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One-Pot Photochemical Ring-Opening/Cleavage Approach for the Synthesis and Decoding of Cyclic Peptide Libraries

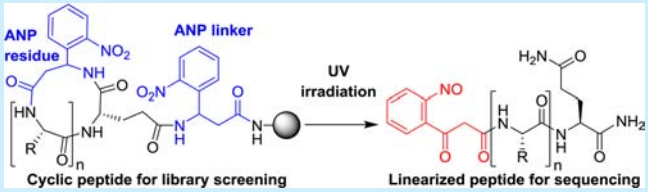
Xinxia Liang,[†,‡,§] Simon Vézina-Dawod,[†,‡,§] François Bédard, Karine Porte,[†,‡] and Eric Biron*[,†,‡]

[†]Faculty of Pharmacy, Université Laval, Québec, Québec G1V 0A6, Canada

[‡]Laboratory of Medicinal Chemistry, Centre de recherche du Centre Hospitalier Universitaire de Québec, 2705 Boulevard Laurier, Québec, Québec G1V 4G2, Canada

Supporting Information

ABSTRACT: A novel dual ring-opening/cleavage strategy to determine the sequence of cyclic peptides from one bead, one compound libraries is described. The approach uses a photolabile residue within the macrocycle and as a linker to allow a simultaneous ring opening and cleavage from the beads upon UV irradiation and provide linearized molecules. Cyclic peptides of five to nine residues were synthesized and the generated linear peptides successfully sequenced by tandem mass spectrometry.



Peptide macrocycles are useful tools in chemical biology and medicinal chemistry to study and modulate protein functions.[1] With great potential as therapeutic agents, they have gained a great deal of interest in drug discovery.[2] Compared to their linear counterparts, cyclic peptides are more resistant to proteases, and their increased conformational rigidity lowers the entropic cost of binding, making them tighter binding to a given macromolecule.[3] The great degree of molecular diversity and complexity that can be accessed by simple changes in their sequence has prompted the use of cyclic peptides in combinatorial chemistry. The one bead, one compound (OBOC) approach, in which each bead carries many copies of a unique compound, has become a powerful tool in the drug discovery process.[4] However, the use of cyclic peptides in combinatorial OBOC libraries has been limited by difficulties in sequencing hit compounds after the screening. Lacking a free N-terminal amine, Edman degradation sequencing cannot be used on cyclic peptides, and complicated fragmentation patterns are obtained by tandem mass spectrometry (MS/MS).[5]

In this regard, a one bead, two compound approach on topologically segregated bilayer beads has been developed.[6] Initially introduced with an enzymatic shaving strategy[7] and later via chemical approaches,[8] topological bilayer segregation offers the opportunity to synthesize two compounds per bead, namely one which is exposed on the bead surface for screening (cyclic peptide) and the other found inside as a tag for sequencing and compound identification (linear peptide). More recently, Lim and co-workers reported a ring-opening strategy on cyclic peptoids to eliminate the need for encoding.[9] The approach involved the introduction of a cleavable alkylthioaryl bridge in the cycle to allow linearization of the molecule after the screening by thioether oxidation followed by nucleophilic displacement of the sulfone to generate a linear peptoid which can be sequenced by MS/MS. An attractive advantage of the ring-opening strategy over encoding methods is the absence of interference by the coding tag during screening since the same molecules are displayed inside and on the surface of the beads. Based on this strategy, we and other groups placed a methionine or a thioether bridge in cyclic peptides or peptoids and as a linker to allow a simultaneous linearization and compound release from the bead upon treatment with cyanogen bromide.[10] A similar approach was applied to cyclic depsipeptides where an aminolysis with NH_3 or hydrolysis with aqueous NaOH was used to cleave the ester bonds and release the linear compound from the bead.[11] Most reported methods require aggressive chemical reagents or postscreening reactions that could lead to side-chain modifications. Based on these strategies, we were looking for an efficient, single-step, and reagent-free ring-opening approach that would be compatible with free amino acid side chains. Here, we report a novel and convenient approach for fast and simple sequence determination of cyclic peptides from OBOC libraries.

