Synthetic Ligands Discovered by in Vitro Selection

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Abstract: The recognition and catalytic properties of biopolymers derive from an elegant evolutionary mechanism, whereby the genetic material encoding molecules with superior functional attributes survives a selective pressure and is propagated to subsequent generations. This process is routinely mimicked in vitro to generate nucleic-acid or peptide ligands and catalysts. Recent advances in DNA-programmed organic synthesis have raised the possibility that evolutionary strategies could also be used for small-molecule discovery, but the idea remains unproven. Here, using DNA-programmed combinatorial chemistry, a collection of 100 million distinct compounds is synthesized and subjected to selection for binding to the N-terminal SH3 domain of the proto-oncogene Crk. Over six generations, the molecular population converges to a small number of novel SH3 domain ligands. Remarkably, the hits bind with affinities similar to those of peptide SH3 ligands isolated from phage libraries of comparable complexity. The evolutionary approach has the potential to drastically simplify and accelerate small-molecule discovery.

Introduction

The evolutionary approach to in vitro molecular discovery was first introduced 40 years ago,1 and since then, has become a routine method for isolating functional biopolymers.2 3 The power of the strategy lies in its use of nucleic acids to serve as amplifiable blueprints that can be translated into biopolymer sequences, enabling iterative functional selections to be carried out with extremely complex molecular libraries. The rare molecules that are fit to survive an imposed selective pressure eventually overtake the library. The simplicity and efficiency of in vitro evolution contrast with the current methods available for synthetic compound discovery, which rely heavily on robotics and high-throughput screening technologies. The largest small-molecule libraries that have been screened comprehensively are many orders of magnitude smaller than a typical in vitro evolution library, and the infrastructure required to screen them precludes their use by most basic science laboratories.

Applying evolution-based search strategies to synthetic chemical libraries could, in principle, dramatically accelerate small-molecule discovery. However, in vitro selection requires that each molecule be encoded by a genetic blueprint. Recently, we reported a strategy that fulfills this requirement for synthetic compounds by encoding their structure in DNA.4 6 Using sequence-directed hybridization5 and DNA-compatible chemical transformations,4 collections of single-stranded DNA (ssDNA) molecules are routed through a split-and-pool combinatorial synthesis. The products are covalent small molecule-DNA hybrids (Figure 1). Because the splitting is based on the DNA sequence, the synthesis of each gene product is uniquely programmed by the attached DNA molecule. Consequently, iterated rounds of library selection and amplification are possible. Using this approach, we previously isolated a known antibody epitope out of a library of one-million synthetic peptides.4 The experiment required six chemical translation steps for ligand synthesis and three generations for library convergence. A similar control was reported by Gartner and co-workers using DNA-templated synthesis.7 In that study, a library of 65 DNA-linked peptide-fumaride macrocycles was synthesized and subjected to one round of affinity purification and amplification, resulting in the enrichment of a DNA sequence encoding a known ligand. Although these efforts clearly illustrate the mechanics of DNA-programmed combinatorial chemistry and selection, they are based on recovering known ligands from “spiked” libraries and do not demonstrate that the technology will yield new binding motifs from wholly unnatural libraries.

The utility of blindly screening combinatorial chemistry libraries in search of new classes of ligands has been debated. One prevalent view is that combinatorial chemistry libraries are only useful if the constituent molecules are narrowly focused around a known lead. Biopolymers, however, are viewed quite differently. Nearly all efforts to isolate biopolymer ligands are based on the idea of using blind libraries: those not known a priori to contain a chemical motif that will bind to the target. It

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Figure 1. DNA Display. A library of ssDNA molecules (top) is chemically translated into synthetic compound-DNA conjugates. The DNA library is split into subpools by hybridization of 20 base codons to complementary oligonucleotide anticodons that are immobilized on separate columns (orange, cyan, pink bars). A distinct chemical transformation is carried out on each subpool, resulting in the covalent attachment of a chemical building block to the DNA (orange, cyan, pink balls). The library is pooled and then split on the basis of the next coding region (green, brown, yellow bars), and distinct chemical transformations are carried out for each subpool. The process is iterated until the entire DNA sequence is read. Each codon can exist at only one coding region. The translated library is subjected to selection for a function of interest (binding to the immobilized gray widget). DNA linked to binders is amplified and used as input for the subsequent round of chemical translation. The entire process is repeated until the library converges. Enriched molecules are identified by DNA sequencing and assayed for function as pure compounds.

