mL) was added to **11** (0.244 g, 0.34 mmol) in a dry reaction flask. The reaction was mixed for 30 min, and the solvents were filtered from the resin. The resin was washed with DMF, methylene chloride (2x), methanol (2x), and DMF once again. A Kaiser test was positive indicating the presence of free primary amines.

[13].

Fmoc-Asp(O*t*Bu)-OH (0.53 g, 1.29 mmol), 1-hydroxybenzotriazole (0.23 g, 1.7 mmol), TBTU (0.55 g, 1.7 mmol), and N-methylmorpholine (0.34 mL, 3.06 mmol) were dissolved in DMF (5 mL) in a dry flask. This solution mixed for 10 min; during this time the color changed from clear to yellow. The color change is indicative of formation of the active ester. At this point **12** (0.244 g, 0.34 mmol) was added, and the reaction mixed for 4 h. The solution was filtered from the resin, and the resin was washed with DMF, methylene chloride (2x), methanol (2x), and DMF once again. A Kaiser test was negative, indicating the absence of free primary amines. A solution of 20% piperidine in DMF (5 mL) was added to the resin (0.244 g, 0.34 mmol) in a dry reaction flask. The reaction was mixed for 30 min, and the solvents were filtered from the resin. The resin was washed with DMF, methylene chloride (2x), methanol (2x), and DMF once again. A Kaiser test was positive indicating the presence of free primary amines.

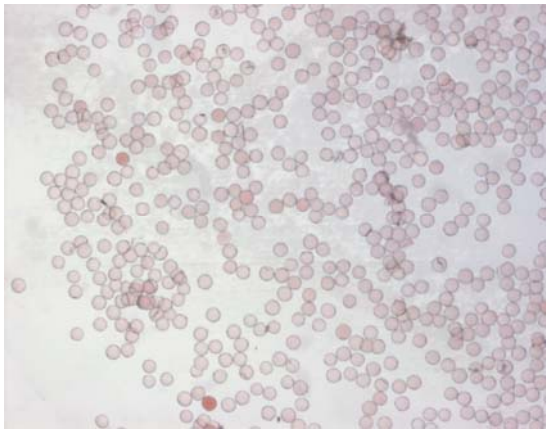
α-Neurokinin Colorimetric Variant (7).

Beginning with **13** (0.244 g, 0.34 mmol) three additional amino acids were coupled to the resin. Fmoc-Thr(O*t*Bu)-OH (0.513 g, 1.29 mmol), Fmoc-Lys(Boc)-OH (0.6 g, 1.29 mmol), and Fmoc-His(Trt)-OH were coupled to the resin using 1-hydroxybenzotriazole (0.23 g, 1.7 mmol), TBTU (0.55 g, 1.7 mmol), and N-methylmorpholine (0.34 mL, 3.06 mmol) in DMF (5 mL). Between additions of each amino acid the Fmoc group was removed using 20% piperidine in DMF (5 mL). Kaiser tests were used to determine effective coupling and Fmoc deprotection. Upon completion of the solid phase synthesis the compound was cleaved from the resin using a TFA/CH_2Cl_2/triethylsilane (45/4/1, 15 mL) solution. The deprotection solution was filtered and collected, and the resin was washed twice more with the deprotection solution. The deprotection solution was then concentrated *in vacuo*. The product was obtained by precipitation with ether, and was subsequently dissolved in water and lyophilized multiple times to obtain a solid red powder (0.261 g, 0.286 mmol, 84%). MALDI-MS, *m/z* 911.4 [M]. HPLC retention time: 17.8 minutes, single peak on spectrum from 0-30 minutes (eluent = water).