Our approach utilizes the photocleavable β-amino acid 3-amino-3-(2-nitrophenyl)propionic acid (ANP)[12] as a linker and within the macrocycle (Scheme 1). The strategy is to use UV irradiation to simultaneously convert cyclic peptides into their linear counterpart and release them from the beads. The generated linear peptides could then be sequenced by MS/MS. To evaluate the ring-opening efficiency upon UV irradiation, a first series of cyclic peptides of different sizes containing various functionalized amino acids and with or without a C-terminal spacer commonly used in OBOC libraries were synthesized on Rink Amide AM polystyrene resin. Scheme 1 illustrates the general procedure for synthesis of the model cyclic peptides.

Linear peptides were synthesized on solid support by standard Fmoc chemistry. After removal of the allyl and Fmoc protecting

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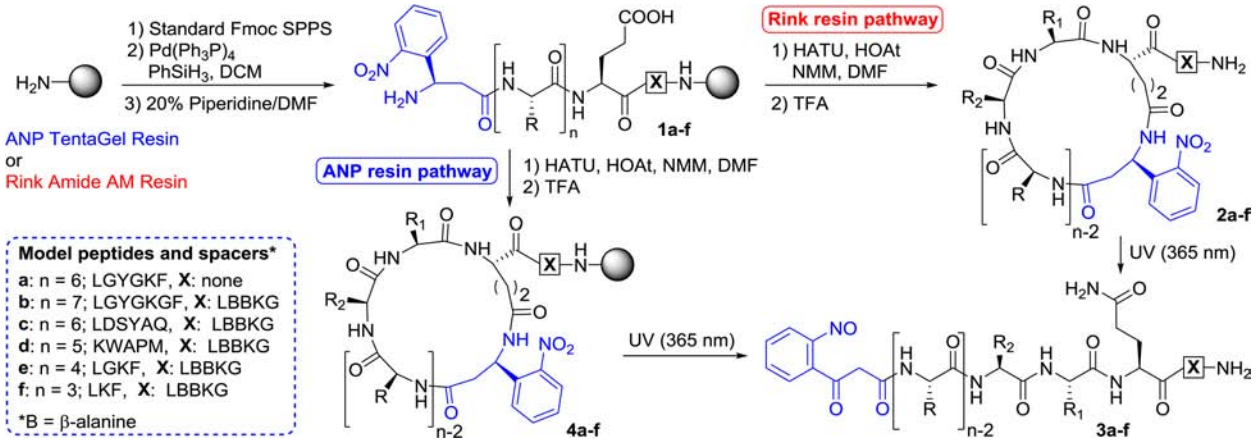
Scheme 1. Synthetic Route to Cyclic Peptides with ANP Residue and Ring-Opening/Cleavage Reaction



groups, the peptides **1a–f** were cyclized on resin with HATU. The chloranil test was used to qualitatively monitor the reaction.[13] After cyclization, the side-chain protecting groups were removed and the compounds cleaved from the resin with a TFA cocktail. The released cyclic peptides **2a–f** were then analyzed by HPLC and electrospray ionization MS (ESI-MS) to confirm the absence of linear peptides (Figure 1A and Figure S3).

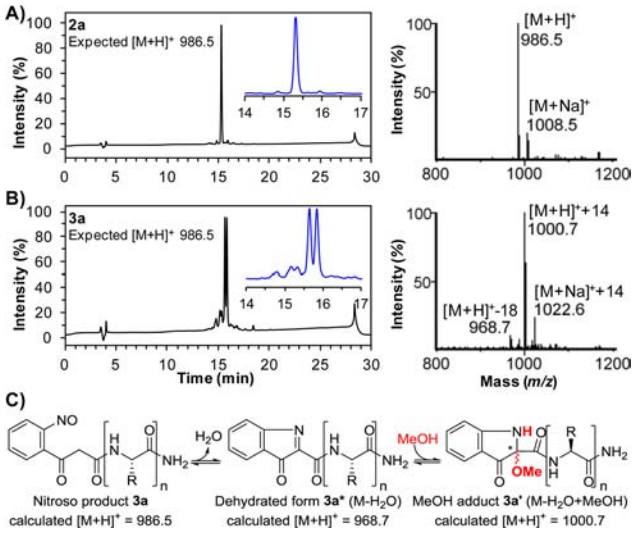


Figure 1. HPLC and ESI-MS profiles of crude products showing cyclization and ring-opening: (A) cyclo[ANP-LGYGKFE]-NH_2 (**2a**) and (B) 3-oxo-3-(2-nitrosophenyl)propionyl-LGYGKFQ-NH_2 (**3a**). (C) Proposed structures for the dehydrated product **3a*** and its adduct **3a′** observed during ESI-MS analysis.

