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High-Throughput Sequence Determination of Cyclic Peptide Library Members by Partial Edman Degradation/Mass Spectrometry

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Abstract: Cyclic peptides provide attractive lead compounds for drug discovery and excellent molecular probes in biomedical research. Large combinatorial libraries of cyclic peptides can now be routinely synthesized by the split-and-pool method and screened against biological targets. However, post-screening sequence determination of hit peptides has been problematic. In this report, a high-throughput method for the sequence determination of cyclic peptide library members has been developed. TentaGel microbeads (90 μm) were spatially segregated into outer and inner layers; cyclic peptides were displayed on the bead surface, whereas the inner core of each bead contained the corresponding linear peptide as the encoding sequence. After screening of the cyclic peptide library against a macromolecular target, the identity of hit peptides was determined by sequencing the linear encoding peptides inside the bead using a partial Edman degradation/mass spectrometry method. On-bead screening of an octapeptide library (theoretical diversity of 160 000) identified cyclic peptides that bind to streptavidin. A 400-member library of tyrocidine A analogues was synthesized on TentaGel macrobeads and solution-phase screening of the library directly against bacterial cells identified a tyrocidine analogue of improved antibacterial activity. Our results demonstrate that the new method for cyclic peptide sequence determination is reliable, operationally simple, rapid, and inexpensive and should greatly expand the utility of cyclic peptides in biomedical research.

Introduction

Cyclic peptides and depsipeptides, due to their conformational rigidity, have enhanced receptor-binding affinities, specificity, and stability relative to their linear counterparts. These features make cyclic peptides attractive leads for drug discovery and excellent molecular probes for biomedical research. Numerous biologically active cyclic peptides and depsipeptides have been found in nature.[1] Rational design and screening of combinatorial libraries have also led to the discovery of cyclic peptides as novel antibiotics,[2] enzyme inhibitors,[3] and receptor antagonists.[4] Today, large libraries of cyclic peptides are readily accessible through solid-phase split-and-pool synthesis.[5] However, screening of these cyclic peptide libraries against biological targets is often bottlenecked by sequence determination of hit peptides. Because cyclic peptides (N-to-C cyclization) have no free N-terminus, they cannot be sequenced by conventional Edman degradation. Sequencing of cyclic peptides by tandem mass spectrometry (MS) has also been challenging, despite numerous attempts.[6] In a mass spectrometer, a cyclic peptide undergoes ring opening at multiple positions to afford a family of mass degenerate ions, each of which further fragments into a complex mixture of shorter peptides, making spectral interpretation very difficult.[6] In the previously reported studies, sequence determination of cyclic peptides was usually achieved by sequencing the corresponding genes (e.g., phage display[3a,d,4d] and intein-mediated cyclization[4e]) or iterative deconvolution.[2b,c,3b,4a,b] The former method is limited to libraries containing natural amino

(1) Reviewed by: Hamada, Y.; Shioiri, T. *Chem. Rev.* **2005**, *105*, 4441−4482.
(2) For examples see: (a) Fernandez-Lopez, S.; Kim, H.-S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. *Nature* **2001**, *412*, 452−456. (b) Kohli, R. M.; Walsh, C. T.; Burkart, M. D. *Nature* **2002**, *418*, 658−661. (c) Qin, C.; Bu, X.; Zhong, X.; Ng, N. L. J.; Guo, Z. *J. Comb. Chem.* **2004**, *6*, 398−406. (d) Dartois, V.; Sanchez-Quesada, J.; Cabezas, E.; Chi, E.; Dubbelde, C.; Dunn, C.; Granja, J.; Gritzen, C.; Weinberger, D.; Ghadiri, M. R.; Parr, T. R. Jr. *Antimicrob. Agents Chemother.* **2005**, *49*, 3302−3310.
(3) For examples see: (a) McBride, J. D.; Freeman, H. N.; Domingo, G. J.; Leatherbarrow, R. J. *J. Mol. Biol.* **1996**, *259*, 819−827. (b) Eichler, J.; Lucka, A. W.; Pinilla, C.; Houghten, R. A. *Mol. Diversity.* **1996**, *1*, 233−240. (c) March, D. R.; Abbenante, G.; Bergman, D. A.; Brinkworth, R. I.; Wickramasinghe, W.; Begun, J.; Martin, J. L.; Fairlie, D. P. *J. Am. Chem. Soc.* **1996**, *118*, 3375−3382. (d) Bratkovic, T.; Lunder, M.; Popovic, T.; Kreft, S.; Turk, B.; Strukelj, B.; Urleb, U. *Biochem. Biophys. Res. Commun.* **2005**, *332*, 897−903.
(4) For examples see: (a) Spatola, A. F.; Crozet, Y.; deWit, D.; Yanagisawa, M. *J. Med. Chem.* **1996**, *39*, 3842−3846. (b) Zang, X.; Yu, Z.; Chu, Y.-H. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2327−2332. (c) Leduc, A.-M.; Trent, J. O.; Wittliff, J. L.; Bramlett, K. S.; Briggs, S. L.; Chirgadze, N. Y.; Wang, Y.; Burris, T. P.; Spatola, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11273−11278. (d) Sakamoto, K.; Yuji, Y.; Hashiguchi, S.; Sugimura, K. *Pep. Sci.* **2003**, *39*, 279−280. (e) Tavassoli, A.; Benkovic, S. J. *Angew. Chem., Int. Ed.* **2005**, *44*, 2760−2763.
(5) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84. (b) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86. (c) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Peptide Protein Res.* **1991**, *37*, 487−493.
(6) Eckert, K.; Schwarz, H.; Tomer, K. B.; Gross, M. L. *J. Am. Chem. Soc.* **1985**, *107*, 6765−6769. (b) Schilling, B.; Wang, W.; McMurray, J. S.; Medzihradszky, K. F. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2174−2179. (c) Ngoka, L. C. M.; Gross, M. L. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 732−746. (d) Siegel, M. M.; Huang, J.; Lin, B.; Tsao, R.; Edmonds, C. G. *Bio. Mass Spectrom.* **1994**, *23*, 186−204. (e) Redman, J. E.; Wilcoxen, K. M.; Ghadiri, M. R. *J. Comb. Chem.* **2003**, *5*, 33−40.

10.1021/ja063722k CCC: $33.50

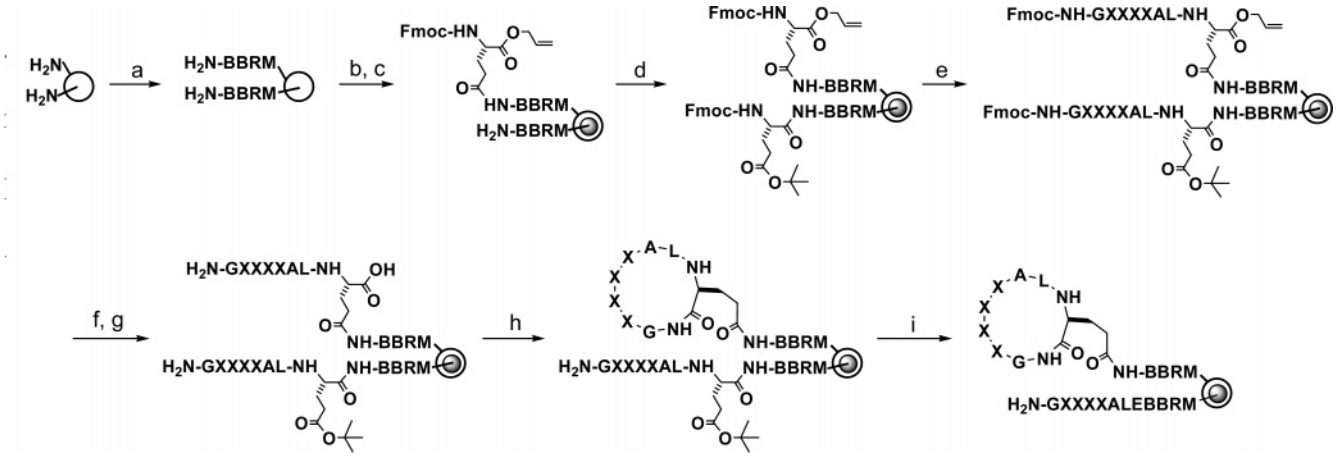

Figure 1. Synthesis of cyclic peptide library. Reagents: (a) standard Fmoc/HBTU chemistry; (b) soak in water; (c) 0.5 equiv of N^{α}-Fmoc−Glu(δ-NHS)−O−$CH_2CH{=}CH_2$ in Et_2O/CH_2Cl_2; (d) excess Fmoc−Glu(^{t}Bu)−OH, HBTU; (e) split-and-pool synthesis by Fmoc/HBTU chemistry; (f) $Pd(PPh_3)_4$; (g) piperidine; (h) PyBOP, HOBt; and (i) TFA.

acids and, in the case of phage display, disulfide-mediated cyclization. The latter is applicable to synthetic libraries containing unnatural building blocks but is highly labor intensive and does not reveal any sequence covariance among binding ligands. Here we report a reliable and inexpensive method for rapid sequence determination of cyclic peptides derived from synthetic combinatorial libraries.