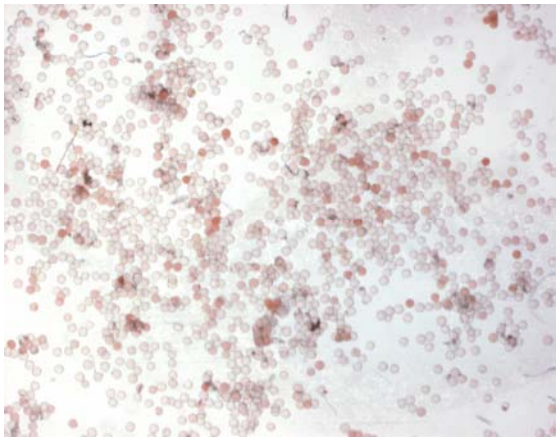
B) Screening of Solid-Supported Library 6 with 7. To identify strong α-neurokinin binding the **6**:**Cu(II)Cl$_2$** complex library was mixed with **7**, and the strongest binding complexes were selected and sequenced to obtain full characterization. A number of concentrations of **7** (70, 90, and 110 μM) were used for screening the receptor library. Portions of the receptor library (5 mg) were added to modified Eppendorf tubes containing a frit and opening on the bottom to extricate solutions. The first step involved incubation of the library resin with Cu(II)Cl$_2$ (1 mM, 200 μL) overnight. The copper solution was extricated and the resin was rinsed multiply with HEPES (10 mM, pH 7.4). Following rinsing, **7** was added at the three different concentrations (70, 90, and 110 μM) in a buffered solution (HEPES, 10 mM, pH 7.4). These solutions were mixed overnight. The beads were rinsed several times with the buffer solution to remove any unbound **7** from the solid-supported receptor complexes. The receptors were placed on cover slips, and images were obtained using an Olympus stereoscope equipped with a charge-coupled device and a video capture card. Figure 1 shows the close angle images captured of the **7**-incubated **6:Cu(II)Cl$_2$** complex library.

At 70 μM concentrations of **7**, 0.1-0.2% of the resin-bound receptors (5 mg) were deep red in color. At 90 μM, approximately 1.7% were deep red, and approximately 4% were darkly stained at 110 μM. Concentrations greater than 110 μM gave widespread deep coloration, and were not worthwhile for detecting the most selective receptors for the α-neurokinin variant **7** (130 μM = ~20%, 150 μM = ~40%).

(A) 70 μM



(B) 90 μM



(C) 110 μM

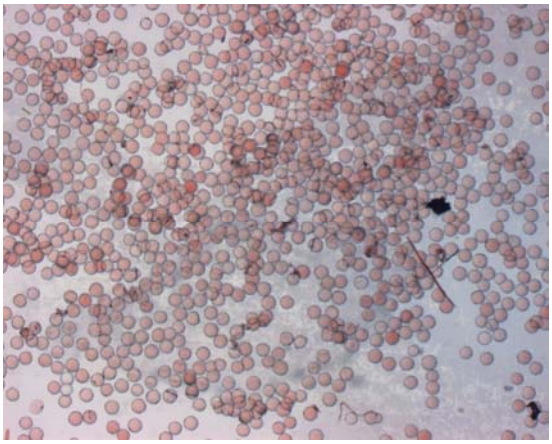
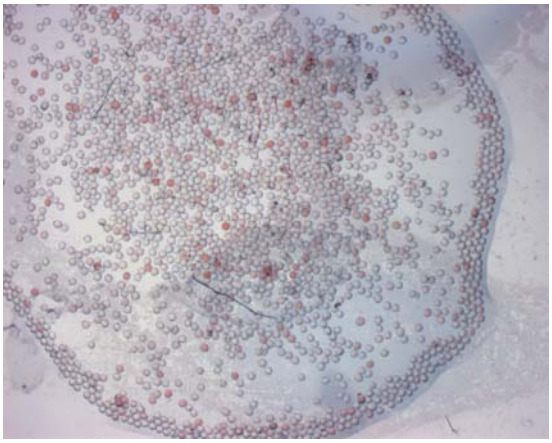


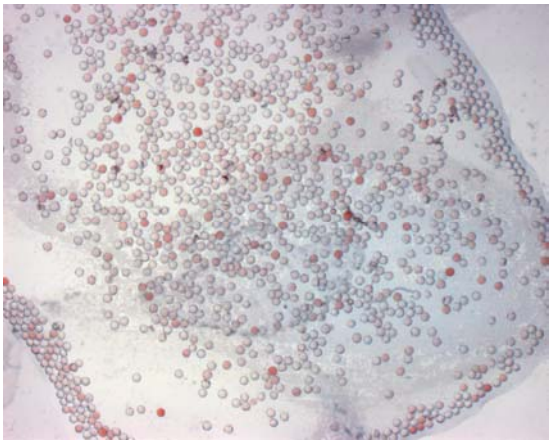
Figure 1. Images of metalated library **6** following incubation with multiple concentrations of α-neurokinin variant **2**. Brightly colored beads indicate strong binding between the receptor:metal complex and **7**. These are close-up images that illustrate the relative binding (coloration) between **6** and **7**.

