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Practical Ring-Opening Strategy for the Sequence Determination of Cyclic Peptides from One-Bead-One-Compound Libraries

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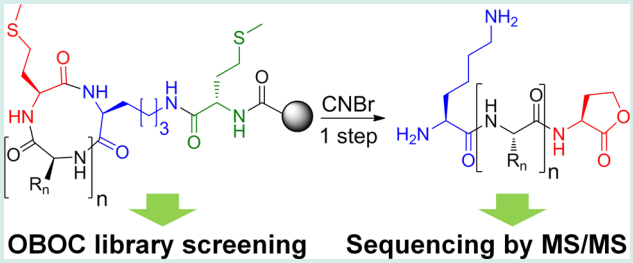
Supporting Information

ABSTRACT: The use of cyclic peptides in one-bead-one-compound libraries is limited by difficulties in sequencing hit compounds. Lacking a free N-terminal amine, such peptides cannot be sequenced by the Edman degradation approach, and complex fragmentation patterns are obtained by tandem mass spectrometry. To overcome this problem, we designed an alternative approach introducing a methionine residue within the macrocycle and as a linker to allow simultaneous ring-opening and release from the resin upon treatment with cyanogen bromide. The methionine linker was inverted relative to the peptide chain to allow the synthesis of cyclic peptides anchored by a lysine side chain and to avoid the presence of two C-terminal homoserine lactones on the released linear peptides. After MALDI-TOF MS/MS analysis, the peptides released from a single bead were sequenced manually and with a de novo sequencing software. The strategy described herein is compatible with commonly used amino acids and allows sequencing of cyclic peptides in one-bead-one-compound libraries, thus reducing the need for encoding.



KEYWORDS: *cyclic peptide libraries, one-bead-one-compound libraries, peptide macrocycles, ring-opening, peptide sequencing*

Combinatorial chemistry has become a powerful tool for discovering potent and selective bioactive compounds for therapeutic and diagnostic applications, and is now an important component of the drug discovery process. Among the different combinatorial methodologies used to prepare and screen large peptide libraries, the one-bead-one-compound (OBOC) approach is one of the most accessible and economical to discover new hits against a target of interest.[1−4]

The OBOC approach exploits the split-and-mix method to generate combinatorial libraries in which each bead displays many copies of a unique chemical entity.[5−7] OBOC libraries containing thousands to millions of different compounds can be readily synthesized and screened simultaneously on-bead against a target of interest. Such libraries have been successfully used to discover ligands and modulators for a wide variety of macromolecular targets.[2,3,8−12]

Cyclic peptides represent an important class of privileged structures, and they have gained a lot of interest in drug discovery.[13,14] Compared to their linear counterparts, cyclic peptides show a greater stability against proteases and a higher selectivity because of their increased conformational rigidity.[14,15] The great degree of molecular complexity and diversity that can be accessed by simple changes in their sequence has prompted the use of cyclic peptides in combinatorial libraries. However, the use of cyclic peptides in OBOC libraries has been limited by difficulties in sequencing hit compounds after screening. Since they lack a free N-terminal amine, such peptides cannot be sequenced via Edman degradation, and yield excessively complex fragmentation patterns with tandem mass spectrometry (MS/MS).[16] Structural elucidation of the selected compounds is a critical step and in this regard, different encoding strategies have been developed for OBOC libraries. The ladder synthesis, in which a small fraction of the peptides are N-terminally capped at each coupling cycle during peptide synthesis before the final cyclization, could be considered an efficient method.[17] However, the latter approach has the disadvantage of displaying the ladder peptides on the bead surface together with the cyclic peptide, thereby causing interference by the coding tags during screening.[8,18] To circumvent this problem, Pei and co-workers used a one-bead-two-compound (OBTC) approach relying on topologically segregated bilayer beads.[19] This strategy offers the opportunity to expose the cyclic peptide on the bead surface for screening while its linear counterpart for sequencing purposes is found inside the bead.[4,12,20−22] The main disadvantage of this approach is that the cyclization step cannot be monitored because of the presence of the linear peptide tag.