Cyclic peptides **2a–f** were then subjected to UV irradiation at 365 nm in MeOH for ring cleavage. HPLC analyses confirmed the transformation of the cyclic peptides into their linear counterparts **3a–f** (Figure 1B and Figure S4). While a mixture of different products was observed in most cases, a doublet-shaped main peak with both peaks having the same mass was noticed for **3a**. This result led us to presume the formation of diastereomers during ring opening. Moreover, even if highly pure products were not observed in the HPLC analyses for other model peptides, ESI-MS spectra showed the presence of a single major molecular species (Figure 1B and Figure S4).

Since the cyclic and linearized peptides have exactly the same elemental composition, the use of MS to differentiate cyclic **2a–f** from linear compounds **3a–f** is theoretically limited. Surprisingly, after MS analysis of the generated linear peptides **3a–f**, the anticipated molecular ion was not obtained, and the most important peak was observed at +14 Da (Figure 1B and Figure S4). This observation led us to suspect the formation of a C-terminal methyl ester as described by other groups[10c,14] or the transformation of the expected N-terminal nitroso product into an indolin-3-one residue.[14] The formation of a methyl ester from the C-terminal amide would have generated a +15 Da adduct and could not explain the presence of diastereoisomers in the HPLC chromatograms. Further analysis of the photocleavage reaction mechanism of *o*-nitrophenyl derivatives led us to propose that the +14 Da peak is generated during UV irradiation from the addition of MeOH (32 Da) on a dehydrated intermediate **3*** ($[M - H_2O + H]^+$) of the nitroso derivative **3** (Figure 1C). The adduct product $M - H_2O$ + MeOH **3′** could be formed by an Ehrlich–Sachs-like reaction and as a result generate two epimers (Figure 1C and Figure S1).[15] This hypothesis was supported by the following results with peptide **3a**: (1) when methanol-d_4 (36 Da) was used as solvent, the adduct peak was observed at +18 Da (Figure S5); (2) addition of $BuNH_2$ (73 Da) to the MeOH (5% v/v) during UV irradiation yielded the adduct peak at +55 Da (Figure S5); (3) only the M − 18 peak was observed in absence of MeOH during MALDI-TOF MS analysis, and (4) all *b* ions (N-terminal fragments) were dehydrated (*b* − 18) while the expected mass was observed for the *y* ions (C-terminal fragments) in the MS/MS spectrum (Figure 2B). Further investigations to understand this reaction are underway.

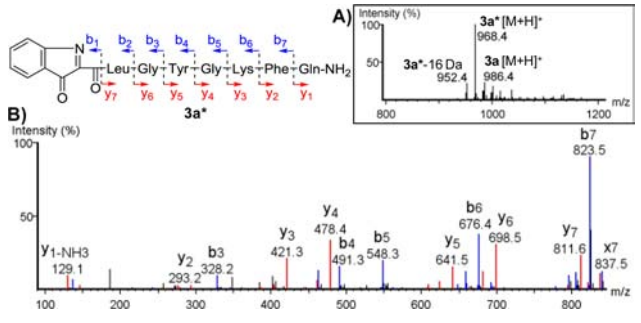


Figure 2. MALDI-TOF MS and MS/MS spectra obtained after dual ring opening/cleavage reaction on a single bead of cyclic peptide **4a**: (A) MS of the crude product; (B) MS/MS of the dehydrated species **3a*** (968.4 Da) $[\mathbf{3a} - 18 + H]^+$.

