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Friendly Strategy to Prepare Encoded One Bead−One Compound Cyclic Peptide Library

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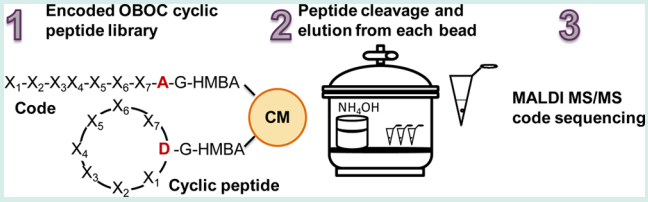
Supporting Information

ABSTRACT: One bead−one peptide libraries allow the screening of suitable ligands for any target protein. Short cyclic peptides are ideal ligands for affinity chromatography because of their high affinity and selectivity for the target protein and stability against proteases. We designed a library synthesis strategy to facilitate the identification of cyclic peptides by MS consisting of (a) sequential incorporation of a mixture of Fmoc-Ala-OH and Fmoc-Asp[2-phenylisopropyl (OPp)]-OH (15:85) to Gly-oxymethylbenzamide-ChemMatrix (Gly-HMBA-CM) resin, (b) synthesis of the combinatorial library on the resin by the divide−couple−recombine method, (c) removal of OPp with 4% TFA, (d) peptide cyclization on solid phase through side-chain Asp and amino terminus, and (e) removal of side chain protecting groups with a 95% TFA cocktail. Peptides were cleaved from the beads with ammonia and the linear code was sequenced by MALDI-TOF MS/MS. The high capacity of ChemMatrix resin together with the sensitivity of MS allows code sequencing from a single bead.



KEYWORDS: *combinatorial chemistry, MALDI-TOF MS/MS, peptide sequencing, affinity, solid phase, ChemMatrix resin, 2-phenylisopropyl, 4-hydroxymethylbenzoic acid, solid-phase synthesis*

Small peptides consisting of a few amino acids represent promising affinity ligands candidates for industrial separations by affinity chromatography. Peptide ligands are much more physically and chemically stable and resistant to proteolytic cleavage than antibodies. They can be readily synthesized by standard chemistry in bulk amounts at a rather low cost under good manufacturing practices. Furthermore, peptides allow site-directed immobilization and high ligand density and the matrices are more robust during elution and regeneration than protein-based affinity matrices such as monoclonal antibodies.[1,2] Particularly, cyclic peptides have higher selectivity and resistance to enzymatic degradation than linear peptides because of their structural constraint which reduces the conformational flexibility, thus potentiating their use as affinity ligands for protein purification.[3]

The application of combinatorial peptide synthesis strategies greatly facilitates the discovery of suitable ligands for any given protein of interest. Among the solid-phase strategies available for peptide library preparation, the divide−couple−recombine (DCR), also known as the split-and-mix method, is the most advantageous.[4,5] This method involves repeated cycles of the following sequence: (i) dividing the solid support (resin beads) into equal portions, (ii) coupling each portion individually with a different amino acid (building block), and (iii) mixing the portions. This procedure assures a theoretically even representation of the library members and a "one bead−one peptide" distribution. To screen these combinatorial libraries, tens of thousands to millions of compound beads are first mixed with the probe molecule. The beads that interact with it will be identified and isolated for peptide structure determination.

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The high sensitivity and mass accuracy of mass spectrometry (MS), together with the speed of the analysis and the large amount of information generated in each experiment made it a technique of choice for peptide sequencing.[6] Although linear peptides may be readily sequenced by MS, cyclic peptides typically yield complex fragment ion mass spectra, arising from ring-opening at multiple positions to afford mass degenerate ions, which undergo subsequent loss of residues.[7] One-bead-two-peptides libraries in which each bead contains both the cyclic peptide and its linear counterpart as a coding tag facilitate MS analysis. For peptide identification by MS a cleavage site to release the peptides from the beads is required. The cyclic peptide as well as its linear counterpart anchored on each bead are released and analyzed together by MS. In current approaches, methionine is commonly used as linker.[8−14] The main drawback is that the selective cleavage of the peptides is performed with CNBr, which is a very hazardous reactive, especially when working with hundreds of individual beads selected from the screening process.[15] We have previously proposed the use of 4-hydroxymethylbenzoic acid (HMBA) linker[16] coupled to ChemMatrix (CM) resin for one-bead-one-peptide library synthesis[17] and developed a suitable cleavage method to release the peptide from the linker HMBA by vapor phase ammonolysis using NH_4OH 30%, which is much more economic and safer than CNBr. The method allows MS analysis from each single bead isolated after one-bead-one-peptide library screening.[18]