Results

Design Strategy and Synthesis of Cyclic Peptide Library. Our strategy is to topologically segregate a resin bead into two different layers; the bead surface displays a cyclic peptide to be screened against a macromolecular receptor(s), whereas the inner core carries the corresponding linear peptide as the encoding sequence, which can be readily determined by partial Edman degradation/mass spectrometry (PED/MS).[7] To test this strategy, a cyclic octapeptide library containing four random residues[8] (theoretical diversity = 160 000) was synthesized on TentaGel resin (90 μm, ~100 pmol peptide/bead) by cyclization between the N-terminus and the α-carboxyl group of a C-terminal glutamate (Figure 1). A linker sequence, BBRM (B = β-alanine), was added to the C-terminus to facilitate CNBr cleavage and MS analysis.[9] During coupling of the C-terminal glutamate to the resin, the beads were segregated into outer and inner layers using a technique pioneered by Lam.[10] Briefly, beads bearing the BBRM linker were soaked in water, drained, and quickly suspended in 55:45 (v/v) dichloromethane/diethyl ether containing 0.5 equiv of a side chain N-hydroxysuccinimidyl (NHS) ester of L-glutamic acid, N^{α}-Fmoc−Glu(δ-NHS)−O−$CH_2CH{=}CH_2$. Because the organic solvent is immiscible with water, only peptides on the bead surface were exposed to and reacted with the activated ester. The beads were washed with DMF, and the remaining free N-terminal amines in the inner core (0.5 equiv) were acylated with Fmoc−Glu(^{t}Bu)−OH. Following the addition of an arbitrary dipeptide Ala−Leu, the random region was synthesized by the split-and-pool method[5] to give a "one-bead one-sequence" library. A glycine was added to the N-terminus to facilitate peptide cyclization. Finally, the N-terminal Fmoc group and the α-allyl group on the C-terminal glutamate were removed by piperidine and $Pd(PPh_3)_4$, respectively. Subsequent treatment with benzotriazole-1-yloxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) cyclized the surface peptides, while the peptides in the bead interior were kept in the linear form.

Cyclization Efficiency and Cyclic/Linear Peptide Ratio. To assess the yield of peptide cyclization, a small aliquot of the above resin (~10 mg, after cyclization but prior to side chain deprotection) was treated with excess benzylamine and PyBOP. The resulting resin was treated with reagent K to remove side-chain protecting groups, and 50 beads were randomly selected for MS analysis. The peptide on each bead was released by CNBr cleavage and analyzed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS. If cyclization of surface peptides was not complete, the remaining free α-carboxyl group on the C-terminal Glu would react with $BnNH_2$ to give an m/z M + 107 peak in the MS spectrum (where M is the pseudo-molecular ion of the cyclic peptide). In addition, the linear encoding peptide in the bead interior should produce a peak at the m/z M + 18 position. Among the 50 beads analyzed, seven showed M + 107 peaks, and their abundance was ≤5% (relative to the M peaks) in six of the cases (Table 1). Figure 2a shows the MS spectrum of a typical bead, on which the cyclization was complete (no M + 107 peak). Figure 2b shows one of the seven MS spectra which had visible M + 107 peaks (5% relative abundance). In a control experiment, an aliquot of the resin before cyclization was treated with excess $BnNH_2$/PyBOP. The MS spectra of the resulting beads all showed intense M + 107 peaks, which dominated the corresponding M peaks (cyclic peptides) in most cases (see Figure 2c for an example). This indicates that the reaction between the α-carboxyl group and $BnNH_2$ was efficient under the experimental conditions. The formation of peptide dimer and/or oligomers was also examined. Out of the 50 beads, dimer formation was observed for eight beads; in all cases the abundance was 12% or less (relative to the M peaks) (Table 1). No oligomer formation was detected on any of the 50 beads. The yield of cyclization and the molar ratio of cyclic/linear peptides on each bead were calculated from the relative abundance of the peaks by assuming equal ionization efficiency

(7) Wang, P.; Arabaci, G.; Pei, D. *J. Comb. Chem.* **2001**, *3*, 251−254. (b) Sweeney, M. C.; Pei, D. *J. Comb. Chem.* **2003**, *5*, 218−222.
(8) Each random position contained 20 amino acids: A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y, and (*S*)-2-aminobutyric acid (C) and norleucine (M) as cysteine and methionine replacements, respectively.
(9) Yu, Z.; Chu, Y.-H. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 95−98.
(10) Liu, R.; Marik, J.; Lam, K. S. *J. Am. Chem. Soc.* **2002**, *124*, 7678−7680.

Table 1. Cyclization Efficiency and Cyclic/Linear Peptide Ratio for 50 Randomly Selected Beads[a]

bead	M (cyclic)	area	M + 18 (linear)	area	M + 107	area	dimer	area	% cyclic peptide	cyclization yield (%)
J3	1130.2	4183	1148.2	4754	ND		ND		46.8	100.0
J4	1292.3	9894	1310.4	12724	ND		ND		43.7	100.0
K1	ND		1349.9	9394	ND		ND		0.0	0.0
K2	1205.2	1141	1223.2	5019	ND		ND		18.5	100.0
K3	1222.2	1774	1240.2	8285	ND		ND		17.6	100.0
K4	1345.2	2053	1363.2	10075	ND		ND		16.9	100.0
K5	1262.4	371	1280.4	5979	ND		ND		5.8	100.0
K6	1180.4	2419	1198.5	4407	1287.5	69	ND		35.1	97.2
K7	1293.5	5081	1311.6	5079	ND		ND		50.0	100.0
K8	1185.5	9988	1203.6	8070	1292.6	533	2388.2	654	51.9	89.4
K9	1260.6	5431	1278.6	8870	ND		2538.5	702	36.2	88.6
K10	1180.4	7893	1198.4	8981	ND		2378.1	338	45.9	95.9
K11	1235.7	9163	1253.7	4111	ND		ND		69.0	100.0
K12	1183.6	4775	1201.6	2582	ND		2384.3	336	62.1	93.4
K13	1177.7	7923	1195.8	8583	ND		2372.6	206	47.4	97.5
K14	1278.6	1478	1296.6	4821	ND		ND		23.5	100.0
K15	1158.7	652	1176.7	8848	ND		ND		6.9	100.0
K16	1176.6	5780	1194.6	6023	ND		ND		49.0	100.0
K17	1239.5	33	1257.6	11951	ND		ND		0.3	100.0
K18	1238.8	14887	1256.9	18708	ND		ND		44.3	100.0
K19	1241.7	2483	1259.7	487	ND		ND		83.6	100.0
K20	1238.8	4238	1256.8	2640	1345.9	30	ND		61.3	99.3
K21	1270.6	15965	1288.5	20582	ND		ND		43.7	100.0
K22	1255.6	2415	1273.6	6850	ND		ND		26.1	100.0
K23	1164.6	5588	1182.6	3487	1271.7	321	ND		59.5	94.6
K24	1168.4	244	1186.4	4563	1275.4	137	ND		4.9	64.0
M1	1313.0	10975	1331.1	4171	ND		ND		72.5	100.0
M2	1262.3	8274	1280.3	4930	ND		ND		62.7	100.0
M3	1238.3	1415	1256.3	6573	ND		ND		17.7	100.0
M4	1251.3	5631	1269.3	1081	ND		ND		83.9	100.0
M5	1322.2	11205	1340.2	309	ND		ND		97.3	100.0
M6	1165.4	7606	1183.4	2267	ND		ND		77.0	100.0
M7	1246.4	9284	1264.5	3525	ND		ND		72.5	100.0
M8	1159.4	5923	1177.4	1148	ND		2336.1	60	83.1	99.0
M9	1245.4	4020	1263.4	8551	ND		ND		32.0	100.0
M10	1278.4	2734	1296.4	2548	ND		ND		51.8	100.0
M11	1280.5	8380	1298.5	4996	ND		ND		62.6	100.0
M12	1212.6	533	1230.7	4437	ND		ND		10.7	100.0
M13	1279.6	2402	1297.6	2118	ND		ND		53.1	100.0
M14	1230.5	8249	1248.6	5791	ND		2478.3	91	58.4	98.9
M15	1149.6	5593	1167.6	7063	ND		ND		44.2	100.0
M16	1200.6	3607	1218.6	5622	1307.7	98	ND		38.7	97.4
M17	1179.6	9623	1197.6	1410	ND		ND		87.2	100.0
M18	1205.7	8445	1223.7	3529	ND		ND		70.5	100.0
M19	1237.7	6410	1255.7	38	ND		ND		99.4	100.0
M20	1226.6	214	1244.6	5045	ND		ND		4.1	100.0
M21	1220.8	2442	1238.8	311	ND		2458.9	152	84.1	94.1
M22	1207.7	5724	1225.7	1930	ND		ND		74.8	100.0
M23	1174.5	4065	1192.6	633	1281.5	21	ND		86.1	99.5
M24	1211.3	2904	1229.3	842	ND		ND		77.5	100.0
average									49.0	96.2