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To our knowledge, besides the rearrangement observed with *o*-nitrotyrosine,[14] such rearrangements or modifications of the *o*-nitrosobenzyl moiety in ANP have not been previously reported. Since ANP is most commonly used as a linker and its *o*-nitrobenzyl analogues as protecting groups, little effort has been made to understand what happens to the *o*-nitrosobenzyl derivative after UV irradiation and characterize the products. For this reason, the postcleavage modifications observed in this study are very attractive as they could be exploited to produce specific adducts to facilitate peptide sequencing. Moreover, a better understanding and control of postcleavage rearrangements or modifications of the *o*-nitrosobenzyl derivative could lead to the development of interesting synthetic approaches to access polysubstituted heterocycles.

To evaluate the efficiency of the simultaneous ring-opening/cleavage strategy, the model peptides **1a−f** were synthesized on TentaGel S NH_2 resin (130 μm) bearing the ANP as linker (Scheme 1). After cyclization and side-chain deprotection, a small amount of resin **4a−f** was subjected to UV irradiation and the released products analyzed by HPLC and ESI-MS. The results confirmed the presence of linear peptides **3′a−f** and showed high degrees of similarity with HPLC profiles and ESI-MS spectra obtained from the Rink resin pathway. Next, a single bead was picked up from resins **4a−f** and exposed to UV irradiation in MeOH. The crude products released from each single bead were immediately subjected to MALDI-TOF MS. In this case, the mass spectra showed very little MeOH adduct **3′** ($M + H^+ + 14$), but the dehydrated product **3*** ($M - H_2O + H^+$) was observed as the major peak (Figure 2A and Figure S6). These results suggest that the adduct product is modified into the dehydrated species when exposed to the laser under high vacuum during ionization in the MALDI instrument. With a wavelength of 355 nm, the ability of the MALDI's Nd:YAG laser to induce photochemical reaction has been shown[16] and used by some research groups for monitoring and structural elucidation of biomolecules.[17] More recently, Luyt's group used this "on-target" approach to sequence linear peptides on beads with a MALDI-TOF instrument equipped with a Nd:YAG laser.[18] In most of these cases, since the *o*-nitrobenzyl groups were used as linkers or protecting groups, only the peak corresponding to the desired molecule was analyzed and the residual photolabile group not characterized further.

In our study, the photosensitive residue remained attached to the peptide N-terminus, and a common pattern containing a series of three peaks was observed on most MS spectra. Formed by a **3** $[M + H]^+$ peak, a **3*** $[3 - 18 + H]^+$ peak, and a **3*** − 16 Da peak (Figure 2A and Figure S6), this signature can be very useful to identify the most efficient molecular ion for sequencing unknown peptides from OBOC libraries. Unfortunately, we were not able to structurally define the **3*** − 16 peak, but MS/MS spectra analyses of **3a−f** showed that this modification also occurs on the ANP residue after UV irradiation and exposition to the Nd:YAG laser. Further studies are underway to characterize this modification. Compared to data obtained by ESI-MS after dual photocleavage of peptide **4d**, a significant level of Met oxidation was observed in the MALDI MS spectrum (Figure S6). With the characteristic isotopic distribution of the sulfur atom, the presence of oxidized species in the MALDI MS spectrum can be considered as a good indicator for the presence of a Met residue in the sequence.

MS/MS analysis of the dehydrated product molecular ions yielded high-quality spectra from which the linearized peptides **3*a−f** could be unambiguously sequenced manually and by using de novo sequencing with the PEAKS software (Figure 2B and Figure S6).[19] In comparison, MS/MS spectra of most **3a−f** $[M + H]^+$ molecular ions could not be efficiently sequenced. The obtained complex fragmentation patterns for **3a−f** suggest that macrocyclic structural isomers **2a−f** remained in the mixture. The presence of a C-terminal spacer in the peptides was very helpful in the sequencing process. With a fixed known mass, it allowed us to initiate sequencing of *y* ions from 458.35 Da and avoid often missing low mass *y* ions. On the other hand, since the identity of the five C-terminal amino acids is known, we were able to properly initiate sequencing of the *b* ions from the precursor molecular ions.

