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Oxazolidinone-Mediated Sequence Determination of One-Bead One-Compound Cyclic Peptide Libraries

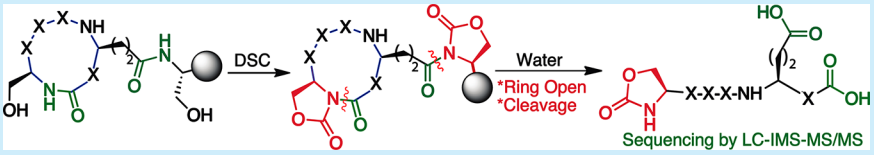
Hader E. Elashal,[†,‡] Ryan D. Cohen,[†,§,‡] Heidi E. Elashal,[†] and Monika Raj*[,▽]

[†]Department of Chemistry, Seton Hall University, South Orange, New Jersey 07079, United States

[§]Department of Process Research and Development, Merck & Co., Inc., Rahway, New Jersey 07065, United States

[▽]Department of Chemistry and Biochemistry, Auburn University, Auburn, Alabama 36830, United States

Supporting Information



ABSTRACT: A novel one-bead one-compound (OBOC) dual ring-opening/cleavage approach for cyclic peptide sequencing was developed. The method selectively modifies serine, cysteine, threonine, and/or glutamic acid to an oxazolidinone-derived moiety, thereby increasing the susceptibility of the modified peptide backbone toward hydrolysis. The resulting linear peptide was then sequenced in 1 min by tandem mass spectrometry on a quadrupole time-of-flight instrument incorporating two-dimensional liquid chromatography and ion mobility spectrometry separation. To evaluate this approach, a library of cyclic peptides was successfully sequenced with 98% overall accuracy, demonstrating its robustness and broad substrate scope.

Cyclic peptides are of considerable interest, because of their powerful potential as therapeutic agents.[1,2] Compared to their linear counterparts, cyclic peptides exhibit high receptor-binding affinity, specificity, and stability because of conformational rigidity.[3] The therapeutic ability of cyclic peptides has prompted the synthesis of large libraries by split and pool methods,[4] followed by high-throughput screening against biological targets.[5] However, the sequencing of cyclic peptides proves to be a challenging task. While enzymatic cleavage is one of the most utilized methods for peptide sequencing, cyclic peptides are resistant to proteolysis by 20−300-fold, as compared to their linear counterparts. This results from both a lower rate of cleavage (k_{cat}) and a weaker interaction with proteases (K_m).[6] Since cyclic peptides lack a free N-terminal amine, they cannot be sequenced via Edman degradation.[7] In addition, tandem mass spectrometry (MS/MS), which is another widely employed sequencing method, affords complex fragmentation patterns that are difficult to interpret.[8,9]

Many attempts have been made in developing methods to overcome the challenges associated with sequencing "hit" macrocyclic peptides. An early approach employed genetic encoding tags to record the synthesis history of cyclic peptides.[10] The Pei and Lam groups developed a one-bead two-compound (OBTC) method that consisted of segregated bilayer beads containing both cyclic peptides and their respective linear sequences.[11,12] While innovative, these approaches are limited by complex synthesis procedures.[11,12] Another major limitation associated with these methods are that they cannot be used for sequencing cyclic peptides obtained from nature without an encoding tag.

More recently, strategies based on opening the macrocyclic ring to its linear counterpart post-screening have been developed. In this manner, Lim and co-workers utilized a thioether bridge, which generates the linear peptide upon oxidation but also oxidizes other functional groups, such as guanidines and primary amines, resulting in complicated MS/MS fragmentation.[13] The Kodadek[14] and Biron[15a] groups used a methionine residue for linearization and cleavage of cyclic peptides/peptoids from the solid support. However, this method was limited by complicated inverse peptide synthesis, side reactions, long cleavage times, and employed toxic CNBr for cleavage. Biron's group then developed a photocleavable moiety, β-amino acid 3-amino-3-(2-nitrophenyl)propionic acid (ANP), for sequencing cyclic peptides, which also generated a mixture of various linear peptide side products and oxidized Met and Trp.[15b]

Herein, we report a simple and convenient dual ring-opening/cleavage approach for rapid sequencing of cyclic peptides from one-bead one-compound (OBOC) libraries that overcomes the challenges of the existing methods. The method employs selective activation of the amide backbone chain at serine for ring-opening and post-screening sequencing. The significance of this approach is that encoding tags or toxic reagents are unnecessary. Moreover, this method is compatible with free amino acid side chains and does not lead to byproducts.