More recently, a strategy based on the "reopening" of the macrocycle after the screening step has emerged to eliminate the need for encoding OBOC libraries.[23,24] In this approach, a cleavable residue is introduced in the cycle backbone to allow

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linearization of the molecule under specific conditions and sequencing of the linear variant by MS/MS. Lim and co-workers reported a ring-opening approach involving the introduction of a cleavable alkylthioaryl bridge in peptoid macrocycles to allow linearization of the molecule by oxidation of the thioether, followed by nucleophilic displacement of the sulfone.[23] Using a similar strategy, Simpson and Kodadek inserted a methionyl residue within a peptoid macrocycle and as a linker to allow the simultaneous linearization and release of the compound from the bead upon treatment with CNBr.[24] A major asset of the ring-opening strategy is that, in contrast to OBTC and ladder synthesis methods, the same chemical entity is displayed inside and on the surface of the bead, eliminating the risk of interference by the coding tags during the screening. Moreover, the cyclization step can be easily monitored to ensure a complete conversion of the linear variant. On the basis of these elegant strategies with peptoids, we looked for an efficient and single step approach compatible with free amino acid side chains and that would allow simultaneous ring-opening of the cyclic peptides and cleavage from the resin. Herein, we report an alternative, straightforward version of the ring-opening approach for a fast and simple sequence determination of cyclic peptides from OBOC libraries.

The cleavable residue plays a critical role in the ring-opening approach and among the different linkers and cleavable residues that are readily available, we were particularly interested in methionine (Met). As any amino acid, Met can be used in standard solid-phase peptide synthesis and its impact on peptide conformation will be limited compared to extended aromatic residues and linkers. Moreover, Met is stable in the acidic, basic or reductive conditions commonly used to remove side chain protecting groups. Finally, the reaction conditions used to cleave Met residues are selective and compatible with other free amino acid side chains. Indeed, Met has been widely used as a linker in OBOC peptide libraries and can be selectively cleaved upon treatment with CNBr to yield a C-terminal homoserine lactone.

Our initial strategy to prepare OBOC cyclic peptides libraries was to use a solid supported Met bearing a side chain anchored Fmoc-Glu-OAll and to perform head-to-tail cyclization after peptide synthesis and selective deprotection of the carboxylic acid. In the ring-opening strategy, the Met residue can be introduced at two different positions in the macrocycle to allow its linearization. The Met residue can be incorporated either as the first residue following the side chain anchored Glu or as the last amino acid of the chain before cyclization (Figure 1). Depending on the Met position, the pattern of the linearized peptides thus yielded will be completely different. First, when Met is coupled directly to the Glu anchor before library synthesis, a linear peptide **1a** with two C-termini each bearing a homoserine lactone is obtained after cleavage with CNBr. Unfortunately, we observed that linear peptides with such a pattern can entail problematic sequencing even when their amino acid composition is known. Next, when Met is coupled as the last residue during peptide elongation prior to cyclization, a linear peptide **1b** with a C-terminus bearing two homoserine lactones is generated. In this case, as observed by Simpson and Kodadek,[24] the cyclization efficiency was moderate and complete ring formation could not be obtained when Met was the N-terminal amino acid. The latter behavior was not observed using the former strategy. Moreover, a linear peptide bearing two homoserine lactones was also generated.

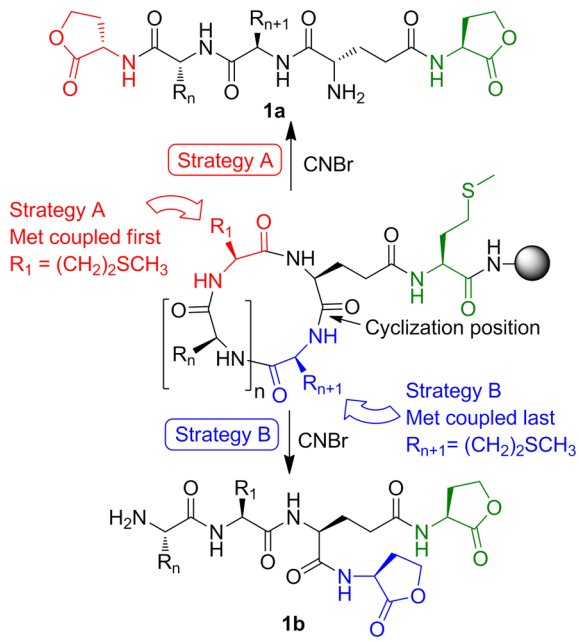

Figure 1. Incorporation of a Met residue in the macrocycle at different positions and the corresponding linear peptide generated after ring reopening and cleavage from the resin.