[a] ND, not detected by MALDI-TOF (threshold = signal/noise >3). Percentage of cyclic peptide (%) was determined with formula area(M)/{area(M) + area(M + 18) + area(dimer)} × 100. Cyclization yield (%) was determined with formula area(M)/{area(M) + area(M + 107) + area(dimer)} × 100.

for cyclic, linear, benzylated, and dimeric peptides (which all have the same amino acid sequence and contain a C-terminal arginine for efficient ionization). With the exception of two beads (K1 and K24), the cyclization yield was typically ≥90% (Table 1). The percentage of cyclic peptides on beads varied greatly, from 0.3% (K17) to 99.4% (M19) (not counting bead K1). However, the calculated average value (%cyclic) for the 50 beads was 49%.

The above cyclization yields were surprisingly high (Table 1) since peptide cyclization had often been described as challenging and having low yields by previous investigators.[11]

In addition, the cyclization yields were based on the signal intensities in MALDI mass spectrometry, which is not commonly used for quantitative analysis. We therefore carried out additional experiments to confirm the observed cyclization yields. First, the percentage of cyclic peptides was determined by ninhydrin tests of the free amine contents for a small amount of the resin before and after cyclization (1.0 mg each). This assay showed that ~50% of the peptides were cyclized (data not shown), in agreement with the calculated value (49%). Second, eight cyclic peptides that had been selected from two different peptide libraries for binding to streptavidin (vide infra) and porcine α-amylase (S.H.J. and D.P., unpublished data) were individually synthesized on Rink LS amide resin (0.2 mmol/g) in the absence of encoding linear peptides. The crude products

(11) See reviews: (a) Lambert, J. N.; Mitchell, J. P.; Roberts, K. D. *J. Chem. Soc., Perkins Trans.* **2001**, *1*, 471−484. (b) Li, P.; Roller, P. P.; Xu, J. *Curr. Org. Chem.* **2002**, *6*, 411−440. (c) Davies, J. S. *J. Pep. Sci.* **2003**, *9*, 471−501.

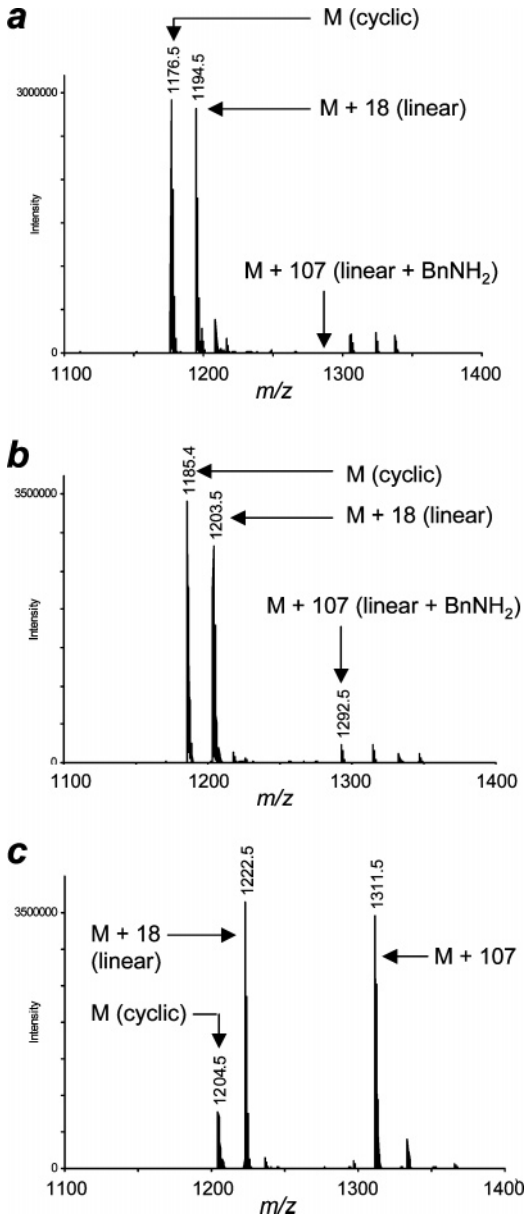

Figure 2. MALDI-TOF MS spectra showing the efficiency of peptide cyclization. TentaGel resin was treated with $BnNH_2$ and PyBOP after (panel a and b) or before peptide cyclization (panel c). Panel a shows the spectrum of a typical bead, where cyclization is complete. Panel b shows an example in which a small amount of benzylated product was observed. Panel c shows that if added prior to cyclization, $BnNH_2$ reacted faster than peptide cyclization.

(after deprotection by reagent K) were analyzed by MALDI-TOF MS. For each of the eight peptides, the desired cyclic peptide was by far the predominant species in the spectrum (Figure S1). No uncyclized peptide (M + 18 peak) was observed in any of the spectra. Peptide dimer formation was observed for four of the peptides and represents the principal impurities (20−30% abundance relative to the cyclic monomer product).[12] Third, two of the above peptides, cyclo(AVWmeFRRVQ) and cyclo(AVWfFRRVQ) (where meF and fF are L-N^{α}-methylphenylalanine and L-*p*-fluorophenylalanine, respectively), and their ∼1:1 (mol/mol) mixtures with their respective linear counterparts were analyzed by HPLC and MALDI-TOF MS (Figures S2 and S3 and Table S1). In each case, the crude sample contained the desired cyclic peptide as the major product (84% and 54% purity, respectively, based on HPLC analysis). The relative ionization efficiency of cyclic vs linear peptides varied with peptide concentration in the MALDI samples. With ∼12 pmol of peptides (linear and cyclic) spotted in each sample, ionization ratios of 1.1 and 0.89 (cyclic/linear) were observed for the above peptides, respectively (Table S1). At ∼6 pmol peptides, the respective ratios were 0.50 and 0.25. During our typical analyses of single beads by MALDI MS (Table 1), ∼7 pmol of peptide was used in each sample. Taken together, the above results indicate that high peptide cyclization yields were achieved during the cyclic peptide library synthesis and the observed variation in the cyclic/linear peptide ratio on different beads was not primarily due to variation in peptide cyclization efficiency. Rather, the difference in aqueous/organic phase partitioning during the bead segregation process was likely a contributing factor; a smaller bead is expected to have a larger fraction of its volume exposed to the organic solvent and therefore a higher percentage of cyclic peptides. Fluctuation in ionization efficiency during MALDI MS was another contributing factor, which was most likely responsible for the "observed" extremely low (0.3% in K17) and high cyclic peptide percentages (99.4% in M19).

Sequence Determination of Cyclic Peptides by PED/MS. Forty beads were randomly selected from the cyclic peptide library, placed in two separate reaction vessels (20 beads each), and subjected to seven cycles of PED,[7] which converted the linear encoding peptide on each bead into a series of progressively shorter peptides (Figure 3a). The beads were then separated into individual microcentrifuge tubes, and the peptides were released by cleavage with CNBr and analyzed by MALDI-TOF MS. A total of 37 beads (92%) produced spectra of sufficiently high quality to allow their unambiguous sequence assignment (Table 2). The MS spectra of the other three beads missed one or more peaks, preventing reliable sequence assignment. A set of 20 MS spectra and their assigned sequences are provided as Supporting Information (Figure S4). Figure 3b shows a representative MS spectrum derived from a single positive bead selected for binding against streptavidin (vide infra). The cyclic peptide produced an intense peak at *m*/*z* 1214.6, whereas the full-length linear peptide bearing an N-terminal nicotinoyl group generated a peak at *m*/*z* 1337.7. The truncated peptides gave a series of peaks at *m*/*z* 1280.6, 1179.6, 1042.5, 945.49, 817.42, and 746.40. From the masses of this peptide ladder, the sequence of the cyclic peptide was determined as cyclo(GTHPQALE)BBRM.[7] We have applied this method to sequence hundreds of cyclic peptides, which contained both naturally occurring and unnatural amino acids (e.g., *D*- and N^{α}-methylated amino acids), and our success rate has typically been ∼90% (S.H.J. and D.P., unpublished results).

