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MALDI-MS Determination of Cyclic Peptidomimetic Sequences on Single Beads Directed toward the Generation of Libraries

Zhiguang Yu,† X. Christopher Yu,‡ Yen-Ho Chu*,†

†Department of Chemistry and ‡Campus Chemical Instrument Center
The Ohio State University, Columbus, Ohio 43210

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Abstract: A series of peptides of various sequences were prepared on TentaGel resin and cyclized *via* either *N*-to-side chain or side chain-to-side chain lactam formation. The cyclization efficiency was quantitated and found to be both ring size- and sequence-dependent. The strategy of partial chain-termination was applied successfully to the sequence determination of a cyclic peptide on a single bead. Our results provide a useful method for the construction of cyclic peptidomimetic libraries and characterization of cyclic ligands.

Combinatorial library methods have been widely accepted as useful tools in identifying antigenic determinants, receptor-binding ligands, enzyme substrates, and enzyme inhibitors.[1,2] However, ligands selected from linear peptide libraries are inferior drug candidates, in part due to their poor oral availability and rapid enzymatic degradation. Currently, the focus in combinatorial chemistry for drug discovery is on peptidomimetic and small non-peptide organic compounds.[3] Cyclic peptides are constrained peptidomimetics with reduced flexibility in conformation and are more resistant to proteolysis.[4,5] Recently, novel cyclic peptidomimetics have been identified to be effective inhibitors for chymotrypsin and HIV-1 protease.[6,7] An endothelin antagonist was also identified from a cyclic pentapeptide library using the method of deconvolution.[8]

Peptide cyclizations on various solid supports *via* lactam formation have been widely reported.[9] We are interested in constructing cyclic peptide libraries on TentaGel support for the discovery of novel ligands for receptors, such as disease-specific monoclonal antibodies, cell wall components, and enzymes involved in drug resistance. TentaGel is known for its good swelling ability in aqueous buffer; therefore high throughput biological screening can be conducted directly on the support to discover lead compounds. Even though it has been reported that the cyclization efficiency of peptides on other supports could be ring size- or sequence-dependent,[9,10] a systematic evaluation of the cyclization yield on the TentaGel is still not available. Such information would be valuable for the successful construction of cyclic peptidomimetic libraries on this support.

In our experiments, a 12-mer epitope, EGVQQEGAQQPA, from *Borrelia burgdorferi* flagellin, a Lyme antigen, was used and studied in this work.[11] A common pentapeptide E(OAl)BBKM (B= β-Ala)[12] was flanked on TentaGel resin (80-100 μm, 0.26 mmol / g), then peptides of various lengths were assembled (Table 1). Upon removal of the allyl group with $Pd(PPh_3)_4$ (100 mg catalyst / 2 mL solution / 100 mg resin; solution: $CHCl_3$ / HOAc / NMM = 37 : 2 : 1, v/v/v) and Fmoc deprotection with piperidine (20% (v/v) in DMF; 2 mL solution / 100 mg resin), all peptides were cyclized on the resin by HOBt/BOP/NMM (1 : 1 : 1) in acetonitrile (5 mol eq / 2 mL solvent / 100 mg resin) *via* *N*-to-side chain (N→S) lactam formation.[9,10] After side chain deprotection,[13] the peptides on resin were treated with cyanogen bromide in 70% formic acid (200 mg / 2 mL solution / 100 mg resin) for 20 hours in the dark and each reaction solution was lyophilized to remove excessive cyanogen bromide.

The reaction mixtures were analyzed using reversed phase HPLC.[14] All significant peaks were collected individually and characterized by MALDI-MS.[15] Among the cyclic peptides studied, the 8-mer sequence gave the highest yield of 76% (Table 1, column 1, and Figure 1). The major side products observed include the linear peptides modified at their Glu side chain carboxyl groups (see below), and intermolecularly cross-linked dimers. In cases that the ring sizes are larger than 11 amino acids or smaller than 6 amino acids, dimers and linear peptide adducts increased noticeably. Under our experimental conditions, trimers and higher oligomers were not observed in the cyclization of all peptides studied.