To circumvent the problems associated with the presence of two homoserine lactone residues for the sequencing of the linearized peptide and the moderate cyclization efficiency obtained when Met is at the N-terminal position, an alternative approach was designed. Our strategy involved the inversion of the Met handle in order to eliminate one of the homoserine lactones in the linearized peptide combined with the introduction of the cleavable Met residue at the C-terminal position (Figure 2). Initially reported by Kappel and Barany for the synthesis of lysine-containing head-to-tail cyclic peptides,[25] inversion of the Met handle allows the release of a free amino group while the homoserine lactone remains attached to the resin. Therefore, the Fmoc-Glu-OAll anchor was replaced by Fmoc-Lys-OAll as a side chain anchored amino acid to allow on-resin peptide cyclization after standard solid-phase peptide synthesis and appropriate deprotection (Figure 2). The introduction of a Met residue directly next to the side chain anchored Lys in the cyclic peptide allows simultaneous ring-opening and peptide cleavage from the resin upon treatment with CNBr, yielding a linear peptide bearing a single C-terminal homoserine lactone and a free N-terminal lysine. The invariable residues at the C- and N-termini can be used as starting points in MS/MS spectrum analysis, thereby significantly helping the sequencing process. Moreover, because of its positive charge, the lysine residue facilitates ionization of the peptide during MALDI MS analyses.

To evaluate the efficiency of the proposed strategy, four model peptides of different size were prepared on Rink Amide AM resin and TentaGel S-NH2 (TG) resin bearing an inverted Met handle **2** (Figure 2). The latter were prepared by treatment of the amino functionalized resins with succinic anhydride in the presence of DIPEA followed by coupling of H-Met-OFm with HATU on the pending carboxyl sites. After removal of the 9-fluorenylmethyl ester with piperidine in DMF, Fmoc-Lys-OAll was anchored via its side chain to the C_α-carboxyl of the resin-bound Met residue with HATU to yield resins **3**. The loadings were determined by quantification of the Fmoc group

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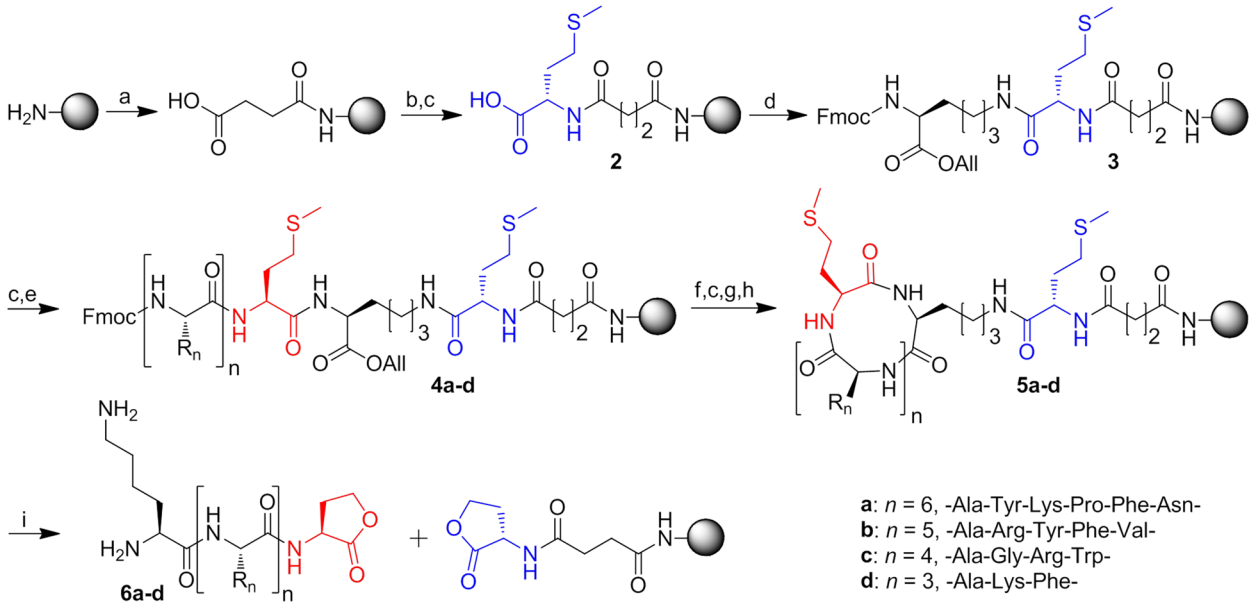

