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A Cleavable Scaffold Strategy for the Synthesis of One-Bead One-Compound Cyclic Peptoid Libraries That Can Be Sequenced By Tandem Mass Spectrometry

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Abstract

Many macrocyclic depsipeptides or related compounds have interesting medicinal properties and often display more favorable pharmacokinetic properties than linear analogues. Therefore, there is considerable interest in the development of large combinatorial libraries of macrocyclic peptidomimetic compounds. However, such molecules cannot be easily sequenced by tandem mass spectrometry, making it difficult to identify hits isolated from library screens using one bead one compound libraries. Here we report a strategy to solve this problem by placing a methionine in both the linker connecting the cyclic molecule to the bead as well as within the cycle itself. Treatment with CNBr both linearizes the molecule at a specific position and releases the molecule from the bead, making its characterization by tandem MALDI mass spectrometry straightforward.

Introduction

Many cyclic peptide and depsipeptides have been found to display potent biological activities and often have better pharmacokinetic properties than their linear counterparts.^{1–5} This has prompted interest in the synthesis and screening of large libraries of cyclic peptides^{6–11} or peptide-like compounds.^{12,13} However, with the exception of genetically encoded cyclic peptides^{7,14–16} a difficulty with screening such libraries is deducing the structure of hit compounds. Because cyclic peptides and peptide-like compounds lack a free N-terminus, Edman sequencing cannot be employed directly. In the mass spectrometer, cyclic oligomers fragment at multiple positions, making the interpretation of tandem MS-MS spectra difficult¹⁷. ‘One bead-two compound’ encoding strategies¹⁸, in which each bead contains both a cyclic and linear version of each compound have been used to mitigate this problem^{19,20}. Another recent innovation is a combined Edman cleavage/mass spectrometric strategy for the identification of cyclic peptides and peptoids.^{19,21}

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Supplementary data

Full experimental procedures and full data on compound characterization can be found online at:

Recently, Lim and co-workers reported a different strategy in which the macrocycle contained a group that could be cleaved selectively, thus linearizing the molecule, which could then be sequenced easily^{22,23}. We describe here a different version of this strategy that incorporates a selectively-cleavable methionine residue into both the cyclic scaffold and the linker connecting the molecule to the bead. This allows for simultaneous site-specific linearization and release of the bead-bound molecule. The soluble species can then be sequenced by tandem mass spectrometry.

Results and Discussion

“Dual methionine” libraries

The first strategy that we explored for the creation of macrocyclic peptoid libraries that can be sequenced by mass spectrometry is depicted in Figure 1. The design involves incorporation of a methionine amino acid unit into the macrocycle as well as the linker arm that tethers the molecule to the bead. When treated with cyanogen bromide, the anticipated cleavage at the C-terminal side of both methionines should provide a soluble, linear molecule that could be sequenced by tandem mass spectrometry.

The library was synthesized on 75 μ m TentaGel beads, derivatized with an invariant linker comprised of Pro-Asp-Nffa-Met-Nlys-(3-aminopropanoic acid)-(2-phenyl isopropyl-protected Glu)-Nmea. The first three residues (Pro-Asp-Nffa) allow for mild acid cleavage of the molecules from the beads and subsequent spotting of the molecules onto maleimide-activated glass slides²⁴, but this feature of the library is not relevant to the present study. The remaining residues provide a point of site-specific cleavage (Met), a positive charge to aid in ionization in the MALDI mass spectrometer (Nlys), two spacers (3-aminopropionic acid and Nmea) and a carboxylate side chain to support macrocyclization with N-terminal end of the molecule (differentially protected Glu). Following this invariant linker, split and pool synthesis using the standard “sub-monomer” route to peptoids²⁵ was employed to construct a one-bead one-compound peptoid library^{26,27} with a theoretical diversity of one million compounds by incorporating the ten amines shown in Figure 2. The library was completed by capping the peptoid chain with methionine, selectively deblocking the phenyl isopropyl group with 2% TFA and, finally, effecting cyclization with PyAOP.