On-Bead Screening for Streptavidin Binding Ligands. As a proof of principle, an aliquot of the above library (50 mg, ∼143 000 beads) was screened for binding to streptavidin, which had been conjugated to alkaline phosphatase (SA−AP). Since the protein conjugate (∼200 kD) is too large to diffuse into the TentaGel beads,[13] only cyclic peptides on the bead surface

(12) We have noted that the peptide cyclization yield (cyclic monomer vs dimer) differed significantly with resin used. TentaGel S NH_2 resin of low loading capacity gave the best results among the resins tested so far.

(13) Vagner, J.; Barany, G.; Lam, K. S.; Krchnak, V.; Sepetov, N. F.; Ostrem, J. A.; Strop, P.; Lebl, M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8194−8199.

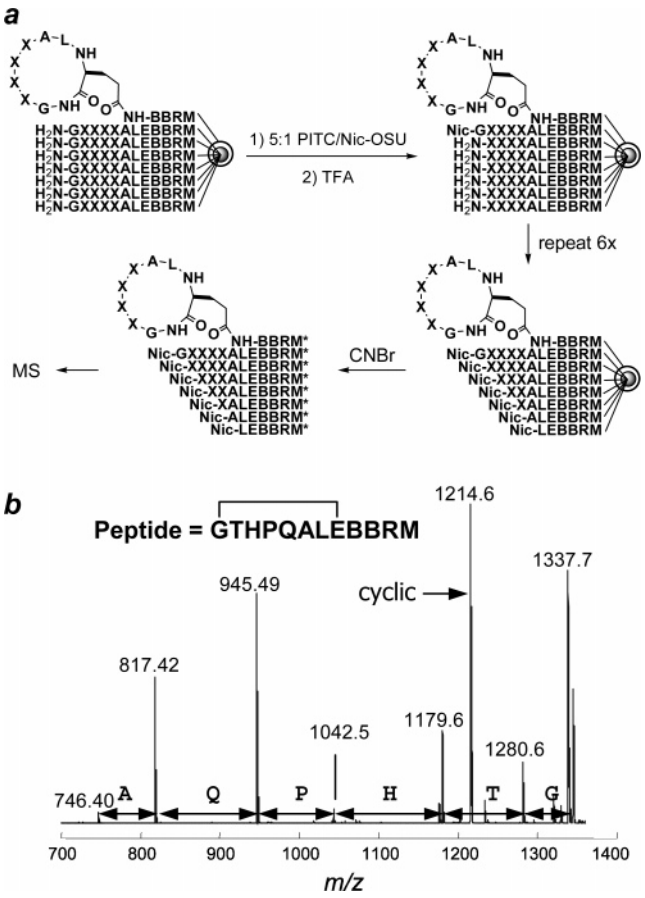

Figure 3. (a) Partial Edman degradation of resin-bound peptide. PITC, phenylthioisocyanate; Nic-OSU, *N*-hydroxysuccinimidyl nicotinate; M*, homoserine lactone. (b) MALDI-TOF mass spectrum of the peptide and its degradation products from a colored bead.

Table 2. Success Rate for Sequencing Resin-Bound Cyclic Peptides by PED/MS

trial no.	selected against SA–AP?	no. of beads analyzed by PED/MS	no. of complete sequences obtained
1	no	20	17 (85%)
2	no	20	20 (100%)
3	yes	11	7 (64%)
4	yes	11	10 (91%)
total (average)		62	54 (87%)

should interact with the protein (no interference from linear peptides). Binding of streptavidin to a bead recruited alkaline phosphatase to the bead surface, and subsequent incubation in the presence of BCIP produced an intense turquoise color on the bead.[5a] Screening of the octapeptide library against 5 nM streptavidin resulted in 22 colored beads. PED/MS analysis of the beads gave 17 complete sequences (77%). The lower success rate for the streptavidin-binding sequences was due to their high contents in proline and tryptophan residues, which produced more complex MS spectra.[7] The MS spectrum derived from one of the colored beads is shown in Figure 3b, from which a sequence of cyclo(GTHPQALE)BBRM was obtained. Inspection of the 17 sequences revealed that streptavidin recognized two different consensus sequences, HP(Q/Y) and WYX (Table 3). Two of the peptides, (GTHPQALE)BK and (GWYHNALE)-BK, as well as their linear counterparts, were individually synthesized and tested for binding to SA−AP. In an SA−AP

Table 3. Cyclic Peptides Selected Against Streptavidin[a]

bead no.	peptide sequence	bead no.	peptide sequence
1	GCHPQALE	11	GWYCIALE
2	GHPQCALE	12	GWYCLALE
3	GHPQYALE	13	GWYHIALE
4	GIHPQALE	14	GWYHNALE
5	GMHPQALE	15	GWYQLALE
6	GTHPQALE	16	GWYTHALE
7	GWHPQALE	17	GYYYKALE
8	GNHPYALE		
9	GRHPYALE		
10	GYHPYALE		

[a] C, (*S*)-2-aminobutyric acid; M, norleucine.

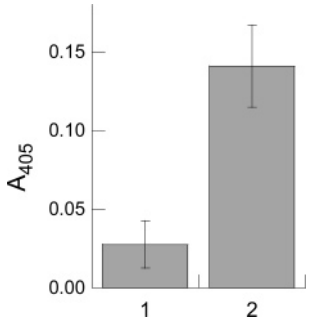

Figure 4. Amount of SA−AP retained by underivatized TentaGel S NH_2 resin (1) and the affinity resin containing cyclic peptide (GTHPQALE) (2). Data shown are the mean ±SD from six sets of experiments.

pull-down assay, TentaGel derivatized with cyclic peptide (GTHPQALE)BK retained significantly more alkaline phosphatase activity than the control resin (underivatized TentaGel), confirming the ability of the cyclic peptide to bind SA−AP (Figure 4). Our attempts to determine the dissociation constant by surface plasmon resonance failed due to weak binding affinity (K_D >10 μM). SA−AP pull-down assays in the presence of the cyclic peptides as competitors gave IC_{50} values of ∼100 and ∼700 μM for peptides cyclo(GTHPQALE)BK and cyclo-(GWYHNALE)BK, respectively (Figure S5). The corresponding linear peptides (GTHPQALEBK and GWYHNALEBK) were less effective competitors (IC_{50} > 1 mM). Other investigators have previously reported tripeptide HPQ as a specific streptavidin-binding ligand.[4b,5a,14]

Design and Synthesis of Tyrocidine Analogues. To test whether our cyclic peptide sequencing method is compatible with solution-phase screening, we designed a 400-member library of tyrocidine A analogues. Tyrocidine A is a cyclic decapeptide antibiotic (Figure 5a compound **1**), whose primary target of action is thought to be the bacterial cell membrane.[15] A major advantage of peptide antibiotics such as tyrocidine is their low rate of drug resistance.[16] However, clinical application of tyrocidine A has been limited by its low specificity toward microorganisms; it also disrupts mammalian cell membranes, as indicated by its high hemolytic activity. Numerous tyrocidine analogues have previously been prepared in attempts to improve its therapeutic index and some of these analogues indeed show significantly improved properties.[2b,c,17−19] We decided to ran-

(14) (a) Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. *Science* **1990**, *249*, 404−406. (b) Giebel, L. B.; Cass, R. T.; Milligan, D. L.; Young, D. C.; Arze, R.; Johnson, C. R. *Biochemistry* **1995**, *34*, 15430−15435.
(15) Prenner, E. J.; Lewis, R. N. A. H.; McElhaney, R. N. *Biochim. Biophys. Acta* **1999**, *1462*, 201−221.
(16) Hancock, R. E. W. *Lancet* **1997**, *349*, 418−422.
(17) Danders, W.; Marahiel, M. A.; Krause, M.; Kosui, N.; Kato, T.; Izumiya, N.; Kleinkauf, H. *Antimicrob. Agents Chemother.* **1982**, *22*, 785−790.