Figure 2. Design and synthesis of cyclic peptides for the tandem ring-opening/cleavage approach. Reagents and conditions: (a) succinic anhydride, DIPEA, DMF; (b) H-Met-OFm, HATU, DIPEA, DMF; (c) 20% piperidine/DMF; (d) Fmoc-Lys-OAll, HATU, DIPEA, DMF; (e) standard Fmoc solid-phase peptide chemistry with HCTU; (f) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2; (g) PyBOP, HOBt, DIPEA, DMF; (h) TFA/H_2O/TIS (95:2.5:2.5); (i) CNBr, CH_3CN/AcOH/H_2O (5:4:1).

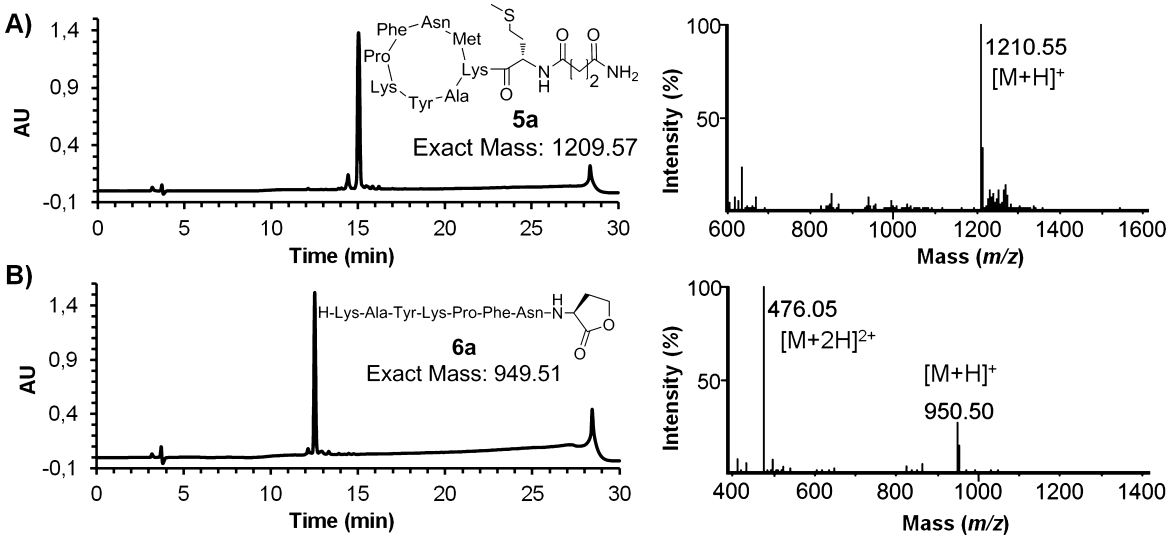

Figure 3. HPLC profiles (λ = 220 nm) and ESI-MS spectra of crude model peptides. (A) cyclo[AYKPFNMK(M-succinamide)] **5a** and (B) H_2N-KAYKPFNh* **6a** after tandem ring-opening/cleavage from TG resin. (h* = homoserine lactone).