is unclear whether these opposite points of view arise because biopolymers are fundamentally different from synthetic compounds (perhaps “privileged”) or because modern combinatorial chemistry libraries are too small to routinely yield potent hits from unfocused libraries. Only experimental data will ultimately resolve this question. Here, we take a step toward an experimental test by applying DNA-programmed synthesis and in vitro selection to a combinatorial chemistry library of 100 million diversity, several orders of magnitude larger than any that has been exhaustively explored to date.

Materials and Methods

Materials. Chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO), Acros (Geel, Belgium), Novabiochem (La Jolla, CA), Chem-Impex International (Wood Dale, IL), TCI America (Portland, OR), or Fisher Scientific (Hampton, NH). Microwave-assisted coupling was carried out with a Panasonic NN-S954WF 1250 W microwave.

The N-CrkSH3 peptide ligand (YPPPALPPKRRR) and all oligonucleotides were purchased from the Stanford PAN Facility (Stanford, CA). Oligonucleotide coupling experiments utilized an unmodified 10-base oligonucleotide of sequence CGGACTAGAG and a reactive twenty-base oligonucleotide of sequence H2 N-X-AGCAGGCGAAT-

Peptoid Coupling. The N-terminal SH3 domain from c-Crk (residues 134–191) was PCR amplified from a GST-fusion construct generously provided by Wendell Lim (University of California, San Francisco). An N-terminal FLAG-tag and nine amino acid Gly-Ser linker were added by subsequent PCR. The resulting FLAG-Crk construct was cloned into pET24a (Ndel and XhoI), which appended a C-terminal His6 tag. Protein was expressed in BL21 DE3 Escherichia coli, inducing with 1 mM IPTG for 5 h at 37 °C. Cells were harvested by centrifugation and sonicated. Filtered lysates were passed over a NiNTA column in 50 mM Tris-HCl pH 8.0, 300 mM NaCl, washed, and eluted in 100 mM NaH2PO4, pH 3.9, 300 mM NaCl. Pooled fractions were dialyzed into 50 mM Tris pH 8.0 and loaded onto a HiTrap flow Q-column. After washing, bound protein was eluted with 50 mM Tris pH 8.0 and 1 M NaCl and concentrated to approximately 2 mg/mL. Protein was over 90% pure by SDS-PAGE and verified by MALDI-MS.

Library Construction and Chemical Translation. We assembled the initial DNA library as described. A fraction of the assembled material was subcloned, and 114 isolates were sequenced to verify sequence degeneracy. New 20-base constant regions (Z0 and Z10) were material was subcloned, and 114 isolates were sequenced to verify sequence degeneracy. New 20-base constant regions (Z0 and Z10) were then added to the ends of the degenerate material via PCR to exclude the possibility of amplifying contaminating DNA from previous library experiments. All library amplification steps used these regions for priming. Single-stranded DNA synthesis, anticodon column synthesis, sequence-directed splitting, DEA-E-Sepharose column packing, anticondon-to-DEAE transfer, and endpoint isolation of the library were carried out exactly as described. One-hundred picomoles of ssDNA was used as starting material for each library round.

Peptoid Coupling. Chemist’s columns were washed with 3 mL of DEA bind buffer (10 mM acetic acid, 0.005% Triton-X100), 1 mL of water, and 6 mL of methanol. The columns were then incubated (2 × 20 min) with 500 µL of 150 mM DMT-MM and 100 mM ClaCOna in distilled methanol in open syringe barrels on a vacuum manifold, and then washed with 3 mL of methanol, 2 mL of 1 M propylamine in methanol, and 3 mL of methanol. Each column was then incubated
with 500 μL of the appropriate amine solution (Table S1, Supporting Information) and microwaved for 20 s at 100% power six times over 30 min in a closed system between two syringes. The columns were allowed to cool for 5 min after each microwave step. Only three microwave steps were used over 30 min for methylamine, ethylamine, ethanalamine, tryptamine, ethylenediamine, and 1,3-diaminopropane. After alkylation, the columns were washed with 1 mL of DMSO and 3 mL of DEAE bind buffer. For oligonucleotide coupling experiments, the columns were then eluted with 2 mL of DEAE elute buffer (50 mM Tris pH 8.0, 1.5 M NaCl, 0.005% Triton X-100) and analyzed by HPLC and MALDI-MS. For library synthesis, a 2 mL PBS wash (and a 2 mL DEAE elute buffer wash after the final chemistry step) was carried out just before subsequent hybridization.