A critical issue is to assess the cyclization efficiency for each molecule, since the chemical nature of the intervening residues can have significant effects on cyclization rates. To do so, we employed a ninhydrin test, which reveals unreacted terminal amines from uncyclized N-terminal methionine residues. To calibrate the assay, aliquots of beads removed from the library prior to the cyclization reaction were counted and analyzed. Kaiser test reagents (equal parts: 5% ninhydrin in ethanol, 80% phenol in ethanol, and 2% 0.001 M aqueous KCN in pyridine; 25 μ l total volume) were added to each tube and the tubes were heated to 95 °C for 7 min. Aqueous ethanol (60% v/v, 225 μ l) was added to each tube. The optical density of the resulting solutions was measured at 590 nm (see Table 1). This provided a benchmark for the intensity of color that would be obtained if no cyclization at all had occurred. The same protocol was then employed to analyze batches of beads that had been subjected to cyclization conditions. As can be seen from Table 1, the average value obtained in these experiments was approximately 30% of that observed from uncyclized beads, suggested that the average overall cyclization efficiency was approximately 70%.

To assess the level of bead-to-bead variability of the yield, a portion of the beads from the cyclization reaction that had undergone the ninhydrin test were examined under a low power microscope. The image (see Fig. 3) suggests that this overall yield represented mostly a mixture of beads that bear peptoids that cyclize in high yield and some that cyclize very poorly, as judged by the intensity of the color. In other words, most of the beads were either

dark blue or yellow. There were relatively few beads whose color was intermediate between the two that suggested a moderate cyclization yield. Therefore, these results suggests that the library is comprised, to a first approximation, of beads displaying almost entirely cyclic peptoids (70%) and beads displaying almost entirely linear peptoids (30%).

Screening of the dual methionine library and sequencing of single hits by tandem mass spectrometry

We next turned to the question of whether the macrocyclic molecules could be cleaved by CNBr and sequenced by tandem mass spectrometry efficiently enough to allow the characterization of “hits” from screening OBOC libraries against labeled proteins. To test this, approximately 500,000 beads from the library described above were blocked with a commercial protein solution, then incubated with streptavidin-coated magnetic particles (Dynabeads). After a suitable incubation, the entire bead population was exposed to a powerful magnet held to the side of the tube containing the library. We anticipated that beads displaying streptavidin-binding peptoids would be retained by the magnet through their non-covalent association with the Dynabeads, while the “non-hits” would settle to the bottom of the tube²⁴. Twenty beads were isolated in this manner and were stripped of the magnetic particles by treatment with 1% SDS. Each bead displaying a putative hit was then placed in a well of a microtiter plate and treated with an acidic solution of cyanogen bromide. After 16 hours, the cleavage mixture was evaporated and the residue was dissolved in acetonitrile/water (50:50) for MALDI mass analysis. Fifteen of the twenty beads (75%) produced clean spectra, which is comparable with results obtained with MS/MS sequencing of hits from linear libraries (Supplementary Figures 1–15).

The mass spectrum of each cleaved peptoid contained three prominent peaks (Figure 4a). The center peak corresponds to the expected cleavage product and could be sequenced, providing the identities of the putative streptavidin-binding peptoids (Figure 4b). The smaller peak at -101 may correspond to loss of the N-acyl homoserine lactone on the glutamic acid or C-terminus. The peak at +46 may correspond to the Metoxidized form of the cyclic compound, which would not react with cyanogen bromide to complete ring opening. However, neither of these possibilities has been confirmed rigorously, as it is not of central importance to our study. We conclude from these experiments that the dual methionine library strategy indeed provides cyclic molecules that can be characterized by tandem mass spectrometry from a single bead.

Preliminary characterization of the streptavidin-binding peptoids

While the focus of this study was the development of efficient protocols for library synthesis and compound characterization, we nonetheless carried out a preliminary characterization of the putative streptavidin-binding compounds.