with the UV method and found to be 0.43 mmol/g and 0.22 mmol/g for the Rink Amide AM and TG resins, respectively.[26] After Fmoc group removal with piperidine in DMF, Fmoc-Met-OH was coupled to the free amino group and the peptides were assembled by standard Fmoc solid-phase synthesis with HCTU as coupling reagent. Afterward, the C-terminal allyl ester of the Lys anchor was selectively cleaved with $Pd(PPh_3)_4$ followed by removal of the N-terminal Fmoc group. Head-to-tail peptide cyclization was performed with PyBOP, and progress of the reaction monitored using the chloranil test.[27] To confirm the presence of cyclic peptides, the Rink Amide AM resin was treated with a TFA cocktail to afford the fully deprotected cyclic peptides **5a**–**d** in solution. HPLC and MS analyses confirmed transformation of the linear peptides into their cyclic counterparts (Figure 3a and Supporting Information, Figure S5). Next, the tandem ring-opening/cleavage reaction was performed on the TG resin bearing the side chain deprotected cyclic peptides by treatment with a solution of CNBr in CH_3CN/AcOH/H_2O. The compounds thus released were analyzed by HPLC and MS, which showed that linear peptides **6a**–**d** have been generated, suggesting that the tandem reaction proceeded successfully (Figure 3b and Supporting Information, Figure S6).

To validate the efficiency of the approach in a one-bead-one-compound context, a single bead was picked up from the TG resins **5a**–**d** and treated with the CNBr solution. The crude products released from each single bead were immediately subjected to MALDI-TOF MS. The mass spectra of linearized peptides **6a**–**d** typically showed the expected molecular ion as the major peak and a sodium adduct at +22 Da (Figure 4 and

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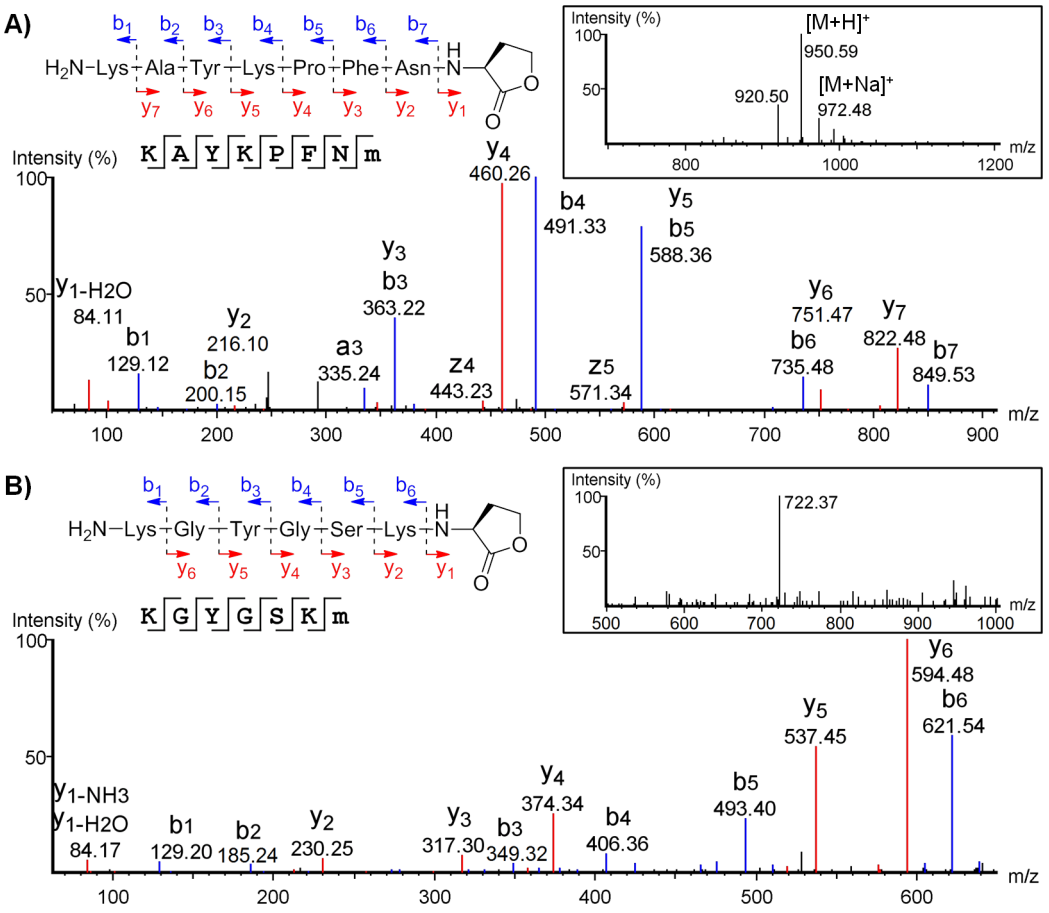