Azide containing amines were synthesized exactly as described. After incorporation into DNA-peptoid conjugates, azide reduction was achieved with 10 mM TCEP, 100 mM Tris-HCl pH 8.0 for 1 h at room temperature. Reduced products were verified by HPLC and MALDI-MS. During library synthesis, this step was carried out just after duplexing the library.

Proline Coupling. Chemistry columns were washed with 3 mL of DEAE bind, 1 mL of water, 3 mL of THF, and 6 mL of distilled DCM. A 22.5 mg portion of Fmoc-L-proline (67 DEAE bind, 1 mL of water, 3 mL of THF, and 6 mL of distilled DCM.

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For control selection experiments, the ligand and competitors constructs were generated by PCR using HPLC purified peptide—oligonucleotide conjugates as forward primers. The constructs were quantified via UV absorbance and ethidium bromide fluorescence using a Typhoon 8600 imager (GE Healthcare, Piscataway, NJ). Selection load and elute amplification products were cleaned up using QIAquick PCR purification kits (Qiagen, Hilden, Germany), digested with BglI and BamHI, analyzed by agarose gel electrophoresis, and quantified via ethidium bromide staining and fluorescence.

Quantitative PCR. Selection inputs and elutes were quantified on a Stratagene (La Jolla, California) Mx3000P quantitative PCR machine using DyNamo HS SYBR Green qPCR reagents (Finnzymes, Espoo, Finland) and 200 nM of primers (Z1 and Z9). Samples were measured in duplicate and were quantified against a standard curve generated from serial dilutions of dsDNA of known concentration (also measured in duplicate). Dissociation curves indicated a single amplification product for all samples.

Sequence Analysis and Clustering. DNA sequences were translated computationally into the single letter peptoid code. Only full reads (all eight coding regions) were considered for further analysis. A similarity matrix for all peptoid 5mers was computed using the Needleman–Wunsch global alignment algorithm in MATLAB, with the gap-opening penalty set to 0.5 and no gap extension penalty. Two-dimensional hierarchical clustering was carried out with Cluster and viewed with TreeView (Michael Eisen, University of California, Berkeley).

Solid-Phase Peptoid Synthesis. Peptoids were resynthesized on Fmoc-Rink amide MBHA resin (Novabiochem) using a microwave-assisted protocol. All amines were used as 1 M solutions in DMSO. Proline residues were incorporated using 10 equiv of Fmoc-L-proline and HATU and 20 equiv of DIEA, twice for 1 h at 37 °C. The resin was then washed with DMF (8 × 3 mL), and the Fmoc group was removed with 20% piperidine in DMF for 20 min, and the column was washed with 3 mL of DMF and then 3 mL of DEAE bind buffer. For oligonucleotide coupling experiments, the columns were eluted with 2 mL of DEAE elute buffer and analyzed by HPLC and MALDI-MS. For library synthesis, a 2 mL PBS wash was carried out just before subsequent hybridization.

Selection. Prior to selection, the ssDNA-peptoid conjugates were duplexed via one-cycle PCR (50 μL) containing 20 μM of a single end primer (Z0), 200 μM of each dNTP, 5 mM MgCl2, 1X Promega Taq reaction buffer, and 5 U of Taq DNA polymerase (95 °C for 2.5 min, 58 °C for 1 min, 72 °C for 15 min). Azide side chains were then reduced, and the library was ethanol precipitated and resuspended in 25 μL of selection buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 0.25 mg/mL BSA, 0.1 mg/mL yeast tRNA). The library was incubated at 4 °C for 1 h with Pansorbin cells to preclear nonspecific binders. After centrifugation, the supernatant was taken (discarding cells), and the library was ethanol precipitated and resuspended in 1 mL of selection buffer containing 500 mM NaCl and once with 500 μL of selection buffer, all at 4 °C. Bound library members were eluted with 2 mL of DEAE elute buffer after the final chemistry step) was carried out just before subsequent hybridization.