This approach utilizes two serine residues: one as a linker and the other within the macrocycle (Scheme 1). Both are

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Scheme 1. Rationale for Sequencing Cyclic Peptides by Ring-Opening/Cleavage Strategy

converted to oxazolidinone (*Oxd*) moieties, thereby making the amide bond susceptible to hydrolytic cleavage at the Ser N-terminus. Earlier attempts at formation of this moiety on Fmoc/Boc-protected peptides generated dehydroalanine.[16] Our group recently reported the application of *Oxd* for thioester synthesis.[17] This methodology simultaneously converts cyclic peptides into their linear counterparts and releases them from the solid support. The linear peptides obtained in solution could then be easily sequenced by MS/MS. Scheme 1 illustrates the ring-opening/cleavage reaction. Notably, only those resin-bound peptides that complete both activation and displacement steps would be released. The released linear peptides generally exhibited high purity, thereby eliminating the need for extensive purification.

To implement this strategy, a serine (trt) trityl residue was introduced as a linker and as part of the cyclic peptide using standard Fmoc chemistry (Scheme 1).[18] After removal of the allyl and Fmoc protecting groups, the peptides were cyclized on resin using standard coupling reagents.[19] Following macrocyclization, the (trt) groups from both serine residues were selectively removed with TFA/DCM)(3:7).[19] Initially, we assessed the serine activation efficiency within a macrocyclic peptide and as a linker. Serine residues were activated with *N,N′*-disuccinimidyl carbonate (DSC) to generate two five-membered oxazolidinone rings on a model cyclic peptide *cyc*(GFSFAE)-S **1a** on solid support (entry 1, Table 1). To optimize formation of two oxazolidinone moieties, various reaction conditions such as time, temperature, and DSC equivalents were explored (see Table S1 in the Supporting Information (SI)). Serine activation was investigated for cyclic peptides on solid support with varying sequences and ring sizes (4−9-membered) (**1b**−**1i**; see entries 1−9 in Table 1).

It is noteworthy that formation of the activated oxazolidinone moiety is independent of the nature of the amino acid preceding serine, and high conversion was obtained with cyclic peptides of various sizes and with Ser located at various positions within the macrocycle (Table 1), which is contrary to the methionine-based ring cleavage methods.[14,15a]

Backbone amide activation is also possible at Thr and Cys, because of similar side-chain functionality exhibited by these residues (**1g**−**1i**; see entries 7−9 in Table 1). The possibility of modification at Ser, Cys, and/or Thr provides an additional advantage of this method affording multiple cleavage sites to aid in the sequencing process. Activation at any such residue provides flexibility in OBOC library construction (i.e., the peptide library includes, but is not limited to only Ser residues). In addition, the resin-bound oxazolidinone- and thiazolidinone-modified cyclic peptides were stable in a desiccator for longer than a month, which is contrary to the previously reported photocleavage method.[15b]

Table 1. Substrate Scope of the Activation of Cyclic Peptides 1a−1i on Solid Support to 2a−2i[a]

entry	cyclic peptides **1**	conversion **2**[b] (%)
1	*cyc*(GFSFAE)-S **1a**	96
2	*cyc*(SFFAE)-S **1b**	85
3	*cyc*(SGVFAE)-S **1c**	89
4	*cyc*(GSFAE)-S **1d**	92
5	*cyc*(GFKSYGLE)-S **1e**	90
6	*cyc*(AFSIGFE)-S **1f**	88
7	*cyc*(AFSFE)-T **1g**	95
8	*cyc*(GFCE)-C **1h**	92
9	*cyc*(AMPFISFPE)-C **1i**	85

[a]Cyclic peptides **1a**−**1i** (50 mg) were reacted with DSC (20 equiv), DIEA (20 equiv), and a crystal of DMAP in DMF for (15 h) at room temperature (rt). [b]Conversions to **2a**−**2i** were calculated using HPLC at 220 nm (see the Supporting Information (SI)).

