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Direct Comparison of Linear and Macrocyclic Compound Libraries as a Source of Protein Ligands

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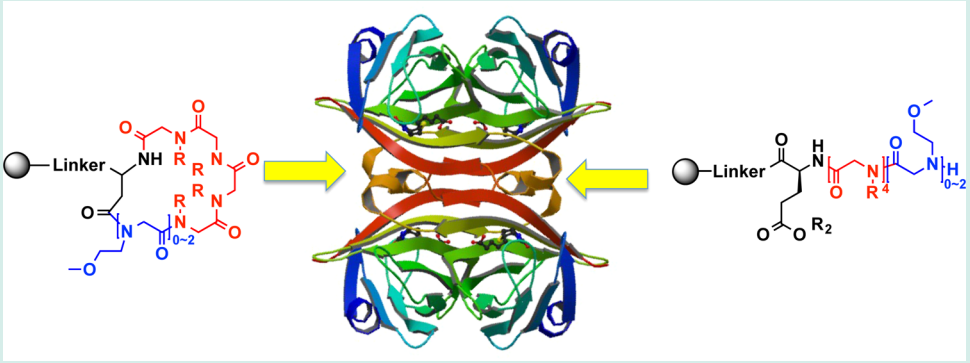
Supporting Information



ABSTRACT: There has been much discussion of the potential desirability of macrocyclic molecules for the development of tool compounds and drug leads. But there is little experimental data comparing otherwise equivalent macrocyclic and linear compound libraries as a source of protein ligands. In this Letter, we probe this point in the context of peptoid libraries. Bead-displayed libraries of macrocyclic and linear peptoids containing four variable positions and 0−2 fixed residues, to vary the ring size, were screened against streptavidin and the affinity of every hit for the target was measured. The data show that macrocyclization is advantageous, but only when the ring contains 17 atoms, not 20 or 23 atoms. This technology will be useful for conducting direct comparisons between many different types of chemical libraries to determine their relative utility as a source of protein ligands.

KEYWORDS: *macrocycle, peptoid, combinatorial library, high-throughput screening*

Many bioactive molecules are identified through screening combinatorial libraries or compound collections. As a result, there has been increasing interest in better understanding what libraries or collections have the most advantageous features for this endeavor. For example, there has been much discussion regarding the desirability of building more "natural product-like character" into libraries,[1] such as increasing the number of stereocenters and decreasing the number of flat, heteroaromatic rings. In another vein, many investigators feel that the best source of compounds to modulate protein−protein interactions[2] are likely to be larger species[1b,3] that occupy chemical space outside of Lipinski's "rule of five".[4] In the context of these "medium-sized" compounds, there is interest in building macrocycles,[5] since this structural feature limits the conformational freedom of what might otherwise be relatively "floppy" molecules.[6] To address these kinds of important questions in combinatorial chemistry, one would ideally run comparable screens against a particular protein target using several different libraries, then characterize all of the hits to determine which one was the best source of ligands. To our knowledge, there are few, if any, such studies in the literature. Here we report a method for doing precisely this and apply it to a comparative analysis of the utility of linear vs cyclic peptoids[5d,7] as well as cyclic peptoids of different ring sizes.

The design and synthesis of the libraries used in this study are shown in Figure 1. A common linker was synthesized on 75 μm TentaGel beads that included methionine to facilitate compound release from the beads postscreening by treatment with CNBr and an alkyne side chain to allow selective labeling of the compound after screening.[8] To the N-terminus of the linker was added a 95:5 mixture of allyl- and t-butyl-protected Fmoc-glutamic acid. To ensure efficient coupling, both diisopropylcarbodiimide (DIC) and ethyl-2-cyano-2-(hydroxyimino) acetate (Oxyma) were employed as coupling reagents. No base was added to prevent potential racemization. HPLC showed that after coupling of the 95:5 mixture of allyl- and t-butyl-protected Fmoc-glutamic acids to a model peptoid, the products indeed contained approximately 95% allyl-protected Fmoc-Glu linker and 5% *tert*-butyl-protected Fmoc-Glu linker