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Table 1. The Effect of Ring Sizes and Sequences in Peptide Cyclization, cyclo(**peptide**)BBKM, on the TentaGel Resin.[a]

Peptide (N→S)	Cyclization Efficiency (%)[b]	Peptide (S→S)	Cyclization Efficiency (%)[b]	Peptide (N→S)	Cyclization Efficiency (%)[b]	Peptide (N→S)	Cyclization Efficiency (%)[b]
AQPAE	12	KAQPAE	47	AAPAE	38	AGAQFPAE	78
AQQPAE	27	KAQQPAE	57	AAAPAE	29	AGAFQPAE	75
AAQQPAE	58	KAAQQPAE	40	AAAAPAE	41	AGFQQPAE	80
AGAQQPAE	76	KAGAQQPAE	48	AAAAAPAE	35	AFAQQPAE	79
AEGAQQPAE	60	KAEGAQQPAE	51	AAAAAAPAE	9	AAAQQPAE	74
AQEGAQQPAE	41	KAQEGAQQPAE	42	AAAAAAAPAE	<5	ADAQQPAE	81
AQQEGAQQPAE	27	KAQQEGAQQPAE	33	AAAAAAAAPAE	<3	A IAQQPAE	68
AVQQEGAQQPAE	<10	KAVQQEGAQQPAE	<7	AAAAAAAAAPAE	<3	AKAQQPAE	78
AGVQQEGAQQPAE	<6	KAGVQQEGAQQPAE	<6	AAAAAAAAAAPAE	<3	APAQQPAE	72
AEGVQQEGAQQPAE	<4	KAEGVQQEGAQQPAE	<5	AAAAAAAAAAAPAE	<3	ASAQQPAE	72

[a] The peptide cyclizations were carried out in acetonitrile at ambient temperature. [b] The cyclization efficiency was dtermined based on the integrated HPLC area of the desired cyclic peptide with respect to all peaks in the chromatogram. With those of low cyclization efficiencies, the major products were uncyclized +69 Da adduct and dimers. Trimers and higher oligomers were not detected by HPLC and MALDI.

Side chain-to-side chain (S→S) cyclization of peptides was also studied in this work. Possibly due to the flexible side chain functional groups, cyclization efficiency is less sensitive to the ring size (Table 1, column 3). However, the cyclization yield drops dramatically at the ring size of 12 amino acids. As no peptides with deletions and other side products were detected, the low cyclization yields of long peptides were not due to the poor quality of their corresponding linear forms.

Since the ring-size dependence of cyclization efficiency may be specific to the epitope sequence and proline has been reported to assist peptide cylization,[9,16] a series of oligoalanyl peptides with the sequence $(A)_nPAE(OAl)BBKM$ (n = 2-11) were synthesized and subsequently cyclized (N→S) on TentaGel resin. Results in Table 1 demonstrated that the cyclization of the oligoalanyl peptides larger than 8 amino acids was poor. Peptides with deletion sequences were detected and became problematic with increasing length, even though double coupling was employed in the synthesis. For the cyclic peptide, cyclo(AGAQQPAE), amino acid substitutions were made at various positions to study the sequence specificity in cyclization (Table 1, column 7). It was found that this template tolerates the substitution of amino acids at various positions with minimum changes in cyclization efficiency. Results in Table 1 clearly demonstrate that an adequately designed template is important for the library construction of conformationally constrained peptides.

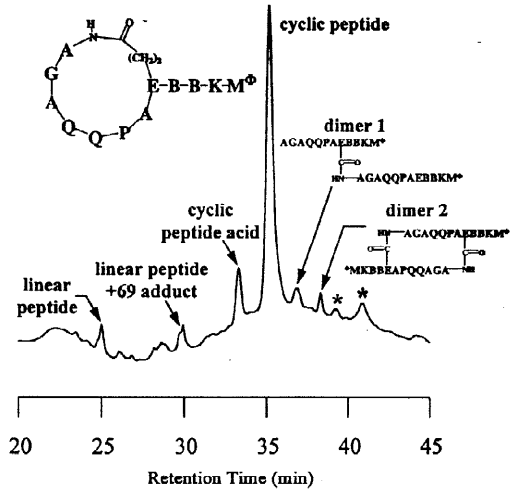

Figure 1. A representative HPLC chromatogram, monitored at 214 nm, of the crude cyclic peptide cyclo(AGAQQPAE)BBKM$^{\Phi}$ (M$^{\Phi}$, homoserine lactone) after cleavage. The asterisk peaks (*) are non-peptides, since no signals were detected at m/z = 500-100,000 by MALDI-MS. The peak intensity of the cyclic peptide acid (i.e., homoserine) increases over time, possibly due to the hydrolysis of the lactone. For experimental conditions, see detail in ref. 14.