Nucleophilic displacement of the oxazolidinone moieties under basic hydrolytic conditions opened the cyclic peptide **2a** to its linear variant and simultaneously released the resulting linear peptide acid *Oxd*-FAEGF **3a** from the solid support. The sequence of the linear peptide *Oxd*-FAEGF **3a** was then determined by MS/MS (see Figure 1 and the SI). To optimize the ring opening and cleavage of the activated cyclic peptide from the solid support, various reaction conditions, such as base and temperature, were investigated (see Table S2 in the SI).

Markedly, the opening and release of the oxazolidinone-activated cyclic peptides **2** from solid support is independent of the nature of the amino acid residues preceding serine and size of the ring (see Table 2). For example, cyclic peptides with bulky amino acid residues next to serine (such as Tyr, Phe, and Lys), and *β*-branched residues (such as Ile) generated linear

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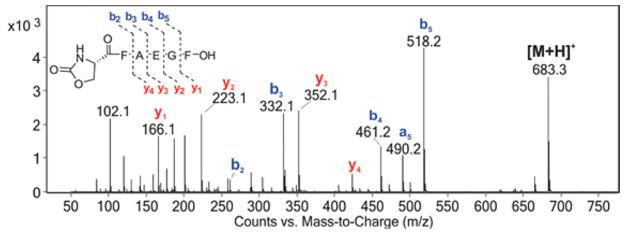
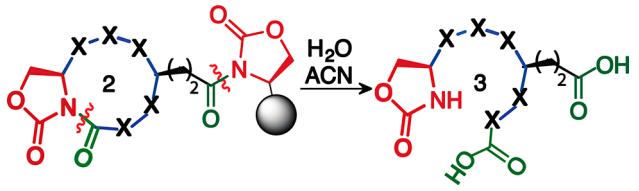


Figure 1. MS/MS spectrum of ring opened *Oxd*-FAEGF **3a**.

Table 2. Substrate Scope of the Ring-Open/Cleavage Strategy on Activated Cyclic Peptides 2a−2i[a]



entry	activated cyclic peptides **2**	conversion **3**[b] (%)
1	*cyc*(GF-*Oxd*-FAE)-*Oxd* **2a**	87
2	*cyc*(*Oxd*-FFAE)-*Oxd* **2b**	85
3	*cyc*(*Oxd*-GVFAE)-*Oxd* **2c**	89
4	*cyc*(G-*Oxd*-FAE)-*Oxd* **2d**	92
5	*cyc*(GFK-*Oxd*-YGLE)-*Oxd* **2e**	90
6	*cyc*(AF-*Oxd*-IGFE)-*Oxd* **2f**	88
7	*cyc*(AF-*Oxd*-FE)- ^{Me}*Oxd* **2g**	95
8	*cyc*(GF-*Thz*-E)-*Thz* **2h**	92
9	*cyc*(AMPFI-*Oxd*-FPE)-*Thz* **2i**	85

[a]Activated cyclic peptides **2a−2i** (50 mg) were treated with H_2O:ACN (1:1) 1 mL and DIEA (40 μL). [b]Conversions to **3a−3i** were calculated using HPLC at 220 nm.

peptides cleanly with high conversion (entries 5−9, Table 2). We did not observe any modification of the unprotected side chains after dual ring-opening/cleavage of cyclic peptides.

The developed methodology is designed to be utilized post-screening of OBOC libraries to determine the sequence of a "hit" compound. Any of the amino acids that are amenable to modification (Ser, Thr, and Cys) will be incorporated into the cyclic peptide library in their native, unmodified forms. Following peptide screening against a target to determine a lead compound, the DSC chemical methodology will be applied to modify the peptide, followed by subsequent hydrolysis to determine cyclic peptide sequence. The methodology will be executed post-screening as to not interfere with binding properties and the stability of the cyclic peptides in screening medium.