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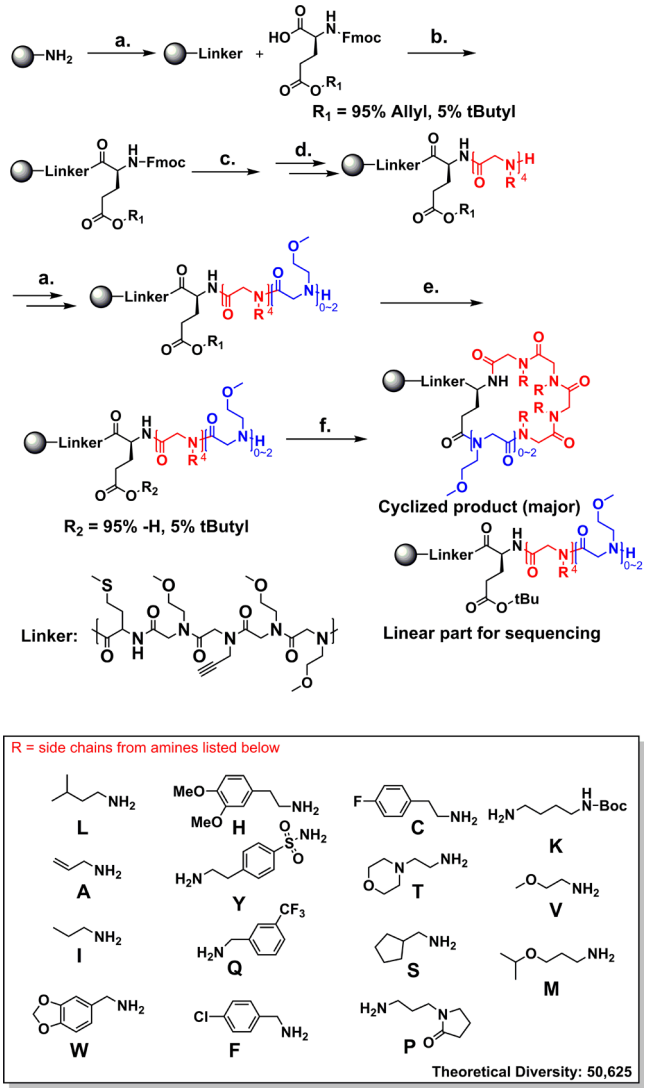

Figure 1. Design and synthesis of the libraries of the three linear and three cyclic molecules employed in this study. All six libraries consist of three regions: (1) linker region (shown in black), (2) variable region (shown in red), and (3) fixed region (shown in blue). The amines employed to construct the variable peptoid region are shown in the box. Each residue was assigned a one letter designator, which is employed in all of the tables. (a) Standard peptoid synthesis (see Supporting Information for details). (b) 4:4:4 equiv of DIC/Oxyma/glutamic acid mixture (95% Fmoc-Glu(OAll)-OH, 5% Fmoc-Glu-(OtBu)-OH) in DMF, 2 h ×2. (c) 20% piperidine in DMF, 15 min ×2. (d) Split-pool synthesis of four peptoid units using 15 primary amines listed in the box. (e) Pd(0)$(PPh_3)_4$, 2 equiv in 37:2:1 $CHCl_3$–AcOH-NMM under argon, 2 h. (f) PyBOP 4 equiv DIEA 10 eq. in DMF, 2h ×2.

(Figure S2.a-b Supporting Information), showing that the nature of the side chain-protecting group did not affect the coupling rate. After removal of the Fmoc group, split and pool peptoid synthesis protocols[9] were employed to add four variable peptoid residues using the 15 amines shown at the bottom of Figure 1 as diversity elements. Following the variable region, the beads were split into three portions and 0,1, or 2 peptoid units derived from methoxyethylamine were added. The beads were then treated with $(Ph_3P)_4Pd(0)$, which removed the allyl protecting group from the Glu residue, but not the *t*-Bu protecting group (Figure S1, Supporting Information). For each of the three libraries, half were treated with PyBOP, HOBt, and DIPEA to effect macrocyclization and half were not cyclized. HPLC and mass spectrometric analysis of the cyclized library demonstrated highly efficient ring closure (Table S1, SI). This protocol resulted in the production of six libraries, all of which were identical in containing the same four variable peptoid positions, each having a theoretical diversity of 50,625 compounds. The cyclic libraries differ only in the number of atoms in the ring (17, 20, and 23 atoms for libraries C1, C2 and C3, respectively) and the libraries of linear compounds differ from them only in that they are not cyclized.