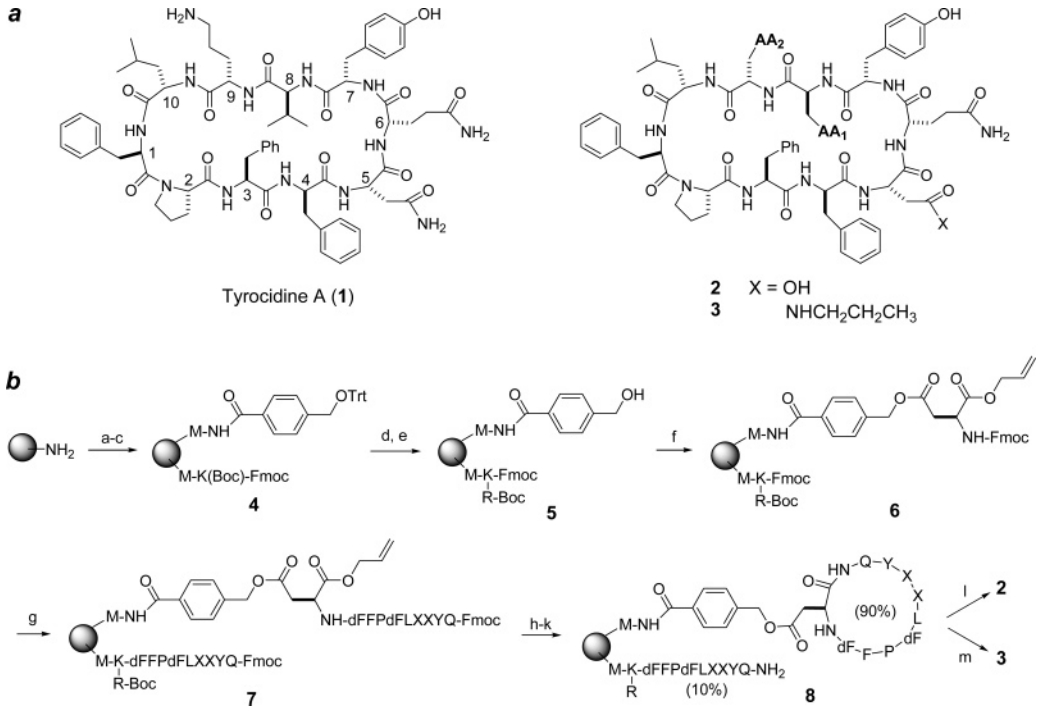

Figure 5. (a) Structures of tyrocidine A and its analogues. (b) Synthesis of tyrocidine analogue library. (A) Fmoc−Met/HBTU; (b) 20% piperidine; (c) 9:1 (mol/mol) Trt−HMBA/Fmoc−Lys(Boc)−OH, HBTU; (d) TFA; (e) Boc−Arg(Pmc)−OH/HBTU; (f) Fmoc−Asp−OAll/DIPC/DMAP; (g) Fmoc/HBUT chemistry; (h) $Pd(PPh_3)_4$; (i) 20% piperidine; (j) PyBOP/HOBt; (k) reagent K; (l) $NaOH/H_2O$; and (m) $CH_3CH_2CH_2NH_2$.

domize positions 8 and 9 of tyrocidine A by replacing the L-valine and L-ornithine residues with 20 L-amino acids (L-ornithine, L-norleucine, and 18 proteinogenic amino acids except for cysteine and methionine). The Val^8-Orn^9 motif is a common feature of many β-pleated cyclic decapeptide antibiotics including gramicidin S, tyrocidine A−E, streptocidin A−D, and loloatin A−D,[19b] but to the best of our knowledge, no extensive structure−activity relationship study has been carried out at either position 8 or 9.

The cyclic peptide library was synthesized on TentaGel macrobeads (280−320 μm, 0.21 mmol/g, and 3.5 nmol/bead) (Figure 5b). A methionine was introduced to the C-terminus to facilitate peptide release by CNBr prior to MS analysis.[9] Next, the resin was reacted with a 9:1 (mol/mol) mixture of O-trityl hydroxymethylbenzoic acid (HMBA) and Fmoc−Lys(Boc)−OH to afford resin **4**. Treatment with TFA removed the trityl group, as well as the Boc group on the lysine side chain. Boc−Arg(Pmc)−OH was then selectively added to the lysine side chain to provide a fixed positive charge to the encoding peptide and facilitate MS analysis. Synthesis of the tyrocidine analogues started with L-Asn at position 5. Its side chain provided a convenient anchor point for attachment to the solid support. To this end, the free hydroxyl group of **5** was acylated with the side chain carboxyl group of N^{α}-Fmoc−Asp−O-allyl to give allyl ester **6**, which upon removal of the allyl group would later provide a C-terminal carboxyl group for peptide cyclization. Subsequent peptide chain elongation employed standard Fmoc/HBTU chemistry and the split-and-pool method for the random residues to afford linear peptide **7**. Sequential removal of the allyl and Fmoc groups followed by cyclization with PyBOP gave resin **8**, on which 90% of the peptides were cyclic and attached to the resin through a labile ester linkage, whereas the other 10% were linear and attached to the resin through a more stable amide linkage. The resin-bound peptides were deprotected by treatment with reagent K.

The cyclization yield was analyzed by three different methods. First, an equal number of beads (20) was randomly selected from the library immediately before and after the cyclization reaction and subjected to ninhydrin tests. Comparison of the absorbance at 580 nm before and after cyclization showed that ~90% of the peptides were cyclized (theoretical yield 90%). Second, 20 beads were randomly picked from the deprotected library (resin **8**), treated with CNBr, and individually analyzed by MALDI-TOF MS. The MS spectra of five representative beads are provided as Supporting Information (Figure S6). For the majority of beads, their MS spectra showed only the cyclic product (*m*/*z* M) and no signal for any uncyclized peptide (*m*/*z* M + 18). A few beads gave small peaks at *m*/*z* M + 18 positions indicating incomplete cyclization, but the intensities of the *m*/*z* M + 18 peaks were <5% relative to the cyclic peptides. Note that the linear peptides are expected to have higher ionization efficiencies than their cyclic counterparts due to the presence of a free N-terminal amine (especially for peptides that do not contain basic residues). Finally, nine selected cyclic peptides were individually synthesized on large scale and analyzed by HPLC and MS (vide infra). For six of these peptides, the desired cyclic products were the predominant species (>50% purity).

Antimicrobial Activity of Tyrocidine Analogues. Approximately 1500 beads were randomly selected from the tyrocidine analogue library and placed into 96-well microtiter plates (one bead/well). The cyclic peptide on each bead was released by cleavage of the ester linkage with a 1 N NaOH solution, while the linear encoding peptide, which was linked

(18) Lin, H., Walsh, C. T. *J. Am. Chem. Soc.* **2004**, *126*, 13998−14003.
(19) (a) Bu, X.; Wu, X.; Xie, G.; Guo, Z. *Org. Lett.* **2002**, *4*, 2893−2895. (b) Qin, C.; Zhong, X.; Bu, X.; Ng, N. L. J.; Guo, Z. *J. Med. Chem.* **2003**, *46*, 4830−4833.

Table 4. Selected Tyrocidine Analogues with Antibacterial Activity

bead no.	peptide sequence[a] AA_1	AA_2
1−3	P	K
4, 5	K	K
6, 7	P	O
8	Y	O
9	F	Q
10	Q	Q
11	M	E
12	S	D
13	Y	L
14	T	Y

[a] M, L-norleucine; O, L-ornithine.

to the resin via a stable amide linkage, was retained (Figure 5b). The crude cyclic peptides were tested for antibacterial activity against Gram-positive bacterium *Bacillus subtilis*. Out of the 1500 samples, 14 resulted in visible growth inhibition of *B. subtilis*. The 14 beads were recovered from the microtiter plates, and the identity of the cyclic peptides was determined by PED/MS sequencing of the remaining linear peptides on the beads. Consistent with the conservation of Orn at position 9 of the natural products,[19b] we found that 8 out of the 14 selected peptides contained a basic amino acid, lysine or ornithine, at this position (Table 4). Among the 10 different sequences selected, Pro^8-Lys^9 appeared three times, whereas Lys^8-Lys^9 and Pro^8-Orn^9 motifs were each selected twice from the library. Nine of the peptides (except for Phe^8-Gln^9) were resynthesized individually on NovaSynTGA resin, which produced the cyclic peptides containing an aspartic acid at position 5. Analytical HPLC analyses showed that six of the peptides were at least 50% pure and two had ~40% purity (see Figure S7 in the Supporting Information). The remaining peptide (Tyr^8-Leu^9) had poor aqueous solubility and HPLC analysis was unsuccessful. Major impurities included an isomer that has the same molecular mass as the desired product but a different retention time on HPLC and a high-molecular-mass species. We tentatively assign the isomer as the cyclic epimer containing a D-aspartic acid at position 5, which was presumably formed during peptide cyclization. The high-molecular-mass species was assigned as the corresponding peptide dimer.