Binding assay. Dissociation constants (Kd) were determined by measuring tryptophan fluorescence perturbation as a function of peptoid concentration essentially as described. The data were fit to the equation

\[
F_b = \frac{K_d + P + L \pm \sqrt{(K_d + P + L)^2 - 4 \times P \times L}}{2 \times P}
\]

where \(F_b\) = fraction protein bound, \(P\) = total protein concentration, and \(L\) = total peptoid concentration. N-CrksH3 concentration was between 0.5 and 1 μM. Measurements were made in triplicate at 22 °C on a FluoroLog-3 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ) with excitation at 295 nm (4 nm band-pass) and emission at 340 nm (4 nm band-pass). Average \(F_b\) values for each peptoid concentration were used to fit the binding curve and extract a \(K_d\) value. Errors for \(K_d\) values are estimated to be between 5 and 15%. Selection buffer was used for all assays.

**Results**

N-Substituted glycines, or peptoids (Figure 2A), comprise an attractive class of molecules for combinatorial discovery.\(^\text{15-17}\) Although structurally similar to peptides, peptoids lack amide hydrogens and are prolate resistant, making them potentially more druggable than peptides.\(^\text{18,19}\) Furthermore, the diversity element in peptoid synthesis is introduced via nucleophilic displacement with a primary amine, of which thousands are available commercially. Extremely large libraries of small peptoids can thus be actualized. Methods for solid-phase peptoid synthesis are well-established, rapid, and highly efficient, making large-scale resynthesis of library hits robust and inexpensive.

Peptoid synthesis is typically carried out in a submonomer format, whereby each residue is constructed in two synthetic steps — bromoacetylation of a secondary amine followed by nucleophilic displacement with a primary amine.\(^\text{20}\) Standard bromoacetylation protocols damage DNA and had to be modified for DNA-programmed peptoid synthesis. We found that chloroacetylation of oligonucleotides bearing a 5′ amine proceeded efficiently and without detectable DNA damage using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and sodium chloroacetate (ClAcONa) in methanol. Subsequent nucleophilic displacement to introduce the peptoid side chain was accomplished in high yield with concentrated amine (\(\sim 1 \text{ to } 3 \text{ M}\)) in DMSO or water. Taken together, the two-step addition of a peptoid residue to aminated DNA (Figure 2B, 2C) could be achieved in under 90 min. Yields for the addition of each residue ranged from 75 to \(>95\%\), depending on the size of the side chain on the secondary amine substrate (supplementary Figure 1, supplementary Table 1).

Side-chain hydroxyl and guanidium groups did not require protection, because no significant side reactions of these functional groups were detected. Side-chain carboxylic acids and primary amines could be introduced as tert-butyloxycarbonyl esters and azides, respectively. Acids were deprotected thermolytically,\(^\text{6}\) and azides were reduced to primary amines using tris(2-carboxyethyl)phosphine (TCEP). DNA-linked peptoids up to eight residues long were synthesized with per residue yields averaging 85−90%, even for sequences containing multiple hindered side chains (Figure 2D).

We wanted to include cyclic N-substituted glycine monomers (e.g., proline) in our peptoid libraries for added side chain and structural diversity. Although DMT-MM could be used to couple Fmoc-proline to DNA bearing a primary amine, acylation of secondary amines proceeded poorly. More stringent in situ acid chloride formation conditions, using trichloroacetimidite and triphenylphosphine in dichloromethane, were successful for coupling proline to even the most hindered peptoids tested (supplementary Figure 2). With subsequent Fmoc deprotection, total coupling time came to 1 h. This acid-free in situ activation strategy may prove to be generally useful for the acylation of hindered amines in a DNA-compatible fashion.

We chose the N-terminal Src homology 3 domain of the c-Crk protein (N-CrkSH3) as our selection target.\(^\text{21}\) SH3 domains are an abundant family of protein fragments that recognize proline-
rich peptide sequences, mediate numerous protein–protein interactions in signaling pathways, and are regarded as potential drug targets. Nguyen and co-workers demonstrated that several SH3 domains, including N-CrkSH3, could tolerate one or two proline-to-peptoid substitutions within native peptide ligands. Furthermore, a well-established, fluorescence-based binding assay exists for N-CrkSH3, facilitating the characterization of potential selection hits.