Cyclic molecules are difficult to sequence by tandem mass spectrometry, thus, the 5% of uncyclized, *t*-Bu-Glu material on the beads was used for structural characterization of the molecules in libraries C1–C3 (Figure S2, Supporting Information).[7d] MALDI mass spectroscopy showed that the encoded macrocyclic compounds from a single bead could be identified and decoded easily after cleavage (Figure S2c–d, Supporting Information). Indeed, when 120 beads were picked randomly from each library, 112 of the compounds could be sequenced unequivocally after release from the bead with CNBr (Table S2, Supporting Information). The ring sizes used in this study were chosen because smaller rings did not cyclize efficiently and, while larger rings could be formed, significant amounts (~15%) of linear dimers were also produced (Table S1, Supporting Information).

Approximately 500 000 beads from each of the six libraries (~10-fold redundancy of each) were mixed together. They were then incubated with a mixture of unlabeled proteins (Starting Block), washed, and then incubated with streptavidin-coated magnetic beads. Peptoids that display ligands for streptavidin were then isolated by exposing the bead population to a powerful magnet and separating them from beads that did not become magnetized.[10] Under the conditions employed, 486 magnetized beads were isolated and placed into the wells of microtiter filter plates (Figure 2). Fluorescein azide was then

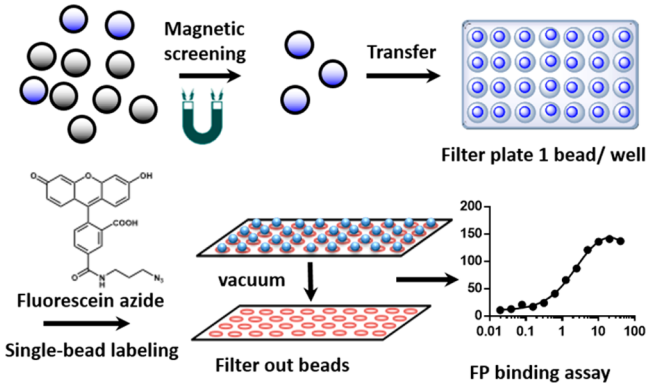

Figure 2. Schematic representation of the screening and hit characterization strategy employed.

added along with a copper catalyst to append the fluorescent probe to the alkyne side chain in the conserved linker.[8] Excess fluorescein azide was washed away and the compounds were released into solution by addition of CNBr to each well. The soluble, fluorescein-labeled compounds were filtered into a new mother plate. A small amount of material was withdrawn for determination of the structure of the compound by MALDI tandem mass spectrometry. The remainder was employed for binding studies (vide infra).

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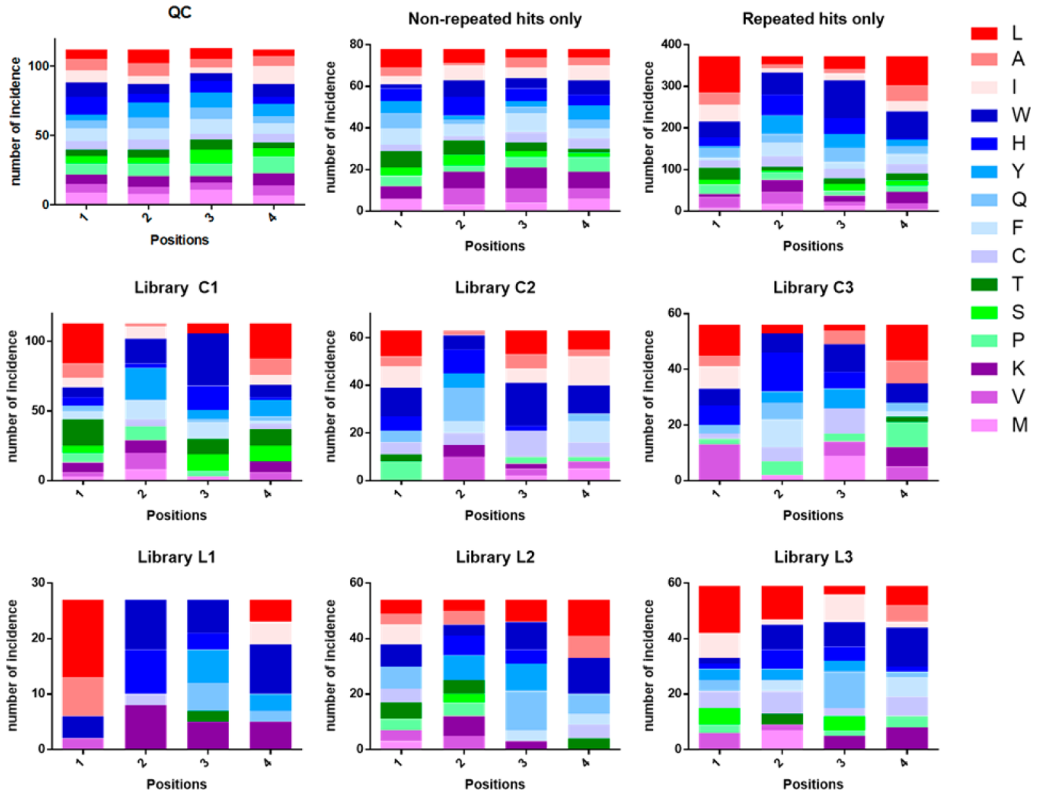