C) Screening of Tripeptide Control Solid-Supported Library. In the control a tripeptide library was developed on NovaSyn tentagel amino resin.[3] This tripeptide library was incubated with **7** at the same concentrations as library **6**. An interesting result ensued, approximately 1-2% of the tripeptide library, regardless of the concentration, were darkly colored as seen in Figure 2. It must be noted that the effective molarity of the tripeptides on the resin is very high, therefore tripeptides that are specifically complementary to **7** are likely colored darkly. Notice that the rest of the resin is nearly colorless. However, with library **6** most of the resin-bound receptors had some color indicating association with **7**. This is likely due to the conserved metal ligand core of all the receptors in the library.

(A) 70 μM



(B) 90 μM



(C) 110 μM

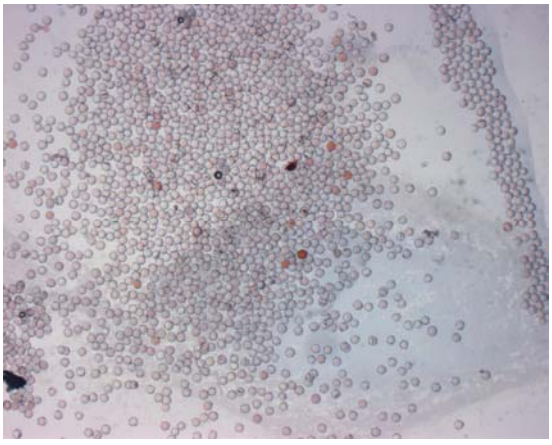
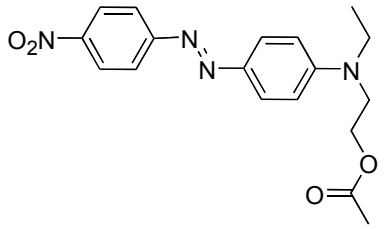


Figure 2. Screening of a tripeptide control library with **7**. Interestingly, no matter what the concentration of **7**, about the same percentage of the control library was darkly stained. It was found later that generally the darkly stained beads have high charge density.

D) Synthesis of Esterified Disperse Red 1. Acetic acid 2-{ethyl-[4-(4-nitro-phenylazo)-phenyl]-amino}-ethyl ester (14).



14

Disperse red 1 (0.2 g, 0.636 mmol) was dissolved in methylene chloride (10 mL) in a dry flask. To the solution was added acetic anhydride (120 μL, 1.27 mmol) and dimethylaminopyridine (.016 g, 0.131 mmol). The reaction was mixed for 4 h and then concentrated *in vacuo*. The product was purified on a SiO_2 column (eluent: 99:1 methylene chloride/methanol) to yield a red solid (0.09 g, 0.252 mmol). ^{1}H NMR ($CDCl_3$): 8.33 (d, 2H), 7.89 (q, 4H), 6.80 (d, 2H), 4.29 (t, 2H), 3.67 (t, 2H), 3.54 (q, 2H), 2.06 (s, 3H), 1.24 (t, 3H). MS (CI^+) *m/z* 357.2 $[M]^+$.

E) Screening Solid-Supported Library 6 with 14. A control was set up by incubating metalated library **6** (5 mg) with an acylated derivative of disperse red 1 (**14**). The control **14** was added to metalated **6** and mixed overnight. As is evident from Figure 3 there were no specific interactions between any of the receptors in the library and **14**.