The aim of this work was to design a new and friendlier strategy to prepare beads with homodetic cyclic peptides encoded with a minimum proportion of a linear analogue of the sequence suitable for one-bead-one-peptide libraries. The method was based on the proper combination of the solid support, the linker for resin-peptide anchorage and protecting groups for Nα and side-chain protection.[19]

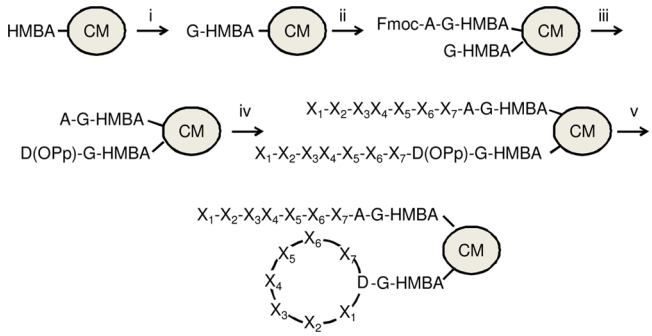
CM was used as solid support and HMBA as linker. CM[20] is a highly cross-linked, amphiphilic resin composed entirely of polyethyleneglycol monomers that contain exclusively primary ether bonds. These bonds facilitate high resin loading, as compared to polystyrene resins, and high chemical and mechanical stability. CM is compatible with both organic and aqueous solvents, which are used for the peptide synthesis and for the screening step respectively, and therefore it is highly suitable for the whole process. The benzyl ester, which forms the HMBA linker with the first amino acid, is stable to piperidine, used for Fmoc group removal, making the linkage applicable for chemical elongation of a peptide sequence with Fmoc strategies. The HMBA inertness to trifluoroacetic acid (TFA) allows side-chain deprotection without releasing the peptide from the resin. The removal of protecting groups is required since screening should be carried out on fully unprotected peptides. At the C-terminal, Gly was incorporated with the aim of increasing the molecular weight of the final peptide in order to facilitate its identification by MS and overcoming the poor cleavage efficiency of Ile and Val esters.[21]

On-resin synthesis of lactam-bridge via cyclization of the amino function of the N-terminal with the side-chain carboxylic acid function of Asp/Glu requires the selective removal of the carboxylic acid protecting groups in the presence of all other permanent protection. Many side-chain protecting groups for the Asp/Glu residues that are orthogonal to other side-chain protecting groups are used such as 2,4-dimethoxybenzyl (Dmb),[22] trimethylsilylethyl (OTmse),[23] allyl,[24] 4-[*N*-(l-(4,4-dimethyl-2,6 dioxocyclohexylidene)-3-methylbutyl)amino]-benzyl (ODmab),[25] and 2-phenylisopropyl (OPp)[26] esters. Once the protecting groups have been removed, the activation of the carboxyl group and subsequent intramolecular aminolysis allows the formation of a lactam bridged cyclic peptide.

In the present approach, Fmoc-Asp(OPp)-OH was added to the peptide sequence for solid phase ring formation. The semipermanent group OPp was cleaved with 4% TFA[26] and an amide linkage was formed between the amino terminus and carboxylic acid function to give homodetic cyclic peptides. Most permanent protecting groups commonly used in solid phase peptide synthesis are stable to 4% TFA. However, these conditions are sufficient to remove the side-chain trityl (Trt) protection of His residue. Thereafter, in the case of using His in the peptide library, Fmoc-His(Boc)-OH must be used instead of Fmoc-His(Trt)-OH. 4-[*N*-(l-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)amino]-benzyl (ODmab), commonly used as a semipermanent protecting group for Asp in solid phase cyclization, cannot be used in the present approach because its cleavage is achieved with hydrazine, reactive which also removes the peptides from the HMBA-CM resin.[27] Although the allyl ester is very often used as orthogonal carboxyl protecting group, it was not selected in the present approach because its removal requires the use of a complex, hazardous and air sensitive cleavage mixture containing Pd(0),[24] which precludes the use of the allyl in those laboratories that are not organic strictly oriented.

To prepare a peptide code together with the cyclic peptide, a minor proportion of Fmoc-Ala-OH was incorporated instead of Fmoc-Asp(OPp)-OH. For the purpose of reducing interference during the screening step because of the interaction of the linear peptides with the target, the minimum proportion of the linear code necessary for its MS analysis was synthesized. When adding 0.2 equiv of Fmoc-Ala-OH to the resin previous to Fmoc-Asp(OPp)-OH coupling, only 0.15 equiv of Fmoc-Ala-OH was incorporated as was measured by Fmoc analysis. Then, Fmoc-Asp(OPp)-OH was coupled to the remaining amino functions (Scheme 1).