Figure 3. Side chain distribution of positions 1−4 in the variable region of the libraries. Top left: distribution of residues in the compounds isolated randomly from the libraries for quality control (QC) purposes. Top middle: distribution of residues in the hit compounds isolated from screening against streptavidin that were isolated only once. Top right: distribution of residues in the hit compounds isolated from screening against streptavidin that were isolated more than once. Middle and bottom rows: distribution of residues in the hit compounds isolated from screening against streptavidin that were isolated from the indicated libraries, showing only repeated hits. See Figure 1 for structure of each residue. Aromatic residues are colored in blue, linear aliphatic residues without a heteroatom are colored in red, residues with a nonaromatic ring structure are colored in green, and linear aliphatic residues with a heteroatom are colored in purple.

Four hundred and fifty of the 486 hits provided strong molecular ions and displayed fragments in the MS/MS that allowed their structures to be determined unequivocally. These data revealed that the hits were comprised of 184 unique sequences (Table S3, Supporting Information). Of these, 106 were compounds that were isolated more than one time (i.e., two or more beads displaying the same peptoid were among the 486 highly magnetized beads). "False positives", that is compounds that appear to bind the target protein well on the bead but display poor, if any, binding to the target protein in solution, are very common in this kind of screening experiment.[11,12] We have shown previously that this is due to significant bead to bead heterogeneity in the density at which compounds are displayed on the surface of the bead. Because of this complication, beads that display extremely poor ligands at an usually high density can appear to be good hits at the bead level, but fail to bind the protein target with acceptable affinity when analyzed in any other environment.[13] Fortunately, we have found that compounds identified multiple times from redundant OBOC libraries are far more likely to represent quality ligands,[13] because it is unlikely that the same poor ligand would be displayed more than once on hyper-dense beads in the population, which are relatively rare.[13] So it was encouraging that many redundant hits were obtained. Indeed, as shown in Figure 3, the distribution of residues in the hits isolated only once in the screen resembled that of the beads chosen randomly from the library for quality control analysis. In contrast, the repeat hits showed strong evidence of residue bias at each position. Moreover, we have found that there is a rough correlation between the affinity of a compound for the target protein and the number of times beads displaying that compound are pulled out of a screen of a redundant OBOC library (Mendes et al., in preparation). With this in mind, the results of the screen, when broken down by library, are interesting (Table 1).

Library C1, which contains molecules of the smallest ring size (17 atoms), yielded the most hits and, more importantly, the most repeat hits. The incidence of compounds isolated 6−8 times from the 10-fold redundant libraries was much higher

Table 1. Results of Screening the Six Libraries against Streptavidin

	CI	C2	C3	LI	L2	L3
total hit beads	132	79	67	38	64	70
unique sequences	46	36	29	20	25	28
repeated hits	27	20	18	9	15	17
hit repeated 8×	1	0	0	0	0	0
hit repeated 7×	3	0	0	0	0	1
hit repeated 6×	4	1	1	0	1	1
hit repeated 5×	3	2	2	1	3	2
hit repeated 4×	4	4	3	2	3	3
hit repeated 3×	5	5	4	2	5	4
hit repeated 2×	7	8	8	4	3	6
total repeated hits	19	16	11	11	10	11

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from library C1 than the others (Table 1). In addition, the fraction of the hits that were isolated more than once was higher from library C1 than the others (85%). In general, the hit statistics from the 20 atom C2 and 23 atom C3 libraries were fairly similar to each other and similar to those of the linear libraries, with the exception of library L1, which seemed to be inferior to the others, at least at this preliminary stage of analysis.