The crude products were tested again for antibacterial activity against *B. subtilis*. Surprisingly, only cyclic peptides containing Lys^8-Lys^9 and Nle^8-Glu^9 motifs inhibited bacterial growth at 32 μg/mL. Suspecting that the negative charge associated with Asp^5 might impede membrane binding, we resynthesized peptides containing Lys^8-Lys^9 and Pro^8-Lys^9 on NovaSynTGA resin and released the peptides from beads by treatment with propylamine (Figure 5b). This procedure resulted in tyrocidine analogues containing an L-N^{δ}-propylasparagine (L-PrAsn) residue at position 5. The crude peptide amides were purified by reversed-phase HPLC. The Pro^8-Lys^9 peptide amide gave only one major peak on a C_{18} column, but the cyclic peptide containing Lys^8-Lys^9 motif produced two peaks of nearly equal intensities with retention times of 21.2 and 22.8 min. MS analysis indicated that both species had the correct molecular mass ($[M + H]^+ = 1356.6$), suggesting that one of them was the desired product, whereas the other was formed as a result of epimerization at Asn^5 during peptide cyclization. To differentiate the two epimers, we resynthesized the cyclic peptide using enantiomerically pure L-PrAsn at position 5 and by starting the peptide synthesis at glutamine.[6] The resulting peptide exhibited a single peak on HPLC and co-eluted with the 22.8 min epimer (containing L-PrAsn at position 5). The purified Pro^8-Lys^9 peptide and the two Lys^8-Lys^9 epimers were tested for antibacterial activity. The L-PrAsn-containing isomer was more potent, having a minimal inhibitory concentration (MIC) of 2−4 μg/mL against *B. subtilis* and 8 μg/mL against *Escherichia coli*. The D-PrAsn epimer and the Pro^8-Lys^9 peptide both had an MIC value of 32 μg/mL against *B. subtilis* and were inactive against *E. coli*. As a comparison, tyrocidine A has an MIC of ~8 μg/mL against *B. subtilis*[19b] and is not known to be active against Gram-negative bacteria. We do not yet understand why some of our peptides showed antibacterial activity in the original screening but failed to inhibit cell growth after individual synthesis. Further analysis of these selected peptides, as well as SAR studies at other tyrocidine positions, are currently underway.

Discussion

We have developed a general method for the rapid sequencing of cyclic peptides derived from combinatorial libraries. Our results demonstrate that it is compatible with both on-bead and solution-phase library screening. Compared to some of the other encoding methods,[20] our method has the advantage of being effectively a direct method since the cyclic and linear peptides always have the same sequence and MS analysis provides information on both the identity and quantity (semiquantitatively) of a cyclic peptide on each bead. Other investigators have attempted to sequence cyclic peptides by tandem MS.[6] To our knowledge, the most powerful MS/MS method for sequencing library-derived cyclic peptides is the method reported by Ghadiri and co-workers.[6e] However, the Ghadiri method achieved only ~77% accuracy; the rest of the sequences were incorrectly assigned by the computer program, compromising the reliability of all sequence data obtained. Our method has a typical success rate of ~90% (defined as unambiguous sequence assignment at all positions), which can be further improved by the use of a traceless capping agent (instead of Nic-OSU),[21] and does not generate any incorrect assignments other than those caused by human error. The incomplete sequences due to missing peaks do not compromise the reliability of the assigned sequences. Moreover, the Ghadiri method was demonstrated with peptides derived from macrobeads (500−550 μm; typically ~100 nmol peptides/bead). It is unclear whether it would work well with the more popular microbeads (~90 μm; ~0.1−1 nmol peptides/bead). Our method works very well with both macro- and microbeads and should in principle work with still smaller beads (e.g., 30 μm). Another advantage of our method over conventional MS/MS methods is its ability to differentiate amino acids of degenerate masses (e.g., norleucine/leucine/isoleucine or lysine/glutamine).[7] This feature will be useful for screening cyclic peptide libraries containing L-, D-, and N^{α}-methylated amino acids, many of which were degenerate in mass. Perhaps the most important attribute of our method is its high-throughput capability. We can routinely sequence >100 cyclic peptides in

(20) Ohlmeyer, M. H. J.; Swanson, R. N.; Dillard, L. W.; Reader, J. C.; Asouline, G.; Kobayashi, R.; Wigler, M.; Still, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10922−10926.
(21) Thakkar, A.; Wavreille, A.-S.; Pei, D. *Anal. Chem.* **2006**, *78*, 5935−5939.
(22) Hu, X.; Nguyen, K. T.; Jiang, V. C.; Lofland, D.; Moser, H. E.; Pei, D. *J. Med. Chem.* **2004**, *47*, 4941−4949.

a single day (20−30 peptides/h) and at an average cost (reagents and instrument time) of <$1 per peptide. Our method does not require a dedicated MS instrument, and the MS analysis can be performed on any MALDI-TOF instrument of sufficient sensitivity and in an automated format. Therefore, it can be readily practiced in any chemical or biochemical laboratories. Our method in its current format is limited to the sequence determination of cyclic peptides from synthetic libraries and does not work with cyclic peptides isolated from natural sources.

On-bead screening of the octapeptide library identifies two types of sequence motifs that can bind to streptavidin, HP(Q/Y) and WYX. The HPQ motif has been repeatedly selected by streptavidin in other studies.[4b,5a,14] Our cyclic peptides bind to streptavidin with high micromolar K_D values, similar to those reported for linear HPQ sequences.[5a,14] Others have reported that cyclization of HPQ sequences resulted in much higher binding affinities (K_D in the nanomolar range).[4b,14b] The discrepancy between earlier reports and our results suggests that the nature of cyclization (e.g., ring size) can dramatically affect the binding affinity of a cyclic peptide ligand, which can be either higher or lower than that of the corresponding linear peptide. Our successful identification of a tyrocidine analogue with improved antibacterial activity demonstrates that the sequencing method is compatible with solution-phase library screening, which can be easily adapted to screen for inhibitors of enzymes or other macromolecular targets. It also suggests that combinatorial synthesis and screening can be an effective method to improve the biological activity of natural products such as cyclic peptide antibiotics.

In conclusion, we have developed an effective method for sequence determination of library-derived cyclic peptides. This should further expand the utility of cyclic peptides in biomedical research and drug discovery.

Experimental Section

Materials and General Methods. Fmoc-protected L-amino acids were purchased from Advanced Chemtech (Louisville, KY), Peptides International (Louisville, KY), or NovaBiochem (La Jolla, CA). N^{α}-Fmoc-L-glutamic acid α-allyl ester (Fmoc-Glu-OAll) was from NovaBiochem. O-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole hydrate (HOBt), and Fmoc−mini-PEG were from Peptides International. Benzotriazole-1-yloxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was from NovaBiochem. All solvents and other chemical reagents were obtained from Aldrich (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), or VWR (West Chester, PA) and were used without further purification, unless noted otherwise. *N*-Hydroxysuccinimidyl nicotinate (Nic-OSU) was from Advanced ChemTech and was recrystallized from ethyl acetate prior to use. Phenyl isothiocyanate (PITC) was purchased in 1-mL sealed ampules from Sigma-Aldrich, and a freshly opened ampule was used in each experiment. Streptavidin−alkaline phosphatase (SA−AP) conjugate (~1 mg/mL) was purchased from Prozyme (San Leandro, CA). TentaGel S NH_2 resin (90 μm, 0.26 mmol/g, and ~100 pmol/bead) was purchased from Peptides International. TentaGel MB-NH_2 resin (280−320 μm, 0.21 mmol/g, and 3.5 nmol/bead) and NovaSynTGA resin (90 μm, 0.23 mmol/g) were from NovaBiochem. Rink Resin LS (100−200 mesh, 0.2 meq/g) was purchased from Advanced ChemTech. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) disodium salt was from Sigma (St. Louis, MO). p-Nitrophenyl phosphate sodium salt was from Research Organics (Cleveland, OH).