To demonstrate the compatibility of our strategy with OBOC libraries, a small cyclic peptide library of varying amino acid composition and ring sizes ranging from 4 to 10 amino acids was prepared on Tenta Gel resin. Post-synthesis, 34 beads were randomly selected, and Ser/Thr/Cys was modified and hydrolyzed to effectively yield the linear counterpart (see Table S3 in the SI). The resulting crude linear peptides were then sequenced by tandem mass spectrometry on a quadrupole time-of-flight (Q-ToF) instrument incorporating two-dimensional liquid chromatography and ion mobility spectrometry separation (LC-IMS-MS/MS) (see Figure 2 and the SI).[20] Pairing reversed-phase ultra-high pressure liquid chromatography and ion mobility spectrometry resulted in an efficient separation time of just 1 min, which would enable the analysis of large

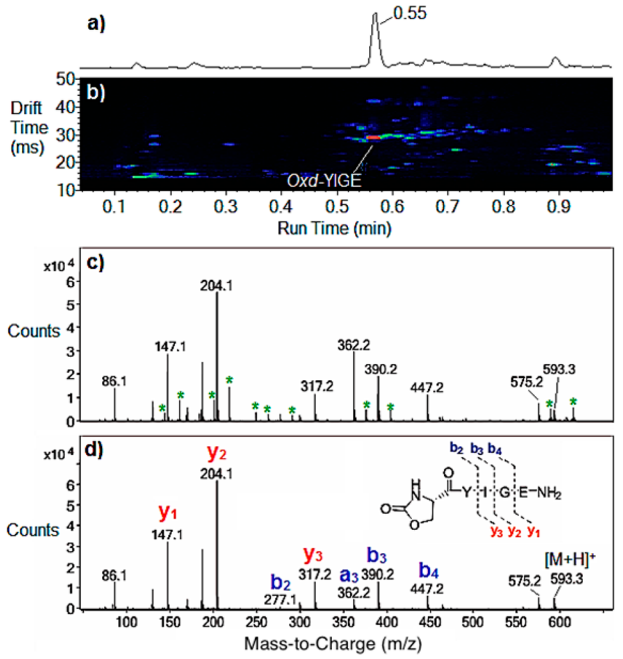
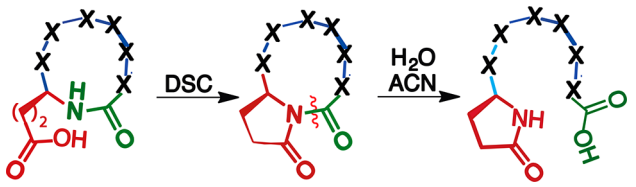


Figure 2. (a) LC-MS separation of *Oxd*-YIGE, (b) 2D separation−ion mobility drift time versus total ion chromatogram, (c) MS/MS spectrum without quadrupole selection of parent ion (interfering signals marked with green asterisks), and (d) MS/MS spectrum including ion mobility filtering to remove chemical interference.

combinatorial cyclic peptide libraries. To increase throughput, MS and MS/MS spectra were acquired in the same run without parent ion selection, and then chemical noise was removed from the MS/MS spectra by using the drift time of the MS base peak as a filter. Spectra were interpreted either manually or, in most cases, via *de novo* sequencing software.

Another unique feature of this approach is that peptides with glutamic acid residues can also be selectively modified to a pyroglutamyl imide moiety with the backbone peptide chain leading to the ring opening of the cyclic peptide at the N-terminus of Glu under hydrolytic conditions (see Scheme 2).

Scheme 2. Rationale for Sequencing Cyclic Peptides with Glutamic Acid via Pyroglutamyl Imide Moiety



We synthesized and sequenced a potent p53 cyclic peptide analogue, which is implicated in the p53/MDM2 interaction[21] and contains two glutamic acid residues (see Figure S4 in the SI).