Alignment of the sequences of the hits revealed that most fall into two groups that differ primarily in the order of Nasp and Npip residues (Figure 2b). Three compounds did not contain these motifs. One compound representing each conserved motif was resynthesized, conjugated to fluorescein, and its binding to streptavidin analyzed by fluorescence polarization binding assays. Notably, the resynthesized compounds cyclized in excellent yield as determined by the ninhydrin test (not shown). Hits 1 and 16 had K_{DS} for monomeric streptavidin (Neutravidin) of $19 \pm 2 \mu\text{M}$ and $12 \pm 1 \mu\text{M}$ respectively (Figure 5). A scrambled version of Hit 1 (Npip-Namy-Nasp-Npip-Nasp-Npip) bound streptavidin with a much lower affinity (Figure 5B). Hit 1 does not bind to a different protein, cholera toxin, and a “non-hit” cyclic compound chosen randomly from the library does not bind streptavidin (Figure 5C). Taken together, these results argue that the peptoids selected in the screen are selective for streptavidin. We also synthesized a linear version of Hit 1 and measured its affinity for

monomeric streptavidin (Figure 5A). The K_D of 14 ± 1 was almost the same as that of the complex between streptavidin and the cyclic peptoid. Clearly, the expectation of higher affinity through cyclization was not realized in this case. While this is only a single datum point and one should be careful not to over generalize the conclusions reached from it, it may be the case that the ring size of the library that we employed in this study is too large to impose significant conformational constraints on the molecule.

Summary

We have developed an effective scheme for the mass spectrometry-based elucidation of cyclic peptoid molecules isolated from one bead one compound combinatorial libraries. This chemistry complements a different ring cleavage protocol reported previously by Lim and co-workers²³. The experiments described here demonstrate that these libraries can be taken through the entire synthesis and screening procedures and provide well characterized, specific protein-binding ligands. In the future, it will be important to adapt this technology to the synthesis of more compact and/or conformationally constrained macrocyclic libraries since the results of the streptavidin-targeted screen suggest that the particular library reported here is not sufficiently constrained to afford ligands with substantially higher affinity than the corresponding linear molecules. This work is underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
The dual methionine strategy for the creation of bead-displayed libraries of cyclic peptoids with a cleavable ring and linker.

Fig. 2. General structure and synthetic route to the combinatorial library of macrocyclic peptoids. The ten amines shown at the bottom of the figure were employed in the split and pool synthesis. The Boc and OtBu protecting groups shown on the amines were removed on resin after completion of the library.

Fig. 3.
Photomicrograph of beads from the cyclic peptoid library after subjecting them to the ninhydrin test.

Figure 4.

(a) General structure of cleaved compounds and the MALDI mass spectrum of cleaved Hit

1. (b) The 15 sequencable compounds identified in the screen.

Figure 5.

Characterization of the protein-binding properties of Hit 1 as measured by fluorescence polarization. A. (▲) Cyclic Hit 1, (●) Cyclic Hit 16, (■) Linear Hit 1 [Nac-Met-Nasp-Nasp-Namy-Npip-Npip-Npip-Nmea-Cys-Fluor]. B. (▲) Cyclic Hit 1 [Nasp-Nasp-Namy-Npip-Npip-Npip], (■) Scrambled [Npip-Namy-Nasp-Npip-Nasp-Npip]. C. (▲) Cyclic Hit 1 binding to streptavidin, (●) Non-hit cyclic compound [Npea-Namy-Nlys-Narg-Nlys-Npea] binding to streptavidin, (■) Cyclic Hit 1 binding to cholera toxin.

Table 1

Results of the ninhydrin tests to determine cyclization efficiency.

	# beads	OD (590 nm)	corrected OD	OD/head	Averages
Blank	0	0.0585			
Uncyclized	90	0.3539	0.2954	3.28	
	110	0.4072	0.3487	3.17	
	140	0.5263	0.4678	3.34	3.26
Cyclized	120	0.1845	0.1260	1.05	
	115	0.1603	0.1018	0.89	
	150	0.2103	0.1518	1.01	0.98
30% uncyclized					