To demonstrate the compatibility of our strategy with OBOC libraries, a small cyclic heptapeptide library was prepared on 100 mg of ANP TG resin bearing the spacer Leu-βAla-βAla-Lys-Gly. The library was prepared by split-and-pool synthesis using standard Fmoc/tBu solid-phase peptide chemistry.[4b,20] Following Glu(OAll), the next six positions within the peptide library were filled by a random combination of 17 L-amino acids (Cys, Gln, and Ile were excluded). Finally, Fmoc-D-ANP-OH was added and the peptide cyclized after selective deprotection as described above. After removal of the side-chain protecting groups, 15 beads were randomly selected and individually irradiated at 365 nm in MeOH for 3 h. The resulting crude peptides were analyzed by MALDI-TOF MS. For each selected bead, the resulting MS spectrum showed the three-peak signature observed for the model peptides with the dehydrated species as the most important peak (Figure 3A and Figure S7).

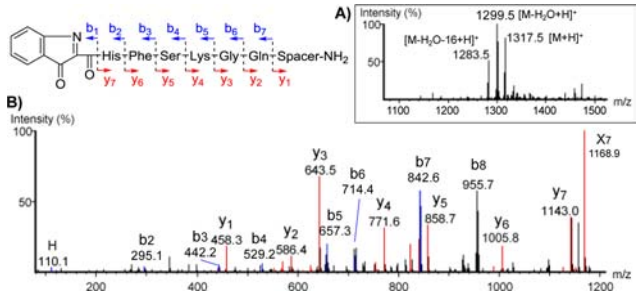

Figure 3. MS and MS/MS spectra of ANP*-HFSKGQLBBKG-NH_2 after dual ring opening/cleavage reaction on a randomly selected bead from the OBOC cyclic peptide library: (A) MS; (B) MS/MS for precursor ion *m*/*z* 1299.5 (B = β-alanine).

The peptides could be unambiguously sequenced by MS/MS of the dehydrated species by manual analysis and/or de novo sequencing with the PEAKS software (Figure 3B and Figure S7).[19] Some beads showed oxidized species in the MS spectrum suggesting the presence of Met in the sequence. In some cases, the oxidized $[M - 18]$ peak was successfully sequenced by MS/MS when Met and Trp oxidation were included as post-translational modification (PTM) during de novo sequencing with the PEAKS software (bead 3, Figure S7). These results demonstrate that the developed dual ring-opening/cleavage strategy is compatible with the side chains of commonly used amino acids and can be used on a single bead to release linear peptides that can be clearly and conclusively sequenced by MS/MS. In addition to not having to use aggressive chemical reagents for ring opening and cleavage, another advantage of the developed approach over some other reported strategies is the presence of a spacer sequence on the released linearized peptide. This spacer facilitates MS/MS spectra analysis since its

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composition is known and constitutes a reliable starting point for sequencing. It can also be exploited to increase the molecular mass and improve ionization of *y* ions by adding positively charged residues in the spacer. The procedure was performed on a freshly prepared library, but it is strongly recommended to protect the library from light during synthesis and handling to prevent opening of the macrocycle or cleavage from the resin. It is also important to consider that hit compounds identified after OBOC library screening will contain an ANP residue within the macrocycle. Therefore, since this photolabile monomer can have an impact on the stability of hit macrocyles in bioassays, the ANP could be replaced by a photostable bioisostere such as 3-amino-3-(2-cyanophenyl)propionic acid (ACP) when hit compounds are resynthesized to avoid ring-opening during binding and biological assays.

In summary, we report the use of a photolabile residue within a macrocyclic peptide and as a linker to allow a one-pot ring-opening/cleavage reaction upon UV irradiation and provide linearized peptides that can be efficiently sequenced by MS/MS. While the ANP linker is well known in combinatorial chemistry, we demonstrated that the generated *o*-nitrosobenzyl product during UV irradiation undergoes a rearrangement leading to an indolin-3-one moiety and adducts that can be used for sequencing. Compatible with commonly used amino acids, the described approach avoids the use of harsh chemical reagents and postscreening reactions to produce a linearized molecule and allows a fast sequence determination of cyclic peptides from OBOC combinatorial libraries by MS/MS. Simple and affordable for any peptide science or combinatorial chemistry laboratory, the described dual ring-opening/cleavage strategy will be useful for the preparation and screening of OBOC macrocyclic peptide libraries.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00296.

Detailed experimental procedures and characterization data for all compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: eric.biron@pha.ulaval.ca.

Author Contributions

§X.L. and S.V.-D. contributed equally.

Notes

The authors declare no competing financial interest.

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