Coupling Rational Design with Libraries Leads to the Production of an ATP Selective Chemosensor

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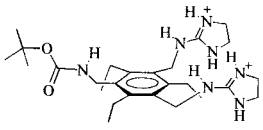
Chemosensors are most often developed using rational design followed by targeted synthesis,[1] while aptamers and antibodies derived from combinatorial techniques routinely reveal biosensors with high affinity and selectivity for various analytes.[2] Yet, the union of designed receptors with combinatorial methods has focused primarily upon screening the receptor against a library of analytes, thereby revealing the selectivity of the receptor/sensor, instead of revealing new receptors/sensors.[3] Further, several recent efforts have focused on immobilizing chemo- and biosensors on or in a variety of solid supports. This allows for incorporation of the sensor into an array.[4] Using ATP as an analyte for proof of principle, we now report that the use of a designed core structure, along with peptide libraries, can produce resin bound sensors with excellent selectivity.

Anion chemosensors have been developed using scaffolds such as **1**.[5] This scaffold places adjacent groups alternating up and down with respect to the plane formed by the benzene ring,[6] creating a preorganized cavity for binding interactions.[7] As a comparison for the study presented herein, we found that the affinity of **1** for ATP is 3.5×10^2 M^{-1} in water. This binding is electrostatic in nature and is not selective for the nucleoside base.

Library **2** was created to derive selective ATP receptors. Tripeptide arms, derived from combinatorial chemistry, were



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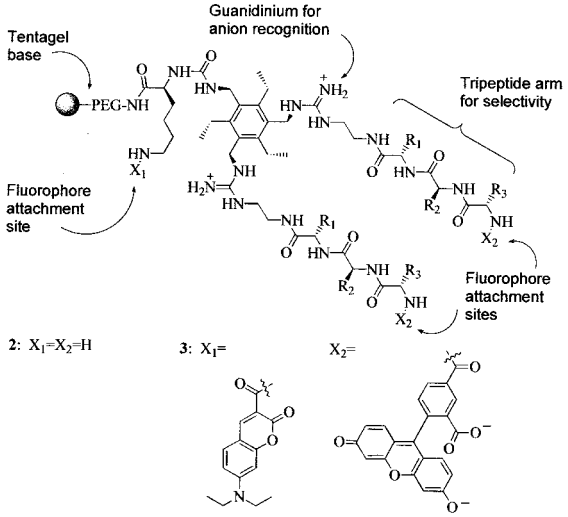

chosen to impart selectivity based upon interactions with the adenine group of ATP. For the ultimate application as sensors, a lysine linker was used to allow for incorporation of fluorophores.[5] Thus, sensor **3** was designed with 5-carboxyfluorescein appended to the ends of the peptide chains, while 7-diethylaminocoumarin-3-carboxylic acid was attached to the lysine either to act as an internal reference or to give a fluorescence resonance energy transfer signal transduction mechanism.[8]

For the discovery of ATP binding receptors, library **2** was prepared first (Scheme 1). After attachment of an orthogonally protected lysine, deprotection of the Fmoc allowed activation with *p*-nitrophenylchloroformate[9] yielding **4**, which in turn allowed introduction of the scaffold to give **5**. Incorporation of the guanidinium linkages using EDC and **6** took advantage of our recently developed solid-phase synthesis procedure for the creation of oligomeric guanidiniums.[10] *N*-terminal Fmoc-protection and acid-labile amino acid side chain protection were used for constructing the two identical tripeptide chains branching off of the guanidinium groups. The split and pool method[11] was used to generate a combinatorial library with a maximum possible 4913 tripeptides.

A model receptor containing the tripeptide *C*′-Gly-Phe-Gly-*N*′ was synthesized on Wang resin in parallel with library **2** in order to confirm the success of the synthetic route. Samples were cleaved with TFA after attachment of the core, after incorporation of the guanidinium groups, after synthesis of the peptide arms, and after final deprotections. High-resolution mass spectra were collected for each of these compounds and peptide microanalysis was performed on the final receptor to confirm the presence of Gly-Phe-Gly.

Fluorescently labeled *N*-methylanthraniloyl-ATP (MANT-ATP) was chosen to screen library **2** for ATP receptors.[12] A portion of

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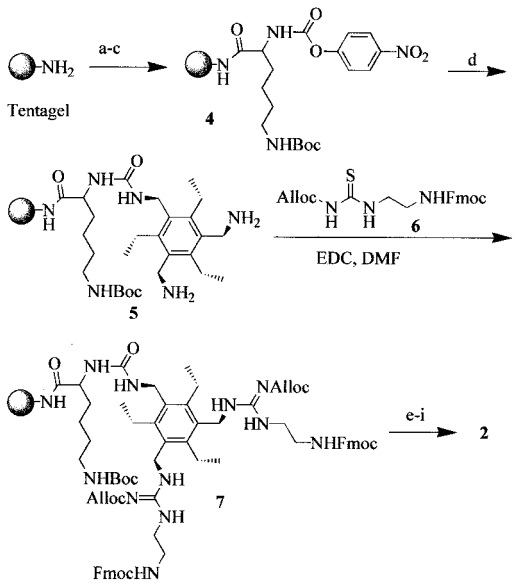
Scheme 1. Library Synthesis[a]



[a] Conditions: (a) HO-Lsy-(Boc)-NHFmoc, DIC, HOBT, DMF, (b) 20% piperidine, DMF, (c) *p*-nitrophenylchloroformate, DIEA, THF/CH_2Cl_2, (d) 1,3,5-triaminomethyl-2,4,6-triethylbenzene, TEA, DMF, (e) 20% piperidine, DMF, (f) three rounds of split and pool library synthesis, (g) 20% piperidine in DMF, (h) $(PPh_3)_4Pd$, *N*,*N*′-dimethylbarbituric acid, TMF, and (i) 90% TFA/4% TIS/4% thioanisole/2% H_2O.