On the basis of previous experiments with DNA-programmed library selections, we pursued an immunoprecipitation-based selection strategy using FLAG-tagged N-CrkSH3, anti-FLAG antibody, and Pansorbin cells, followed by a competitive elution. When DNA bearing a peptide that binds to N-CrkSH3 was diluted into a solution of DNA bearing a nonbinding peptide and subjected to selection, an N-CrkSH3-dependent enrichment of nearly 1000-fold was observed for the ligand-DNA conjugate.

We designed a 100 million-member 8-mer peptoid library for selection experiments. The DNA-library construct included eight coding regions, with ten mutually exclusive codons at each region, and a 5′ primary amine as a synthetic handle. Rather than choosing from thousands of possible side chains at random, we opted to examine an N-CrkSH3 peptide cocrystal structure to assist our library design, assuming that a peptoid might bind to the target in a fashion similar to a peptide. The local environment of each side chain in the model...
The backbone structure of a bound decamer CrkSH3 (PDB accession code 1CKA) peptide ligand was copied and rotated 180° around an axis perpendicular to the helix axis and passing through the Cα of the central leucine (residue 5). The rotated structure was aligned with the original structure of the bound peptide and allowed to translate/rotate to minimize the deviation between (1) peptide carbonyl bonds and peptoid amide nitrogen/hydrogen bonds and (2) peptide Cα-Cβ bonds and peptoid amide nitrogen/hydrogen bonds.

(26) The backbone of a bound decamer CrkSH3 (PDB accession code 1CKA) peptide ligand was copied and rotated 180° around an axis perpendicular to the helix axis and passing through the Cα of the central leucine (residue 5). The rotated structure was aligned with the original structure of the bound peptide and allowed to translate/rotate to minimize the deviation between (1) peptide carbonyl bonds and peptoid amide nitrogen/hydrogen bonds and (2) peptide Cα-Cβ bonds and peptoid amide nitrogen/hydrogen bonds.


was assessed qualitatively and used to choose four monomer sets (Figure 4A). All of the sets, with the exception of the N-terminal set, share at least five side chains, and a great deal of steric and chemical diversity is maintained at each position.

A fully degenerate DNA library was chemically translated into peptoid-DNA conjugates and subjected to selection for N-CrkSH3 binding. The genetic material of the library members isolated after selection was amplified via polymerase chain reaction (PCR) and used to generate the ssDNA input for the subsequent round of chemical translation. We repeated this process, propagating the library through five rounds of chemical translation and selection. As an assay of bulk library convergence, quantitative PCR was used to measure the fraction of the translated library that survived each round of selection (Figure 4B). Initially, about 0.1% of the library was recovered, consistent with our signal-to-noise estimates of 1000:1 for the control selection experiments (Figure 3B). By the third round of the selection, an increase in percent survival was detected. This trend continued, and survival exceeded 1% for the fifth round, suggesting that the library was enriched for ligands. We also assessed the genetic makeup of the library after each selection step by subcloning and sequencing approximately 80 isolates. Clear signs of sequence convergence were apparent by the third library generation, in which roughly one-third of the isolates could be clustered into five sequence groups (the groups included exact matches, single mutants, and double mutants). This trend continued over the next two generations.

We thoroughly characterized the sixth generation affinity-matured library by sequencing 960 clones. Out of the 840 full-sequence reads, we observed only 215 unique sequences, 112 of which occurred at least twice. We computed a similarity matrix for all 840 peptoid 8-mers and clustered the sequences on the basis of their similarity scores, creating a phylogeny of synthetic compounds (Figure 4C). The data are essentially an evolutionarily produced structure—activity profile. Over 97% of the sampled sequences had at least five identical residues to a neighbor in the clustergram. A number of dominant sequence families are apparent, representing chemically and functionally related compounds highly enriched from the initial population.