Figure 4. MALDI MS and MS/MS spectra of peptides after tandem ring-opening/cleavage on a single bead. (A) H_2N-KAYKPFNh* **6a**, MS/MS for precursor ion *m/z* 950.59. (B) H_2N-KGYGSKh* released from a bead randomly selected from the OBOC cyclic heptapeptide library, MS/MS for precursor ion *m/z* 722.37. (h* = homoserine lactone).

Supporting Information, Figure S7). In the case of peptide **6a**, a second unknown minor peak at −30 Da was observed. With peptide **6c**, a second minor peak at +16 Da showed oxidation of the Trp residue. MS/MS analysis of the molecular ions yielded high-quality spectra from which the linearized peptides **6a**–**d** could be sequenced manually and also by using de novo sequencing with the Peaks software (Figure 4a and Supporting Information, Figure S7).[28]

To demonstrate the compatibility of our strategy with OBOC libraries, a small cyclic heptapeptide library was prepared on 100 mg of TG resin bearing a side chain anchored Fmoc-Lys-OAll **3**. After coupling Fmoc-Met-OH to the resin, the library was prepared by split-and-pool synthesis using standard Fmoc/tBu solid-phase peptide chemistry. The next four positions within the peptide library are filled by a random combination of sixteen L-amino-acids. Met and Cys were excluded for reactivity reasons while Gln and Ile, which are isobaric with Lys and Leu, respectively, were not used for the sake of simplicity at the sequencing step. The penultimate position (i.e., relative to the Lys anchor found at the NH_2 end of the linearized heptapeptide) was randomly filled by either of three aliphatic L-amino acids with the least hindrance (namely, G, A, and L) to promote cyclization.[29] Following peptide cyclization and side chain deprotection as described above, five beads were randomly selected and individually treated with the CNBr solution in CH_3CN/AcOH/H_2O. The resulting crude peptides were analyzed by tandem MALDI-TOF MS. For each selected bead, the resulting MS spectrum showed the presence of peptides that could be unambiguously sequenced by MS/MS of the most important peak by manual analysis or de novo sequencing with the Peaks software (Figure 4b and Supporting Information, Figure S8). The procedure was performed on a freshly prepared library and no notable oxidation byproducts were observed. Nevertheless, inadvertent oxidation of the methionine residues may happen during storage, manipulation or screening and, in addition to significantly decreasing the yields, might prevent opening of the macrocycle. To avoid this problem, it is strongly suggested to treat the cyclic peptide library with a reducing solution to reduce any oxidized methionine residues prior to the tandem ring-opening/cleavage reaction.[12]

In conclusion, a straightforward and effective alternative approach was developed to allow sequence determination of cyclic peptides from OBOC combinatorial libraries. The results from this work demonstrate that the tandem ring-opening/cleavage strategy developed herein is compatible with 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. The procedure described herein for the synthesis of the Met handle and the preparation of unencoded cyclic peptide libraries is simple and affordable for any peptide science or combinatorial chemistry laboratory.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, compounds characterization including NMR spectra, HPLC profiles, mass spectra and peptide sequencing results by MALDI-TOF MS/MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DIPEA, *N*,*N*-diisopropylethylamne; ESI, electrospay ionization; HATU, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCTU, 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MS, mass spectrometry; NMM, *N*-methylmorpholine; OBOC, one-bead-one-compound; OBTC, one-bead-two-compound; PyBOP, benzotriazol-1-yl-oxy-tris(pyrrilidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TG, TentaGel

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