Supramolecular Chemistry

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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**

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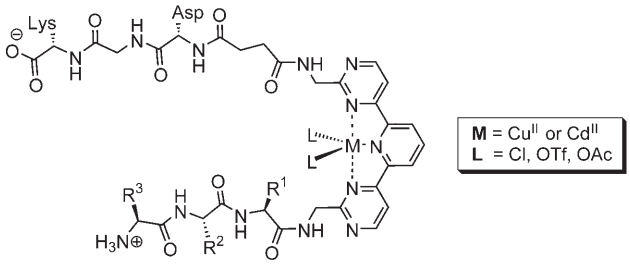
Complex odors and flavors are discriminated by cross-reactive receptor proteins that act in tandem to provide diagnostic signals.[1] This strategy has provided inspiration for the creation of differential sensing arrays.[2,3] These arrays have the potential to discriminate challenging analytes such as proteins and peptides. Peptide recognition has been the subject of a tremendous body of work, and recently systems capable of short peptide recognition, as well as sequence-selective and stereoselective peptide recognition, have been developed.[4]

Tachykinins, exemplified by α-neurokinin and substance P, provide a particularly challenging group of biomolecule analytes. They are neurotransmitter peptides which are implicated in pain transmission in the mammalian brain.[5] Toward the goal of detecting tachykinins, we targeted α-neurokinin analogues His-Lys-Thr (**1**) and His-Lys-Thr-Asp (**3**).[3d] The tripeptide His-Glu-Thr (**2**) was also targeted for comparison. Peptides **1** and **2** differ only by a single amino acid, while peptides **1** and **3** differ in kind by one amino acid. α-Neurokinin (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-C(O)-NH_2; **4**) and substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-C(O)-NH_2; **5**) completed the set of targeted analytes. These tachykinins share a C-terminal sequence of Phe-X-Gly-Leu-Met-C(O)-NH_2 which is conserved throughout this family of peptides. We report here the use of library **6** as differential receptors for discriminating these five peptides, and the discovery that the discriminatory capabilities of the receptors in an array are significantly improved by prescreening this library for the desired targets.

Library **6** has a metal-binding ligand with two appended peptides.[3d,6] The metal center imparts selectivity toward peptides terminating in His, such as α-neurokinin.[6] One peptide arm was consistently Lys-Gly-Asp, while the second was added through combinatorial chemistry and provides the differential recognition character.[3d] We previously showed



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6a = R^1=Ala, R^2=Asp, R^3=Ile
6b = R^1=Thr, R^2=Phe, R^3=Thr
6c = R^1=Thr, R^2=Met, R^3=Phe
6d = R^1=Asp, R^2=Asp, R^3=Ser
6e = R^1=Pro, R^2=Lys, R^3=Met
6f = R^1=Arg, R^2=Met, R^3=Met

that 30 randomly chosen members of library **6** (incubated with $Cu(OTf)_2$)[7] successfully differentiated similar tripeptides. Slopes at a preselected region of indicator-uptake kinetic traces were calculated for the receptors and the dimensionality of the data set reduced using principal component analysis (PCA). However, the majority of the variance in the data (91%) was on a single PC axis, and the PC factor loading values for all 30 receptors were similar. This indicated that the library members did not have significant selectivity between, or cross-reactivity for, the targets.

To target a peptide family as large and complex as the tachykinins in an array setting, the discriminatory capabilities of the potential receptors had to be improved. We hypothesized that by prescreening library **6** we would find receptors possessing affinity for the tachykinins, but also likely possessing differential reactivity between members of this class of analytes, thereby leading to an array of receptors which would serve our purpose. Screening libraries of receptors has been done previously to find the single best receptor for a specific target,[8] but screening to uncover a series of receptors for discrimination of structurally similar members within a class of analytes is a new concept.

Bead-supported library **6** was screened with a colorimetric variant of α-neurokinin (**7**) to identify strong binding receptors. The α-neurokinin variant was a conjugate of the first four amino acid residues of α-neurokinin with an aspartic acid modified with Disperse Red 1. Library **6** (5 mg) was preincubated with $CuCl_2$ (1 mM, 200 μL), followed by addition of **7** (70 μM, in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) 10 mM, pH 7.4).