Given the extensive convergence of library sequences, we wanted to directly examine the ability of peptoids encoded by enriched DNA sequences to bind N-CrkSH3. To that end, we used standard solid-phase peptoid synthesis protocols to prepare related compounds highly enriched from the initial population. 28 Carrying the library forward additional rounds might have produced tighter binding ligands, but at the expense of ligand diversity. The discovery of several novel ligand families for one naïve target suggests that large synthetic libraries should contain binding motifs for many targets.

Discussion

Clear patterns appear in the sequences of the validated ligands (Figure 5B and C). Complete conservation of tris-(2-aminoethyl)-amine as the side chain (W) at the N-terminal peptide residue suggests that these positive charges play an important role in N-CrkSH3 binding. Ionic interactions between acidic amino acids in SH3-domain loop regions and basic ligand residues flanking the polyproline segment are known to be crucial for the affinity and orientation of peptide binding in a
number of SH3 domains.27,29,30 Our peptoid ligands all possess a sequence of three to four relatively small side chains (methyl, ethyl, propyl, cyclopropyl, or ethanol) in the central portion of the peptoid chain and bulky substituents at the first and/or second residue. This does not simply reflect the monomers used at each position, as coding regions utilizing the same initial monomer set (e.g., regions 4, 5, 7, and 8) converged differently. The pattern presumably represents the structural features accommodated by the N-CrkSH3 binding site.

The chemical composition of the ligand families also speaks to the robustness of the technology. In controls, the β-branched side chains (residues C, F, G, I, and M; particularly the larger ones F, G, and I) exhibit lower coupling yields (75–90%) than the other residues. With the exception of isopropylamine (M), all of the β-branched residues are well represented in the converged sequence families (Figure 5B,C). Thus, coupling efficiencies do not dominate the outcome of the selection.

Four of the largest sequence clusters do not encode ligands, as determined by a tryptophan fluorescence perturbation binding assay. Possible explanations for this result include (1) DNA contributions to binding, in either a sequence-specific or nonspecific manner; (2) selection of minor unintended synthetic products, which are not present in the resynthesized and purified compound preparations; and (3) selection of ligands that do not induce a perturbation of tryptophan fluorescence. We ruled out sequence-specific DNA binding for the most enriched nonbinding sequences (Crktoids 6 and 7) using a control similar to that depicted in Figure 3. Specifically, when Crktoids 6 and 7 were synthesized on heterologous DNA sequences and subjected to selection, the peptoid-DNA conjugates were enriched 20–50 fold over background. Given the different behaviors of the Crktoids, we view small-molecule in vitro selection as being most useful for narrowing the focus of a molecular search problem from a very large library (>109) to a manageable number of molecules, which can then be assayed as purified compounds by a direct screening approach. In this example, we started with a library of 100 million distinct molecules, but performed only ten binding assays to discover six new ligand families.

The $K_d$ values for the peptoid hits are similar to those of natural SH3 ligand peptides31 and those derived from phage display libraries,32 despite the fact that the ligand scaffold is unnatural. A comparison of our result to the existing in vitro selection literature is informative. In the well-studied example of selecting linear peptides that bind to streptavidin, phage libraries of $10^8$–$10^9$ complexity routinely give ligand $K_d$ values ≥50 μM, whereas mRNA display33 and ribosome display34 libraries of $\sim10^{13}$ complexity give ligand $K_d$ values in the low nanomolar range. If a similar relationship between affinity and complexity applies to synthetic compound libraries, then compound collections of $10^{13}$ complexity should routinely produce nanomolar hits. The systematic comparison between chemical libraries and biopolymer libraries is only now possible: the ability to exhaustively test large populations of synthetic products is a unique capability afforded by chemical evolution technologies. Efforts are currently underway to generate DNA-programmed small-molecule libraries with complexities exceeding $10^{13}$.

The DNA-programmed combinatorial chemistry approach has the potential for widespread use because of its simplicity. We applied it here to a compound collection 2 orders of magnitude more complex than large HTS libraries and discovered unprecedented peptoid ligands. The molecules that emerged in this work were unknown at the outset. A priority going forward will be to expand the range of novel chemical classes that are amenable to DNA-programmed synthesis. If successful, these efforts should make custom small-molecule reagents as available as antibodies currently are, with applications in chemistry, biology, material science, and medicine.

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Supporting Information Available: Figures S1–S2, Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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