Synthesis of N^{α}-Fmoc−Glu(δ-*N*-hydroxysuccinimidyl)−O−CH_2-$CH{=}CH_2$. Fmoc−Glu−OAll (1.0 g, 2.4 mmol) and *N*-hydroxysuccinimide (0.34 g, 2.9 mmol) were dissolved in 40 mL of dichloromethane (DCM), and the mixture was stirred vigorously for 30 min at room temperature to dissolve most of the *N*-hydroxysuccinimide. Then, 0.55 g of dicyclohexylcarbodiimide (2.7 mmol) was added and the reaction was allowed to proceed at room temperature overnight. The mixture was filtered to remove the white precipitate (*N*,*N*′-dicyclohexylurea), and the filtrate was washed with saturated $NaHCO_3$ solution, brine, and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (80% ethyl acetate in hexane) to afford a white solid (0.85 g, 69%). ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.05−2.21 (m, 1H), 2.26−2.44 (m, 1H), 2.60−2.75 (m, 2H), 2.78 (br s, 4H), 4.21 (t, J = 6.8 Hz, 1H), 4.32−4.53 (m, 3H), 4.64 (d, J = 5.7 Hz, 2H), 5.21−5.39 (m, 2H), 5.62 (d, J = 8.2 Hz, 1H), 5.80−5.99 (m, 1H), 7.27−7.44 (m, 4H), 7.60 (d, J = 7.6 Hz, 2H), 7.76 (d, J = 7.6 Hz, 2H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 25.5, 27.3, 47.1, 53.0, 66.3, 67.0, 119.3, 119.9, 125.0, 127.0, 127.7, 131.1, 141.2, 143.6, 167.8, 169.0, 170.8; HRESI-MS: $C_{27}H_{26}N_2O_8Na^+$ ([M + Na]$^+$), calcd 529.1581, found 529.1566.

Synthesis of Cyclic Octapeptide Library. The cyclic peptide library was synthesized on 2.0 g of TentaGel S NH_2 resin (90 μm, 0.26 mmol/g). All of the manipulations were performed at room temperature unless otherwise noted. The linker sequence (BBRM) was synthesized with 4 equiv of Fmoc-amino acids, using HBTU/HOBt/*N*-methylmorpholine (NMM) as the coupling reagents. The coupling reaction was typically allowed to proceed for 2 h, and the beads were washed with DMF (3×) and DCM (3×). The Fmoc group was removed by treatment twice with 20% piperidine in DMF (5 + 15 min), and the beads were exhaustively washed with DMF (6×). To spatially segregate the beads into outer and inner layers, the resin was treated with 20% piperidine in DMF (5 + 15 min), washed with DMF and water, and soaked in water overnight. The resin was drained and suspended in a solution of N^{α}-Fmoc−Glu(δ-*N*-hydroxysuccinimidyl)−O−$CH_2CH{=}CH_2$ (0.26 mmol, 0.50 equiv) and diisopropylethylamine (1.2 mmol or 2.0 equiv) in 30 mL of 55:45 (v/v) DCM/diethyl ether. The mixture was incubated on a carousel shaker for 30 min at room temperature. The beads were washed with 55:45 DCM/diethyl ether (3×) and DMF (8×) to remove water from the beads and then treated with 2 equiv of Fmoc−Glu(^{t}Bu)−OH plus HBTU/HOBt/4-methylmorpholine in DMF (90 min). Next, the dipeptide Ala−Leu was added to the resin using standard Fmoc/HBTU chemistry. For the synthesis of random positions, the resin was split into 20 equal portions and each portion (100 mg) was coupled twice with 5 equiv of a different Fmoc-amino acid/HBTU/HOBt/NMM for 2 h. To differentiate isobaric amino acids during MS sequencing, 5% (mol/mol) Ac-Gly was added to the coupling reactions of Leu and Lys, whereas 5% Ac-Ala was added to norleucine reactions.[7] After the four random positions were synthesized, a glycine was added to the N-terminus of all peptides to facilitate the cyclization reaction. Next, the allyl group on the C-terminal glutamate was removed with a solution containing tetrakis(triphenylphosphine)palladium (1 equiv), triphenylphosphine (3 equiv), formic acid (10 equiv), and diethylamine (10 equiv) in anhydrous THF overnight at room temperature. Anhydrous THF was obtained using Solvent Purification System from Solv-Tek (Berryville, VA). The beads were sequentially washed with 0.5% diisopropylethylamine in DMF, 0.5% sodium dimethyldithiocarbamate hydrate in DMF, DMF (3×), DCM (3×), and DMF (3×). The N-terminal Fmoc group was then removed in 20% piperidine, and the beads were washed with DMF (6×), 1 M HOBt in DMF (3×), DMF (3×), and DCM (3×). For peptide cyclization, a solution of PyBOP/HOBt/NMM (5, 5, 10 equiv, respectively) in DMF was mixed with the resin and the mixture was incubated on a carousel shaker for 3 h. The resin was washed with DMF (3×) and DCM (3×) and dried under vacuum for >1 h. Side chain deprotection was carried out with reagent K (7.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in TFA) for 1 h. The resin was washed with TFA and DCM and dried under vacuum before storage at −20 °C.

Determination of Cyclization Efficiency and Molar Ratio of Cyclic/Linear Peptides. To determine the yield of cyclization, ~10 mg of resin before or after cyclization (but prior to side chain deprotection) was treated with excess benzylamine ($BnNH_2$) and PyBOP/HOBt/4-methylmorpholine (16 equiv) for 1.5 h. Afterward, the resin was subjected to side chain deprotection with reagent K and 50 beads were randomly selected and placed into individual microcentrifuge tubes. The peptide on each bead was released from resin by cleavage with CNBr and analyzed by MALDI-TOF MS (see below for detailed procedure). Ninhydrin assay was performed by the addition of 300 μL of 76% phenol in ethanol (w/v), 300 μL of 20 μM KCN in pyridine, 300 μL of 5% ninhydrin in ethanol (w/v) to ~1.0 mg of resin, and incubating at 110 °C for 5 min. The absorbance at 580 nm was measured on a Perkin-Elmer Lambda 25 UV/visible spectrometer, and the background was subtracted from the absorbance of the mixture without resin.

Library Screening for Streptavidin Binding. In a micro-BioSpin column (0.8 mL, Bio-Rad), 30 mg of the cyclic peptide library was swollen in DCM and extensively washed with DMF and then water. The resin was incubated in a blocking buffer (30 mM Hepes, pH 7.4, 150 mM NaCl, 0.01% Tween 20, and 0.1% gelatin) overnight with gentle mixing at room temperature. The resin was drained and resuspended in 800 μL of a screening buffer (30 mM Hepes, pH 7.4, 150 mM NaCl, and 0.01% Tween 20) containing ~5 nM SA−AP (1:1000 dilution of commercially available solution) for 2.5 h incubation at 4 °C. The resin was drained and washed twice with 800 μL of SA−AP buffer (30 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10 mM $MgCl_2$, and 70 μM $ZnCl_2$) and twice with 800 μL of SA−AP reaction buffer (30 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, 20 μM $ZnCl_2$). The resin was transferred into a well on a 12-well plate (BD Falcon) by rinsing with 9 × 100 μL of the SA−AP reaction buffer. Upon the addition of 100 μL of 5 mg/mL BCIP in the SA−AP reaction buffer, intense turquoise color developed on positive beads in ~30 min when the staining was quenched by the addition of 3 mL of 8 M guanidine hydrochloride. The resin was washed extensively with water and transferred into a 35-mm Petri dish from which the positive beads were picked manually with a pipet under a dissecting microscope. The screening procedure was repeated once with 20 mg of the cyclic peptide library.

Peptide Sequencing by PED/MS. Selected resin beads (anywhere from 1 to 1 million beads) were pooled and subjected to partial Edman degradation in a single reaction vessel as described previously.[7] The beads were suspended in 160 μL of pyridine/water (v/v 2:1) containing 0.1% triethylamine and mixed with an equal volume of 0.2% (w/v) Nic-OSU and 5−9% (v/v) PITC in pyridine. The reaction was allowed to proceed for 6 min with mixing, and the beads were washed with methanol, DCM, and TFA. The beads were treated twice with ~300 μL of TFA for 6 min each. After the resin was washed with DCM, pyridine, and pyridine/water (2:1), the cycle was repeated *n* times, where *n* equals the number of residues to be sequenced. During the final cycle, the N-terminal amine was treated with Nic-OSU in the absence of PITC. For MALDI-TOF analysis, the degraded beads were treated with ~1 mL of TFA containing ammonium iodide (10 mg) and dimethylsulfide (20 μL) on ice for 30 min to reduce any oxidized Met. After being washed with water, the beads were transferred into microcentrifuge tubes (1 bead/tube) and each treated with 20 μL of 70% TFA containing CNBr (40 mg/mL) overnight in the dark. The solvents were evaporated under vacuum to dryness, and the peptides released from the bead were dissolved in 5 μL of 0.1% TFA in water. One microliter of the peptide solution was mixed with 2 μL of saturated 4-hydroxy-α-cyanocinnamic acid in acetonitrile/0.1% TFA (1:1), and 1 μL of the mixture was spotted onto a MALDI sample plate. Mass spectrometry was performed at Campus Chemical Instrument Center of The Ohio State University on a Bruker III MALDI-TOF instrument in an automated manner. The data obtained were analyzed by either Moverz software (Proteometrics LLC, Winnipeg, Canada) or Bruker Daltonics flexAnalysis 2.4 (Bruker Daltonic GmbH, Germany).