As cyclic peptides often contain no free *N*-termini and Edman degradation is unsuitable for sequence determination, we decided to develop methods based on a previously reported strategy of partial chain-termination for the rapid identification of cyclic peptides from a single bead.[17] Cyclic peptides of cyclo(AEGAQQPAE)BBRM and cyclo(KAEGAQQPAE)BBRM were synthesized as the representative (N→S) and (S→S) cyclic peptides, respectively (Figure 2). At each underlined positions in the sequences, 10% (molar ratio) acetylglycine, the chain-terminating agent, was mixed with the Fmoc-protected amino acid. Cyclic peptides and their sequentially terminated linear peptide fragments were released from a single bead in a microtube using cyanogen bromide in formic acid. After lyophilization, the residue in each tube was dissolved in 0.1% TFA (20 μL) and 5% of the peptide solution was used and analyzed by MALDI-MS. Unexpectedly,

each terminated peptide in the mixtures gave two peaks carrying higher m/z values (+27 and +69 Da) if DMF was used as the solvent for cyclization. These increases in m/z were common to all terminated peptides but not to the full length cyclic peptides. We hypothesized that the side chain carboxyl group of Glu was first activated during the cyclization and the free *N*-terminus in the full length peptide then rapidly reacted with the activated carboxyl group to form a lactam bridge. Since the terminated peptides have their *N*-termini blocked by acetyl groups, the activated carboxyl group of Glu could only capture amine impurities from the reagents and form adducts (i.e., the +27 Da peak was from the adduct formed with dimethylamine, a trace impurity commonly found in DMF, and the +69 Da peak was from the adduct formed with morpholine, a possible trace component in *N*-methylmorpholine). Results from both capillary electrophoresis and MS-MS experiments supported our hypothesis.[18]

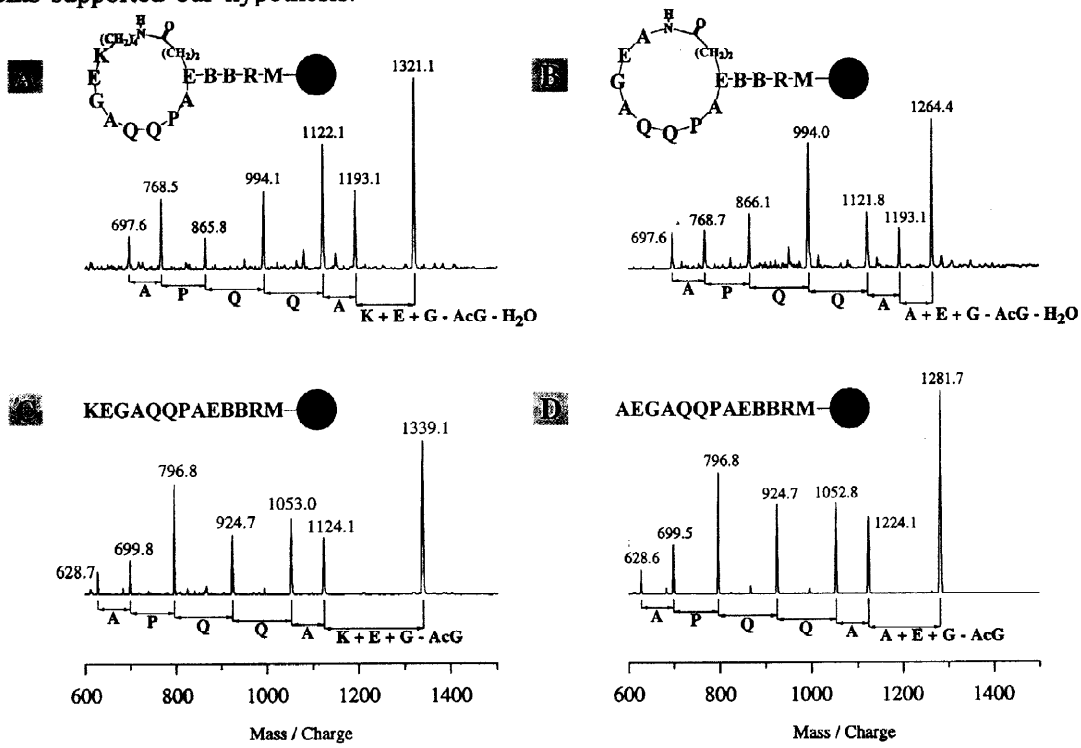

Figure 2. MALDI mass spectra of peptides released from a single bead containing (A) cyclo(KEGAQQPAE)BBRM (the S→S cyclization) and (B) cyclo(AEGAQQPAE)BBRM (the N→S cyclization), and its corresponding linear form: (C) KEGAQQPAEBBRM and (D) AEGAQQPAEBBRM. Sequences of peptides were decoded using the method of partial-chain termination.