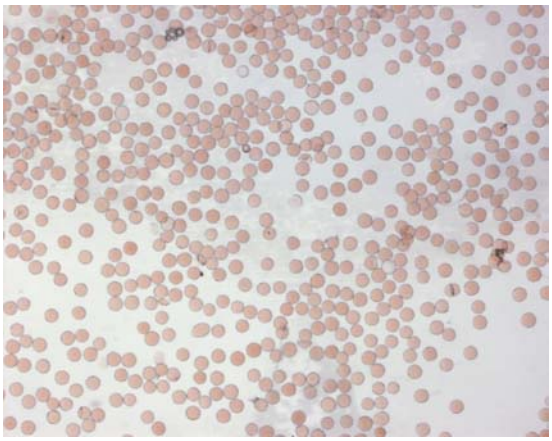


Figure 3. Images of metalated library **6** following incubation with 70 μM **14**.

F) Edman Degradation of Selective Binding Members. To learn more about the nature of selective binding interactions between metalated receptors of **6** and **7**, as well as selective binding interactions between tripeptides in the control library and **7**, a number of the strong binders were selected for sequencing characterization. Six of the darkly colored beads of metalated library **6** from the 70 μM incubation with **7** were obtained and sequenced using Edman degradation, and three darkly colored tripeptides from the control library were sequenced. The results from the sequencing are shown in Figure 4.

The three tripeptides sequenced from the tripeptide control library all have high charge density. Interestingly, both cationic and anionic tripeptides were found. This is interesting because **7** likely has a +2 charge at pH 7.4 (the pH of the screening studies). The tripeptide Glu-Glu-Glu likely bound through strong electrostatic interactions. However, the two cationic peptides likely associated via cation-π interactions with the disperse red 1 portion of **7**.