Synthesis of Individual Streptavidin-Binding Peptides. Each peptide was synthesized on 200 mg of Rink Resin LS (0.2 mmol/g) in a manner similar to that employed for the library construction except that spatial segregation was not necessary. After the addition of the last amino acid, the resin was split into two equal aliquots. One aliquot was used for cyclization, whereas the other was used to synthesize the linear peptide as a control. For the preparation of cyclic peptides, the allyl group on Glu was first removed and then the Fmoc group was removed, prior to cyclization. The condition for cyclization was identical to that used during library construction, and the progress of cyclization was monitored by ninhydrin tests. After cleavage and deprotection as previously described, the crude peptides were purified by reversed-phase HPLC on a C_{18} column, and their identity was confirmed by MALDI-TOF mass spectrometric analyses.

SA−AP Pull-Down Assay. Cyclic peptide (GTHPQALE) plus a miniPEG linker (Peptides International) and a C-terminal methionine was synthesized on 90 μm TentaGel S NH_2 resin as described above. Fifteen milligrams of the resin was suspended in 1.5 mL of blocking buffer (30 mM Hepes, pH 7.4, 150 mM NaCl, 0.01% Tween 20, and 0.1% gelatin), and ~20 μL aliquots (~200 μg resin) were transferred to individual wells of a 96-well plate (Nalge Nunc, Rochester, NY). After overnight incubation, each well was supplemented with 150 μL of a screening buffer (30 mM Hepes, pH 7.4, 150 mM NaCl, and 0.01% Tween 20) containing 2 nM SA−AP (1:2500 dilution of the commercial sample) and 0−800 μM competitor peptide. The mixture was incubated for 30 min with gentle shaking. The resin in each well was transferred to a micro-BioSpin column, drained, and quickly washed twice each with 300 μL of SA−AP buffer and 300 μL of SA−AP reaction buffer. Next, 100 μL of SA−AP reaction buffer containing 10 mM *p*-nitrophenyl phosphate was added to the resin and the mixture was incubated for 1.5 h with gentle shaking. The reaction was stopped with the addition of 900 μL of 1 M NaOH, and the absorbance at 405 nm was measured. In the absence of competitor peptide, the affinity resin containing cyclic peptide (GTHPQALE) retained significantly higher amounts of SA−AP than underivatized TentaGel resin (Figure 4). The presence of competitor peptides inhibited the binding of SA−AP onto the affinity resin and the concentration of a competitor peptide at which 50% of SA−AP binding was inhibited (IC_{50} value) was estimated from the binding curves.

Synthesis of the Tyrocidine Analogue Library. The peptide library was synthesized on 1.0 g of TentaGel MB-NH_2 resin (280−320 μm, 0.21 mmol/g). The C-terminal methionine was coupled onto the resin with 4 equiv of Fmoc-amino acids using HBTU/HOBt/NMM. After removal of the Fmoc group, the resin was coupled with 1.05 equiv of a 9:1 (mol/mol) mixture of $TrtOCH_2C_6H_4COOH$ and Fmoc−Lys(Boc)−OH at room temperature for 4 h. The trityl and Boc groups were removed with TFA/TES/EDT/DCM (90:2.5:2.5:5) for 30 min. The beads were exhaustively washed with DCM (5×) and DMF (3×) and then coupled with N^{α}-Boc−Arg(Pmc)−OH (0.2 equiv) using standard Fmoc/HBTU chemistry. The resin was dried under vacuum overnight, swollen in anhydrous DCM for 20 min, and coupled twice with Fmoc−Asp−OAll (5 equiv), diisopropylcarbodiimide (5 equiv), and 4-dimethylaminopyridine (0.1 equiv) in DCM/DMF (9:1) (3 h each time). Any remaining hydroxyl group was capped with Ac_2O/DMAP in DCM. After removal of the Fmoc group, the coupling of Fmoc-amino acids was carried out using standard Fmoc/HBTU chemistry. For the synthesis of random positions, the resin was split into 20 equal portions and each portion (50 mg) was coupled twice with 5 equiv of a different Fmoc-amino acid/HBTU/HOBt/NMM for 2 h. To differentiate isobaric amino acids during MS sequencing, 5% (mol/mol) Ac-Gly was added to the coupling reactions of Leu and Lys, whereas 5% Ac-Ala was added to norleucine reactions. After the addition of Tyr and Gln, the allyl group on the C-terminal aspartic acid was removed using a solution containing tetrakis(triphenylphosphine)palladium (1 equiv), triphenylphosphine (3

equiv), formic acid (10 equiv), and diethylamine (10 equiv) in anhydrous THF overnight at room temperature. The beads were washed sequentially with 0.5% diisopropylethylamine in DMF, 0.5% sodium dimethyldithiocarbamate hydrate in DMF, DMF (3×), DCM (3×), and DMF (3×). The N-terminal Fmoc group was then removed in 20% piperidine, and the beads were washed with DMF, 1 M HOBt in DMF (3 × 10 min), and DMF (3×). For peptide cyclization, a solution of PyBOP/HOBt/NMM (5, 5, 10 equiv, respectively) in DMF was mixed with the resin and the mixture was incubated on a carousel shaker for 3 h. After the resin was washed with DMF (3×) and DCM (5×), side chain deprotection was carried out with reagent K. The resin was washed with DMF and DCM and dried under vacuum before storage at 4 °C. To determine the yield of cyclization, 20 beads were randomly picked up to do the ninhydrin test using the beads before cyclization as control. Ninhydrin assay was performed by the addition of 300 μL of 76% phenol in ethanol (w/v), 300 μL of 20 μM KCN in pyridine, and 300 μL of 5% ninhydrin in ethanol (w/v) to 20 beads heating at 120 °C for 5 min. The absorbance at 580 nm was measured on a Perkin-Elmer Lambda 25 UV/visible spectrometer, and the background was subtracted from the absorbance of the mixture without resin.

Antimicrobial Assays. Resin beads were manually placed in 96-well microtiter plates (1 bead/well). The cyclic peptide was detached from each bead by overnight treatment with 25 μL of 1 N NaOH at room temperature. After neutralization with 25 μL of 1 N HCl, the pH of the solution was adjusted to 7.5 by the addition of 10 μL of 500 mM sodium phosphate buffer (pH 7.5). The resulting stock solution (60 μL) should contain ~50 mM cyclic peptide on the basis of the loading capacity of the beads (3.5 nmol/bead) and an estimated peptide synthesis yield of 80%. Antibacterial assays were performed using the standard microtiter plate assay method.[20] For each sample, 19 μL of the stock solution was added to a 1000-fold diluted overnight culture and the resulting culture (total volume of 100 μL) was incubated for 6 h at 37 °C and examined visually for bacterial growth. MIC is defined as the lowest peptide concentration that caused no visible cell growth.

Large-Scale Synthesis of Tyrocidine Analogues. Each cyclic peptide was synthesized on 75 mg of NovaSynTGA resin (90 μm, 0.23 mmol/g) in a manner similar to that employed for the library construction. After the cyclization and drying the beads overnight, the cyclic peptides were released from beads and side chain deprotected using reagent K. The cyclic peptides were precipitated in ether and dried under vacuum. The purity of the cyclic peptides was assessed by reversed-phase HPLC on a C_{18} column, and their identity confirmed by MS analysis. Most of the peptides had >50% purity. To synthesize cyclic peptides with an asparagine at position 5, the beads after cyclization were washed thoroughly with DMF and DCM and dried under vacuum. The cyclic peptides were released from the resin by treating with neat propylamine for 6 h at room temperature. After evaporating the excess propylamine under vacuum, the peptides were deprotected with reagent K (room temperature, 1.5 h). The crude peptides were purified by reversed-phase HPLC on a C_{18} column, and their identity was confirmed by MALDI-TOF mass spectrometric analyses.

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Supporting Information Available: Additional experimental details and MS spectra and HPLC chromatograms of cyclic peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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