We developed a general one-bead one-compound (OBOC) method for sequencing cyclic peptides obtained from combinatorial libraries. Our method has high substrate scope, and ring-opening of cyclic peptides can be achieved at serine, threonine, cysteine, and glutamic acid residues, which is a significant advantage over the current OBOC approaches that rely only on one residue (i.e., Met or an unnatural moiety). This method is compatible with free amino acid side chains and

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is applicable for sequencing of cyclic peptides obtained from nature, which is a significant advantage over well-established methods. In addition, we developed a high-throughput LC-IMS-MS/MS method that can sequence over 1000 cyclic peptides in 1 day. The main attributes of our method are its simplicity, facile synthesis of cyclic peptide libraries, and high substrate scope without major side product formation. This robust method will better enable the search for cyclic peptides as novel therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b00717.

Supporting figures, experimental procedures, and analytical data for new compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mzr0068@auburn.edu.

ORCID

Monika Raj: 0000-0001-9636-2222

Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Joo, S. H. *Biomol. Ther.* **2012**, *20*, 19−26.
(2) McGregor, D. P. *Curr. Opin. Pharmacol.* **2008**, *8*, 616−619.
(3) Yudin, K. *Chem. Sci.* **2015**, *6*, 30−49.
(4) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84.
(5) Lam, K. S.; Lebl, M.; Krchňák, V. *Chem. Rev.* **1997**, *97*, 411−448.
(6) Millward, S. W.; Fiacco, S.; Austin, R. J.; Roberts, R. W. *ACS Chem. Biol.* **2007**, *2*, 625−634.
(7) Edman, P.; Begg, G. *Eur. J. Biochem.* **1967**, *1*, 80−91.
(8) Eckart, K.; Schwarz, H.; Tomer, K. B.; Gross, M. L. *J. Am. Chem. Soc.* **1985**, *107*, 6765−6769.
(9) Redman, J. E.; Wilcoxen, K. M.; Ghadiri, M. R. *J. Comb. Chem.* **2003**, *5*, 33−40.
(10) Scott, P.; Abel-Santos, E.; Wall, M.; Wahnon, D. C.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 13638−13643.
(11) Joo, S. H.; Xiao, Q.; Ling, Y.; Gopishetty, B.; Pei, D. *J. Am. Chem. Soc.* **2006**, *128*, 13000−13009.
(12) Liu, R.; Marik, J.; Lam, K. S. *J. Am. Chem. Soc.* **2002**, *124*, 7678−7680.
(13) Lee, J. H.; Meyer, A. M.; Lim, H. S. *Chem. Commun.* **2010**, *46*, 8615−8617.
(14) Simpson, L. S.; Kodadek, T. *Tetrahedron Lett.* **2012**, *53*, 2341−2344.
(15) (a) Liang, X.; Girard, A.; Biron, E. *ACS Comb. Sci.* **2013**, *15*, 535−540. (b) Liang, X.; Vézina-Dawod, S.; Bédard, F.; Porte, K.; Biron, E. *Org. Lett.* **2016**, *18*, 1174−1177.
(16) De Marco, R.; Tolomelli, A.; Campitiello, M.; Rubini, P.; Gentilucci, L. *Org. Biomol. Chem.* **2012**, *10*, 2307−2317.
(17) Elashal, H. E.; Sim, Y. E.; Raj, M. *Chem. Sci.* **2017**, *8*, 117−123.
(18) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86.
(19) Elashal, H. E.; Cohen, R. D.; Raj, M. *Chem. Commun.* **2016**, *52*, 9699−9702.
(20) Valentine, S. J.; Kulchania, M.; Barnes, C. A. S.; Clemmer, D. E. *Int. J. Mass Spectrom.* **2001**, *212*, 97−109.
(21) Fasan, R.; Dias, R. L. A.; Moehle, K.; Zerbe, O.; Vrijbloed, J. W.; Obrecht, D.; Robinson, J. A. *Angew. Chem., Int. Ed.* **2004**, *43*, 2109−2112.

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