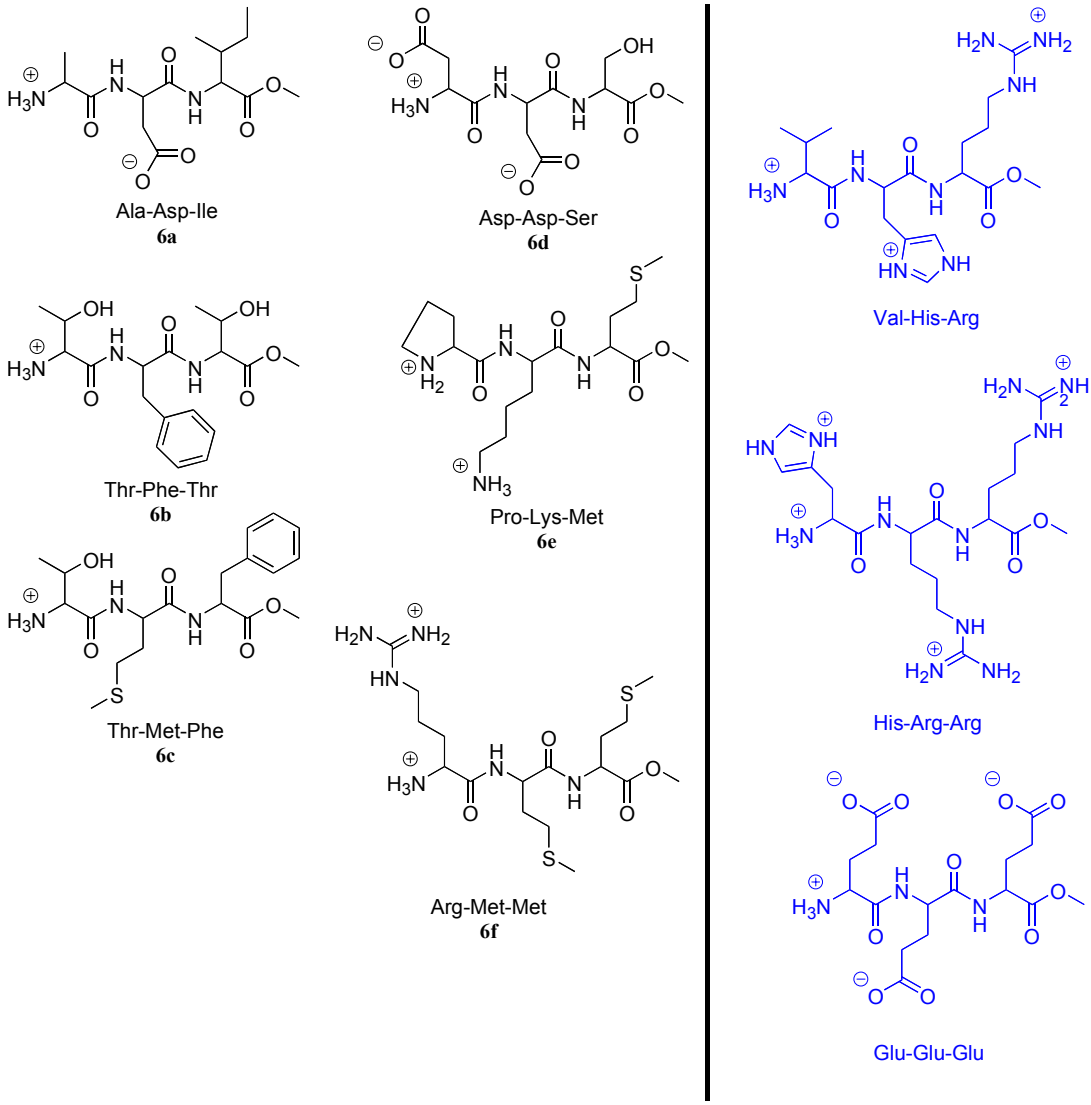


Figure 4. Sequencing results for selective binders of **7** from library **6** and the tripeptide control library (blue).

G) Resynthesis of 6a-6f in Solution.[1] Synthesis of the receptors **6a-6f** on 2-chlorotrityl chloride resin (0.161 g, 0.221 mmol) parallels syntheses described for **7**, and it also follows the solid-supported synthesis described in reference 1, so only a minimal description is provided. The first three amino acids: Lys(Boc)-OH (0.47 g, 0.995 mmol), Fmoc-Gly (0.296 g, 0.995 mmol), and Fmoc-Asp(O*t*Bu)-OH (0.409 g, 0.995 mmol) were coupled to Novasyn 2-chlorotrityl-2-chloride resin. This was followed by addition of mono-fluorenylmethylsuccinic acid (0.294 g, 0.995 mmol) and the mono-Fmoc protected core (0.512 g, 0.995 mmol).[1] The coupling agents used were HOBt (0.122 g, 1.22 mmol), TBTU (0.36 g, 1.22 mmol) and N-methylmorpholine (0.30 mL, 1.63 mmol). Between coupling steps the Fmoc and Fm protecting groups were removed using 20% piperidine in DMF (5 mL). The additional amino acids coupled to develop **6a-6f** were: Fmoc-Ile (0.35 g, 0.995 mmol), Fmoc-Thr(O*t*Bu)-OH (0.39 g, 0.995 mmol), Fmoc-Phe (0.37 g, 0.995 mmol), Fmoc-Ser(O*t*Bu)-OH (0.38 g, 0.995 mmol), Fmoc-Trp(Boc)-OH

(0.52 g, 0.995 mmol), Fmoc-Met (0.37 g, 0.995 mmol), Fmoc-Glu(O*t*Bu)-OH (0.42 g, 0.995 mmol), Fmoc-Ala (0.31 g, 0.995 mmol), Fmoc-Arg(Pbf)-OH (0.65 g, 0.995 mmol), and Fmoc-Pro (0.33 g, 0.995 mmol). The receptors were cleaved from the resin using trifluoroacetic acid/triisopropylsilane/ethane dithiol/water (90/2.5/2.5/5, 20 mL). The cleavage reactions were run for 5 h, and the cleavage solution was filtered and collected. The resin was rinsed with the cleavage solution two additional times. The combined cleavage solutions were reduced *in vacuo*, and the products were precipitated with cold ether. The receptors were redissolved in water and rinsed multiple times with methylene chloride and ether. The water was removed using lyophilization. The products were once again dissolved in water and washed with methylene chloride and ether, and the water was removed with lyophilization. The products were characterized using low resolution MS, ESI, MALDI, and HPLC (1.0 mL/min, 30 min, ran from 100% water to methanol/water (95:5)):

(6a) MS (CI^+) *m/z* 1008 $[M]^+$. MALDI-MS, 1008.58 [M]. HPLC retention time = 1.683 min.

(6b) MS (CI^+) *m/z* 1045 $[M]^+$. ESI (+C) 1044. HPLC retention time = 2.067 min.