Library members which displayed the strongest binding affinity for **7** were identified from images obtained using an

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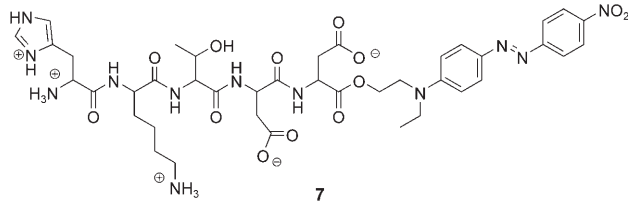
Supporting information for this article (including full experimental and data processing details) is available on the WWW under http://www.angewandte.org or from the author.



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Olympus stereoscope equipped with a charge-coupled device and a video capture card (Figure 1). Only 0.5% of the beads were intensely colored, thus indicating selectivity among the

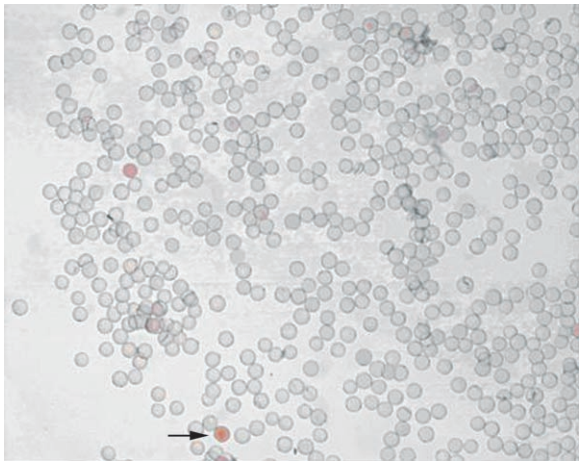

Figure 1. Image of 1/8 of the screened library. Only one bead displays good association with **7** as evidenced from the strong coloration.

beads. To verify that the affinity was not due to binding of the azo-dye portion of **7**, library **6** was mixed with $CuCl_2$ and incubated with an ester variant of Disperse Red 1. Not a single bead displayed binding to the Disperse Red 1 variant, thereby indicating that binding was due to interactions between **6** and the peptide portion of **7**.

Six intensely colored beads were selected and sequenced by Edman degradation. This revealed sequences **6a**–**6f**. It is evident that a number of hydrophobic groups (Met, Trp, Phe, Ala, and Ile) were important for binding **7**, in addition to acidic (Asp) and polar residues (Ser and Thr).

In this study, an equilibrium assay was developed to assess the binding of the peptides to the receptors as monitored in a 96-well plate, rather than an "on-bead" kinetic assay in a silicon chip, which was previously employed.[3d] This required the synthesis of **6a**–**6f** for use in solution, and a combination of solid- and solution-phase methods was used (see the Supporting Information). The expected variations in the binding constants of the peptide–receptor complexes were based on a previous study from our research group performed on an analogous copper receptor with amino acids and tripeptides; binding constants obtained by spectroscopy varied from 10^6 to less than $10^2\,M^{-1}$ in 1:1 methanol/buffered water at pH 7.4.[6] The optimal array was created using receptors **6a**–**6f** (267 μM), each metalated in separate wells with $CuCl_2$ (534 μM), $Cu(OTf)_2$ (267 μM), and $Cd(OAc)_2$ (267 μM). The use of six ligands, two metals, and two different counterions for the Cu^{2+} ions, led to an array of 18 members. Peptides **1**–**5** (267 μM) were then added to each ligand/metal/counterion complex in HEPES (10 mM, pH 7.4). UV/Vis spectra were taken of each well before and after the addition of peptides **1**–**5**. An example is given in Figure 2 for **6d**/

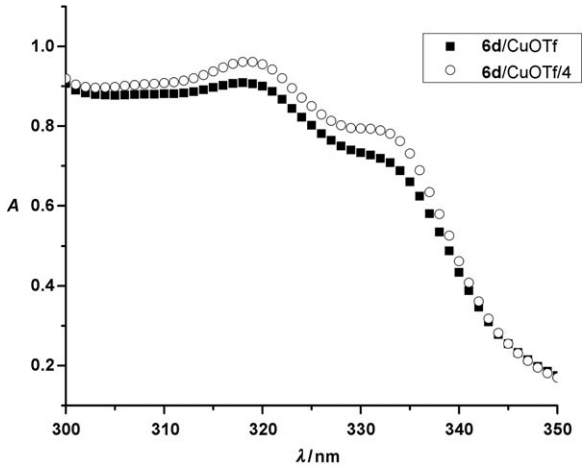

Figure 2. UV/Vis absorbance spectra obtained before and after addition of **4** to **6d**/CuOTf.