To characterize the affinity of each of the hits, both redundant and nonredundant, for streptavidin the fluorescein-labeled compounds were titrated with increasing amounts of the protein in a microtiter plate-based fluorescence polarization assay.[8] This powerful protocol, developed by Auer and co-workers, allows the determination of equilibrium dissociation constants without the need for hit resynthesis, which would be overwhelming in an experiment such as this. Table S3 in the Supporting Information shows these crude K_D values derived from this experiment for all of the hits tested. In general, the binding data confirm the expectations that one might have drawn from the screening statistics shown in Table 1. The smallest ring cyclic library, C1, indeed provided the greatest number of unique structures with K_D < 20 μM for streptavidin (Table 2) with 4, vs only one in the other libraries except L1, which had zero. Similarly, 20 unique compounds from library C1 exhibited average K_Ds below 50 μM while libraries C2 and C3 had 7 and 5 such compounds, respectively and the linear libraries L1−L3 had 2,5 and 9 such hits, respectively (Table 3).

Table 2. Characterization of the Best Hits from Each Library[a]

Best Hit	Avg. K_D	Repeat	$K_{D\text{-resyn}}$	Sequence				Library
KYG-1181	4.1	8X	8.8	L	W	W	W	C1
KYG-1171	7.2	7X	8.2	I	W	W	Y	C1
KYG-1172	7.7	7X	12.1	T	P	W	A	C1
KYG-1173	11.8	7X	15.4	T	P	W	L	C1
KYG-1181	-	-	14.5*	L	W	W	W	C1
KYG-1171	-	-	14.1*	I	W	W	Y	C1
KYG-1481	-	-	113	L	W	W	W	linear 1181
KYG-1471	-	-	>200	I	W	W	Y	linear 1171
KYG-1261	17.4	6X	24	P	V	A	I	C2
KYG-1251	39.4	5X	48	C	K	L	M	C2
KYG-1252	33.0	5X	50	W	C	C	W	C2
KYG-1241	25.7	4X	61	I	H	W	C	C2
KYG-1361	26.3	6X	36	V	H	W	P	C3
KYG-1351	7.3	5X	15.4	L	C	C	K	C3
KYG-1651	-	-	45	L	C	C	K	linear 1351
KYG-1352	75.5	5X	80	H	W	V	W	C3
KYG-1341	34.0	4X	42	L	H	H	L	C3
KYG-2151	26.5	5X	22	A	W	K	W	L1
KYG-2141	66.1	4X	71	L	K	Y	L	L1
KYG-2142	58.0	4X	88	W	K	W	W	L1
KYG-2131	81.2	3X	99	L	H	Q	Y	L1
KYG-2261	17.4	6X	22	T	Y	W	L	L2
KYG-2251	49.7	5X	59	W	P	Q	W	L2
KYG-2252	83.5	5X	122	Q	A	Y	W	L2
KYG-2253	63.1	5X	97	C	V	L	A	L2
KYG-2371	9.0	7X	11.2	I	H	Q	F	L3
KYG-2671	-	-	91	I	H	Q	F	cyclic 2371
KYG-2361	26.2	6X	19	S	L	I	W	L3
KYG-2351	32.0	5X	54	L	C	K	K	L3
KYG-2352	37.4	5X	69	L	W	H	L	L3

[a]All K_D values are in μM. See Figure 1 for residue abbreviations. In sequence code, aromatic residues are shown in medium gray, linear aliphatic residues without heteroatom are shown in light gray, residues with non-aromatic ring structure are shown in deep gray, and linear aliphatic residues with heteroatom are shown in white. Asterisk (*) indicates K_D measured at presence of 1 mM biotin.

Table 3. Number of Hits with K_D Values below 20 μM and 50 μM

	C1	C2	C3	L1	L2	L3
number of hits with raw K_d value						
<20 μM	27	4	6	2	3	7
<50 μM	83	31	23	7	15	27
number of unique sequences with average K_d value						
<20 μM	4	1	1	0	1	1
<50 μM	20	7	5	2	5	9

The four highest affinity streptavidin ligands from each of the six libraries were resynthesized and HPLC-purified to allow for detailed characterization. As expected, this included the compounds isolated the most times from the redundant libraries.[13] As shown in Table 2, the K_Ds measured carefully using purified compound correspond well to the average K_D values derived from the high-throughput FP assay. The best compounds from the cyclic library C1 were approximately 8 μM streptavidin ligands, whereas the best ligands from the library of larger macrocycles, C2 and C3, had K_D values of 24 and 15 μM, respectively. However, the other hits from libraries C2 and C3 were significantly poorer ligands (Tables 2 and 3), showing that the library of smaller ring macrocycles was indeed the best source of high affinity streptavidin ligands.