(6c) MS (CI^+) *m/z* 1074 $[M]^+$. HPLC retention time = 1.700 min.

(6d) ESI (+C) 1040. HPLC retention time = 1.983 min.

(6e) MALDI-MS, 1051.6 [M]. HPLC retention time = 2.750 min.

(1f) MALDI-MS, 1114.7 [M]. HPLC retention time = 2.017 min.

H) Sequence Properties of Peptides 1-5.

Table 1. Sequence properties of peptides **1-5**.

Peptides	Hydrophobicity[a]	Theoretical pI	Molecular Weight g/mol
1	0.658	5.24	385.4
2	0.898	8.76	384.4
3	0.926	6.75	499.5
4	5.292	6.74	1134.3
5	6.389	11.00	1348.6

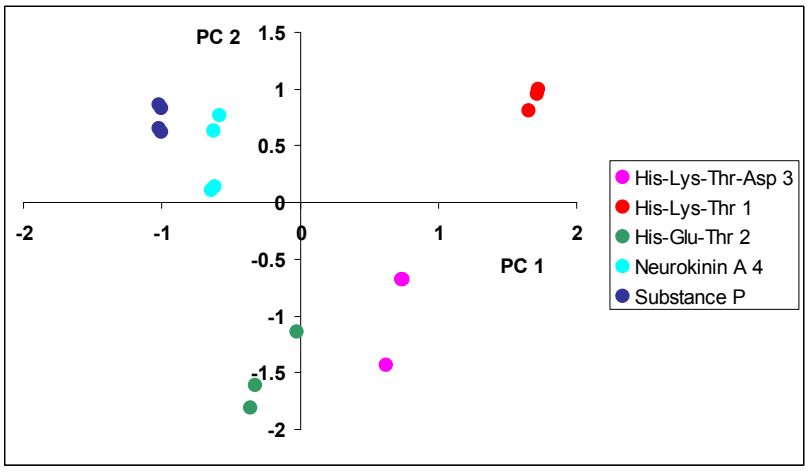
[a] measured using the Black scale[4]

I) Array Preparation. An array was created by adding receptors **6a-6f** (267 μM) to the wells. Each receptor was metalated in separate wells with $Cu(II)Cl_2$ (534 μM), Cu(II)OTf (267 μM), and Cd(II)OAc (267 μM). Oligopeptides **1-5** (267 μM) were added to each receptor/metal complex, the solutions were buffered with HEPES (10 mM, pH 7.4), and the total volume of each well was set (300 μL). A wavelength scan was taken of every well from 300-355 nm. Additionally, scans were taken of each receptor/metal complex without any analytes. Three data points were collected for each well at 315, 321, and 333 nm. The absorbance values of the corresponding receptor/metal complexes were subtracted from the respective absorbance value of a metal/receptor/oligopeptide complex (ΔAbs values). This created a data set of 54 ΔAbs values for each oligopeptide tested. Furthermore, substrates **1-3** were tested triply, and substrates **4** and **5** were tested four times.

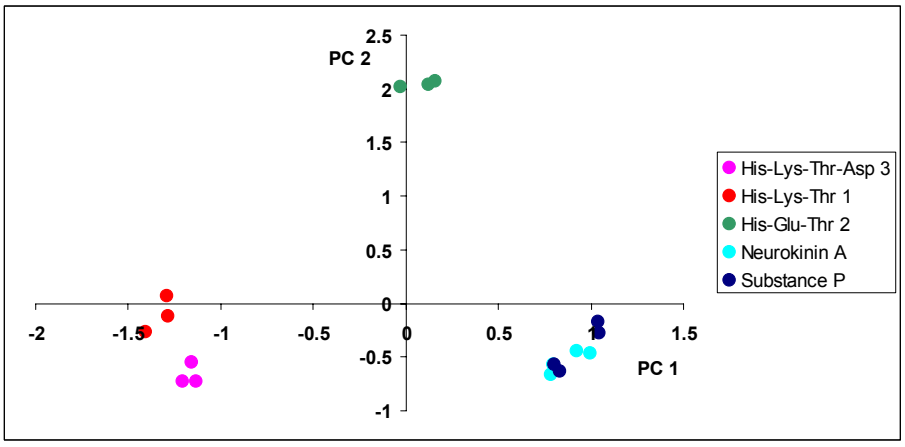
I) Principal Component Analysis. The program Statistica 4.0 from StatSoft was employed for PCA analysis. The program 'Factor Analysis' was selected, and all 54 variables were used in the analysis. The eigenvalue was set to 1.0, and the number of possible factors was set to six, though only four were determined by the program. Two-dimensional plots were created by Microsoft Excel, and three-dimensional plots were created by SigmaPlot.