Table 1. Sequencing Results[a]

active beads	inactive beads
His-Ala-Asp	**His-Phe-Gly**
Glu-Pro-Thr	Ser-Ala-Asp
Thr-Val-Asp	Trp-Asn-Glu
Met-Thr-His	
Asp-Ala-Asp	
Ser-Tyr-Ser	

[a] Sequences in bold were selected for resynthesis. 17 Fmoc amino acids were used. Cys, Lys, and Arg were excluded from the library.

the library was equilibrated with MANT-ATP (0.25 mM) in HEPES buffer (10 mM, pH 7.1) for 8 h and then washed with HEPES buffer. The resin was transferred to a microscope slide and illuminated with 366 nm UV light. It was possible to distinguish a range of fluorescence intensities among the library members from no fluorescence to highly fluorescent. Approximately 15% of the library members fell into the "highly fluorescent" category. Several of the highly fluorescent "hits" as well as a few nonfluorescent "misses" were sequenced. The sequencing results are shown in Table 1.

Three of the "hits" and one "miss" were arbitrarily chosen for resynthesis (see Table 1). The Boc group was removed after peptide synthesis, followed by DIC/HOBT coupling of 7-diethylaminocoumarin-3-carboxylic acid. Finally, the terminal Fmoc groups were removed, and a DIC/HOBT coupling of 5-carboxyfluorescein gave the "hits" and one "miss" in the form of **3**.

To assess the recognition and sensing behavior of the "hits" and "miss", a series of fluorescence studies were performed using a thin layer of beads sandwiched between two layers of gold mesh on a glass slide. The beads equilibrated rapidly upon addition of analyte (2−3 min was allowed) and the background variance was limited to approximately 2%. The "miss" did not show any emission modulation in response to the addition of ATP upon excitation of coumarin or fluorescein. In addition, there was no change in the extent of FRET for any of the "hits" upon addition of ATP. This may be due to there being an average distance between the fluorophores within the beads which does not significantly change upon binding ATP. However, all but one hit (Thr-Val-Asp) exhibited a fluorescence modulation upon excitation of fluorescein. The lack of response from one "hit" shows that screening against a derivatized analyte (MANT-ATP in this case) will not guarantee that the "hits" are successful sensors when synthesized with attached fluorophores. Either this "hit" binds the MANT portion of MANT-ATP or there is no significant microenvironment change around the fluorophores of Thr-Val-Asp receptor **3** upon binding ATP.

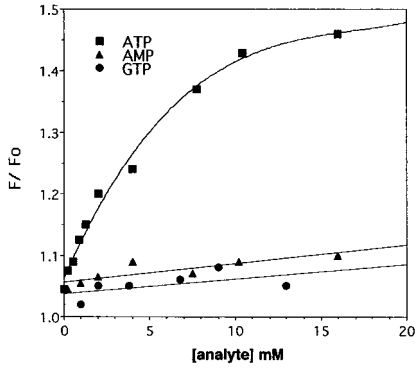


Figure 1. Comparison of the binding isotherms for Ser-Tyr-Ser **3** with ATP, AMP, and GTP. Fluorescence binding studies were performed in 200 mM HEPES buffer, pH 7.4.

Importantly, a large spectral response upon addition of ATP was observed with the Ser-Tyr-Ser sensor **3** (Figure 1). The increase in fluorescein emission is possibly due to a higher local pH around the fluorescein upon binding of ATP. The data for the sensor were plotted as F/F_0 vs $[ATP]_{total}$. We find a binding constant of 3.4×10^3 M^{-1}, approximately 10 times that of the core structure **1** alone.[13]

Our primary goal was to determine if the addition of the peptide arms would result in selectivity for ATP. Since the Ser-Tyr-Ser **3** sensor gave the largest fluorescence change upon addition of ATP, it was chosen for further studies with AMP and GTP. This peptidic library member exhibited very high detection selectivity for ATP over these structurally similar potentially competing analytes (Figure 1). The lack of response to AMP suggests the necessity for triphosphates to bind strongly to the guanidinium entities of **3**, while the lack of response to GTP indicates the specificity for nucleotide bases imparted by the tripeptide arms. The combination of serine and tyrosine suggests π-stacking between the phenol of tyr and adenine and hydrogen bonding interactions between the serine OH and/or the ribose or adenine. In summary, these studies have demonstrated that the union of a proven core with combinatorial methods, followed by the attachment of fluorophores, can create resin bound chemosensors with excellent selectivity.

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Supporting Information Available: Characterization of **6**, analysis of solid-phase synthetic intermediates, and description of technique for analyzing the emission of monolayers of beads (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Due to the differences in microenvironments within and on the surface of the tentagel beads, a range of different binding constants for each bead would best describe the binding in these systems. Hence, we think of the binding constant as an average binding constant.