Experimentally, we were able to eliminate most, if not all, of the +27 Da adduct if dimethylsulfoxide or acetonitrile were used as the solvent for peptide cyclization (Figure 2). As all MS peaks from terminated peptides show systematic shifts in m/z, the sequence of a cyclic peptide on a single bead can be unequivocally determined (Figure 2).

In conclusion, our systematic evaluation of peptide cyclizations demonstrated that a well designed template is compulsory for the successful construction of cyclic peptidomimetic libraries on TentaGel supports. Both the ring size and peptide sequence are important in determining the cyclization yields. The method of partial chain-termination and MALDI-MS analysis can be applied to determine sequences from cyclic peptidomimetic libraries.

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12. The methionine residue was engineered to mediate cleavage of peptides from the resin and the lysine (or arginine) was included to introduce a common protonation site for ease of MS detection and, if necessary, post-cleavage modification (e.g., fluorescent tag labeling). Two β-Ala residues were used as a spacer.
13. Side chains of peptides were deprotected using a cleavage cocktail of 82.0% TFA, 4.0% water, 4.0% thioanisole, 6.0% mercaptoacetic acid, and 4.0% phenol at rt for 4 hours, and the resin was washed with ethyl ether to remove scavengers.
14. A Supelcosil LC-318 C_{18} column (25 cm x 4.6 mm, 5 μm) was used to separate cyclic peptides using a 0.5% min^{-1} linear gradient, running from 100% A (0.1% TFA in water) to 25% B (0.1% TFA in acetonitrile), at a flow rate of 0.5 mL min^{-1}. All separations were monitored at 214 nm.
15. The MALDI-MS analysis was conducted using a Kratos Kompact MALDI-III instrument. Saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50:50 water/acetonitrile was used as the matrix. Sample (1.0 μL) was mixed with the matrix solution (1.0 μL) on a slide for MS analysis. The instrument was calibrated externally with substance P and leucine-enkephalin.
16. Proline can assist the cyclization process and, in some cases, the cyclization can not be achieved without Pro in the sequence. For example, we found that, under our experimental conditions, the NH_2-AVQQEGAE(OH)BBKM peptide was not cyclized on the TentaGel support.
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18. To test our hypothesis, the peptide Ac-GE(OAl)BBRM was synthesized on TentaGel. After the allyl group was removed, the resin was split into three fractions. To each of them, a DMF solution of HOBt/BOP/NMM was added. To two of them, additional morpholine and aqueous dimethylamine were used to individually synthesize the artificial +69 and +27 adducts. They were analyzed by capillary electrophoresis (Beckman P/ACE 5510) and MS-MS (PE Sciex API300). CE was conducted in a 57-cm uncoated capillary, running at a voltage of 30 kV and using 50 mM tris-acetate (pH 8.0) as the eletrophoresis buffer. Tandem mass spectrometry were performed using the triple quadrupole mass spectrometer. Ionspray and orifice voltages were set at 4300 and 60 eV, respectively, and the ring voltage was at 400 eV. Ultra pure nitrogen (99.999%) was used as curtain as well as collision gas, and collision energy was set at 65 eV. Collision cell pressure was maintained at 2.53×10^{-3} torr. Identical results were obtained in both experiments for the artificial and experimentally obtained adducts.
CE migration time: +27 and +69 Da adducts coeluted at 2.7 min, and the endoosmotic flow appeared at 3.2 min. Major MS-MS primary fragments (Da): 46.0 ($X+H^+$), 129.0 (a_2-Ac-G+H^+), 228.0 (a_2), 256.2 (b_2), 327.3 (b_3), 398.4 (b_4) for the +27 Da adduct; and 88.0 ($X+H^+$), 171.0 (a_2-Ac-G+H^+), 270.5 (a_2), 298.2 (b_2), 369.3 (b_3), 440.4 (b_4) for the +69 Da adduct. These two adducts have the same secondary fragments (Da) at 211.0 (b_2-X), 282.0 (b_3-X), and 353.0 (b_4-X). X represents dimethylamine for the +27 Da adduct, and morpholine for the +69 Da adduct.