$CuOTf_2$ and **4**. Absorbance data at 315, 321, and 333 nm (wavelengths of peaks or large changes) with and without peptide were subtracted to yield the difference (ΔA values). This created a data set of 54 ΔA values for each peptide, and ΔA values as large as 0.3 absorbance units were observed. Peptides **1**–**3** were tested three times, while **4** and **5** were tested four times.

The UV/Vis data indicated that there were differences in the extent of the binding between the peptides and the metalated receptors, such that a unique pattern of absorbance changes was created for each peptide. A representative histogram showing peptide patterns created by complexes formed between receptors (**6c**, **6e**, and **6f**) with metal salts ($CuCl_2$, $CuOTf_2$, and $CdOAc_2$) at 315 nm is shown in Figure 3.

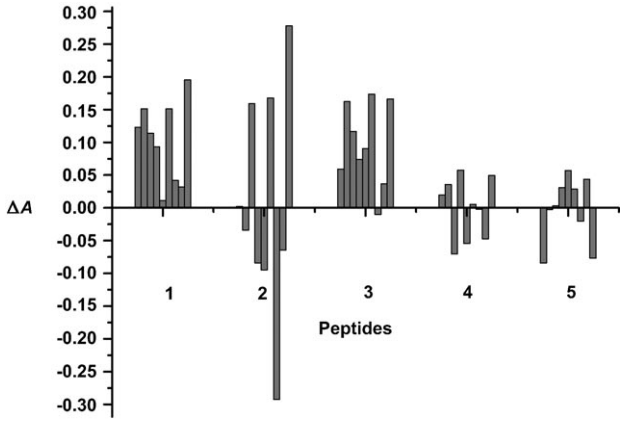

Figure 3. Fingerprints created for peptides **1**–**5** by receptors **6c**, **6e**, and **6f**, and $CuCl_2$, $CuOTf_2$, and $CdOAc_2$ at 315 nm.

These patterns facilitated the evaluation of the data set by PCA.

PCA was performed on a data matrix consisting of ΔA values at the wavelengths of choice for receptors **6a**–**6f** (columns: 54 variables = receptors × metal complexes × wavelengths) and analytes (rows: 18 variables = peptides × experimental trials) to discern patterns for the recognition and the discrimination of the peptides. Four principal components were extracted which accounted for approximately 98% of the variance. A plot of the factor scores of the analytes for the first three principal components (PC1, PC2, and PC3) is shown in Figure 4 A. Excellent classification of the analytes and clustering of the experimental trials within analyte groups, the desirable attributes of a PCA, are the noteworthy features of this plot. Therefore, although the bead-supported library **6**/$CuCl_2$ was screened with only a single analogue of the target peptides, the resulting array gave excellent pattern-based disrimination of all the peptides. The power of the protocol is demonstrated by the ability to differentiate the subtly different small peptides (**1**, **2**, and **3**) as well the large tachykinins (**4** and **5**). Additionally, a significant amount of the variance was distributed throughout PCs 1–3, thus indicating a high degree of cross-reactivity between the receptors in this array. This is a marked improvement over our previous study in which the maximum amount of the variance (91%) was carried by PC 1.

Closer inspection of this PCA data set by examination of plots of PC 2 versus PC 1, PC 3 versus PC 1, and PC 3 versus PC 2 (Figure 4 B–D) revealed discernable trends with respect to sequence properties (see Table 1 in the Supporting Information) and analyte PC axes dependencies of the peptides. With the exception of **2**, the peptides are generally distributed along PC 1 in accordance with their hydrophobicity. Plots of PC 2 versus PC 1 and PC 3 versus PC 1 reveal that PC 2 is able to discriminate the tripeptides whereas PC 3 is able to discriminate the tripeptides as well as α-neurokinin from its analogues (peptides **1** and **3**). No correlation or axis dependency relating to the theoretical p*I* value of a peptide was ascertained. This finding meant that the response of the array was not based solely on electrostatic interactions between the receptors and peptides.