As mentioned in the manuscript, PCA plots were created using only the receptors with a single metal salt. Both the two- and three-dimensional plots are shown in Figure 5. As can be seen most notably in the Cd(II)OAc plot, the data points for substance P and α-neurokinin seem to be separated in two different clusters. This was due to running two trials one day, and two trials a second day. A processing error was found for the two trials on day two. The problem was in the creation of the background absorbance plots of the receptor:metal complexes without analytes. Applying a background correction provided the PCA plot seen in Figure 5 of the manuscript. Additional receptor:metal complexes increase the variance described in the additional PC axes. Shown in Figure 6 is the two-dimensional plot without correction. Despite the slight aberrations in the α-neurokinin and substance P data, the clustering and spatial separation is still excellent.

(A) $Cu(II)Cl_2$ Only.



(B) Cu(II)OTf Only.



(C) Cd(II)OAc Only.

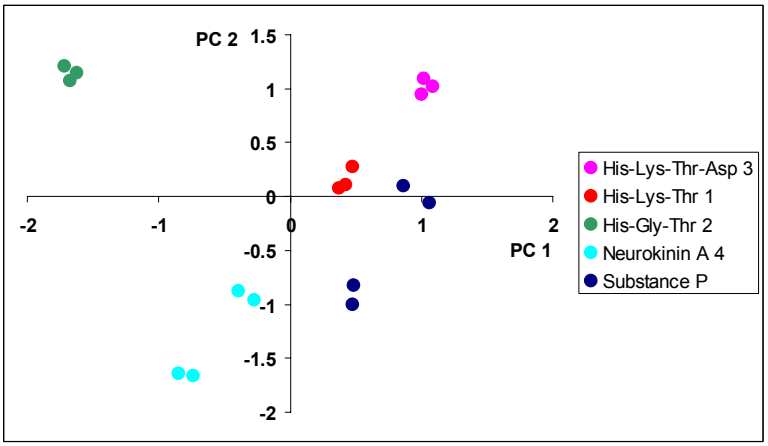


Figure 5. (A) PCA plots created using data taken only from receptors **6a-6f** complexed with Cu(II)Cl_2. (B) PCA plots created using data taken only from receptors **6a-6f** complexed with Cu(II)OTf. (C) PCA plots created using data taken only from receptors **6a-6f** complexed with Cd(II)OAc.

(A) Full two-dimensional plot, no correction.

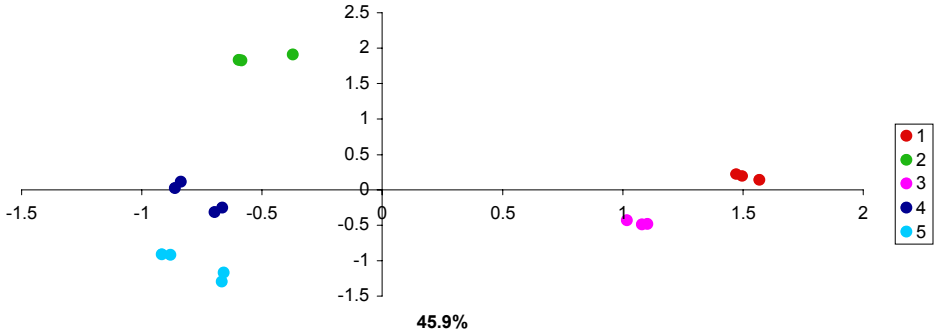


Figure 6. A two-dimensional PCA plot using all receptors **6a-6f** and metal salt complexes without corrections is shown.

References:

(1) A. T.Wright, E. V.Anslyn, J. T. McDevitt, *J. Am. Chem. Soc*. **2005**, *127*, 17405-17411.

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