The linear libraries were all inferior to cyclic library C1 but there was not a substantial difference between the linear hits and those obtained from the larger ring libraries C2 and C3. Indeed, the linear library L3 provided two hits, KYG-2371 (K_D = 11 μM) and KYG-2361 (K_D = 19 μM) that showed a higher affinity for streptavidin than any of the compounds in cyclic libraries C2 or C3. Clearly the presence of a macrocycle did not confer an advantage to libraries C2 and C3 relative to their linear counterparts. Interestingly, the two highest affinity hits from library L3 did not have any obvious sequence similarity to the highest affinity hits from library C1 (Table 2). The linear analogues of the two best hits from library C1, KYG-1181 and KYG-1171, were synthesized and tested for binding to streptavidin. As shown in Table 2, a drastic loss of affinity resulted from linearization. KYG-1481, the linear analogue of KYG-1181, bound streptavidin more than 10-fold more weakly than its cyclic counterpart (K_D = 113 μM). The binding of KYG-1471, the linear analogue of KYG-1171, to streptavidin was barely detectable at the protein concentrations used (K_D > 200 μM). In contrast, when the linear version (KYG-1651) of the best hit from either the C2 or C3 libraries (KYG-1351) was synthesized and evaluated, it also displayed reduced affinity relative to its cyclic analogue, but the decrease was much less dramatic (~3-fold). Finally, going in the opposite direction, the macrocyclic analogue of the best linear hit, KYG-2371, was synthesized. This compound, KYG-2671, bound to streptavidin with about an 8-fold poorer affinity than its linear cousin (Table 2). This provides yet another data point supporting the view that macrocyclization is not always beneficial. Presumably, the linear molecule KYG-2371, must bind streptavidin in a conformation that is disfavored by macrocyclization, though we cannot rule out the alternative possibility that a free N-terminus is involved in the binding event.

While not central to the focus of this study, we asked if the two best ligands from library C1 (KYG-1181 and KYG-1171)

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recognize the biotin-binding site of the streptavidin. To do so, the titration of labeled cyclic peptoids with streptavidin was carried out in the presence or absence of saturating levels of biotin. The results were virtually identical (Table 2), showing that these peptoids bind a surface of streptavidin distinct from that of the biotin-binding site, which is unusual.[5d,14]

In summary, we have demonstrated a convenient, quantitative method to compare the utility of different OBOC libraries as a source of ligands for a given protein. This kind of analysis is made possible by the powerful protocol of Auer and co-workers to determine binding constants of screening hits in solution by fluorescence polarization spectroscopy without the need for resynthesis.[8] The use of redundant libraries containing multiple copies of each compound is also important.[13] This is because there can be considerable fluctuation between the K_D values measured with compound from a single bead in the high-throughput assay between identical molecules (Table S3, Supporting Information). Fortunately, the average K_D measured for multiple copies of the same compound corresponded well to the K_D measured for resynthesized and purified molecules. This methodology should be applicable to the comparison of any libraries that can be created by solid-phase split and pool synthesis.[15]

In this study, we focused on the question of whether macrocyclization of peptoid chains would facilitate the isolation of higher affinity ligands for a given protein target. Note that this is quite a different question than simply selecting a cyclic molecule from a screen and asking if that ligand binds just as well in the linear form, or vice versa.[5d,16] To our knowledge, this is the first time strictly comparable cyclic and linear libraries have been compared in a quantitative fashion. The results are quite clear. A macrocyclic library proved to a superior source of ligands relative to its linear counterpart, but only in the case of the smallest ring size of 17 atoms (library C1). Larger rings of 20 and 23 atoms (libraries C2 and C3, respectively) did not display a significant advantage over their linear congeners. Presumably, the 17-membered ring is small enough to begin to impose significant conformational constraints on the molecule. Of course, this study focused only on a single protein target, streptavidin. Further studies of other protein targets will be necessary before general statements can be made with confidence. Moreover, we used peptoids, which are particularly floppy molecules with few conformational constraints in their linear form. Other types of oligomers with more significant intrinsic conformational constraints in the backbone might provide different results. Indeed, one might predict that larger ring sizes would confer advantages in these cases. Now that this method for quantitative comparisons of the "goodness" of a library for a particular target has been established, such questions can be addressed experimentally.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details of cyclization, encoding, and binding measurements and sequences of quality control beads and hits. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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