The most influential variables in the discrimination of peptides **1**–**5**, as measured by factor loading values along the PC axes, were determined to be complexes formed from receptors **6b** and **6d** with $Cu(OTf)_2$ and $CuCl_2$. This result is consistent with findings that Asp residues form hydrogen bonds and/or ion pairs with the N-H groups on the main chain of peptides and lysine side chains, respectively. We postulate that the polar Ser and Thr residues also participate in hydrogen-bonding interactions with these peptides, which are key to the recognition of these analytes. The presence of the Phe residue also suggests that hydrophobic contacts play a role. The preference for the copper salts is undoubtedly due to their histidine-binding properties; four of the five peptides terminate with a histidine residue.

We speculated that in accordance with array-sensing principles, a reduced library consisting of receptors **6a**–**6f** and a single metal salt would be a less competent discriminating agent than the full array of receptors and metal complexes. To

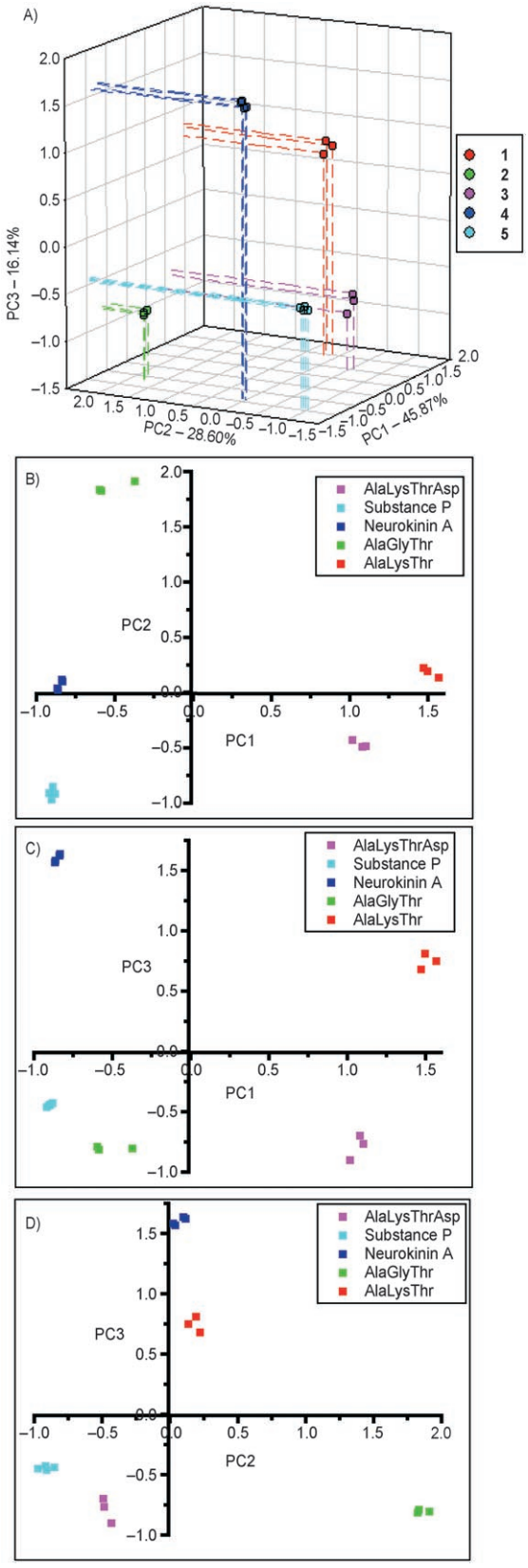

Figure 4. A) PCA scores plot showing PC 1 versus PC 2 versus PC 3 for peptides **1**–**5**. PCA scores plots of B) PC 2 versus PC 1, C) PC 3 versus PC 2, and D) PC 3 versus PC 1.

test this hypothesis, PCA was performed on these reduced libraries (Supporting Information). In the case of $Cd(OAc)_2$ and $CuCl_2$, classification of all peptides was achieved, but with reduced spatial separation between peptide groups and clustering of experimental trials within each peptide group. Interestingly for $CuOTf_2$, there was a dramatic reduction in both spatial separation and clustering, such that tachycinins **4** and **5** were not discriminated.

In summary, the implemention of a prescreening process, and the addition of diversity through metal salts, created an array of differential receptors with extraordinary discriminatory capabilities. Short peptides, considered to be one of the most challenging set of analytes, which varied in length and kind by only a single amino acid, as well as tachykinins, which share a high degree of sequence identity, were all successfully classified. It is not yet clear if prescreening improves differential sensing schemes in general, but this is currently under investigation in our laboratories with schemes that monitor or quantify minor post-translational peptidic modifications.

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