Scheme 1. Synthetic Strategy for the Preparation of the Cyclic Library with a Coding Tag[a]



[a]Reagents and conditions: (i) (a) Fmoc-Gly-OH (3 equiv), DIPCDI (4 equiv), DMAP (0.1 equiv) in DMF (two times), (b) piperidine/DMF (1/4), (c) DMF; (ii) (a) Fmoc-Ala-OH (0.2 equiv), TBTU (0.2 equiv), DIPEA (0.4 equiv) in DMF, 30 min, (b) DMF; (iii) (a) Fmoc-Asp(OPp)-OH (3 equiv), TBTU (3 equiv), DIPEA (6 equiv), (b) piperidine/DMF (1/4), (c) DMF; (iv) (a) library preparation (divide−couple−recombine); (v) (a) selective OPp cleavage with TFA/CH_2Cl_2 (4/96), (b) cyclization PyBOP (4 equiv), HOAT (4 equiv), DIPEA (8 equiv), (c) side-chain deprotection with TFA/TIS/H_2O (95/2.5/2.5).

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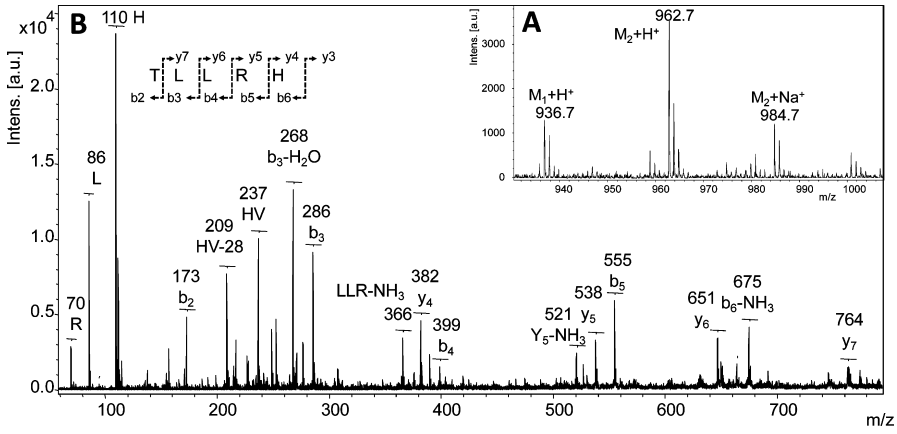

Figure 1. (A) MALDI mass spectrum of the peptides H-ATLLRHVAG-NH_2 ($[M_1 + H]^+$, m/z = 936) and cyclo-(1,8)-ATLLRHVDG-NH_2 ($[M_2 + H]^+$, m/z = 962) from the library cyclo-(1,8)-$AX_1X_2X_3X_4X_5VDG$, with the corresponding linear code $AX_1X_2X_3X_4X_5VAG$. One bead was placed into a micro tube, which was placed in a drying chamber together with a flask containing NH_4OH. Cleaved peptides were eluted with 10 μL of AcOH/CH_3CN/H_2O (3/4/3) and 1 μL of sample was loaded onto the sample plate, air-dried at room temperature and then 1 μL of matrix solution was added to the spot without mixing. (B) MALDI MS/MS mass spectrum of the linear peptide code H-ATLLRHVAG-NH_2. To prepare a peptide code together with the cyclic peptide a minor proportion of Fmoc-Ala-OH was incorporated instead of Fmoc-Asp(OPp)-OH by adding 0.2 equiv of Fmoc-Ala-OH to the resin previous to Fmoc-Asp(OPp) coupling.

Peptides bounded through HMBA to the resin beads can be released with rather strong nucleophiles. CM contains exclusively primary ether bonds and so is stable to nucleophilic conditions. The ammonia vapor has the advantage over other nucleophiles such as NaOH that it is easily removed by evaporation, thus avoiding the presence of contaminants that could interfere with the MS peptide analysis.

The small proportion of the code (noncycled Ala containing peptide) was enough for MS analysis due to the high sensitivity of the MS technique together with the high loadings of hydroxymethylbenzamide-ChemMatrix (HMBA-CM) resin (0.63 mmol/g substitution). High quality mass spectra were obtained. Peptide signals were high and the cyclic peptide sequence could be deduced from the tandem mass spectra of the linear peptide. Figure 1 shows the resulting matrix-assisted laser desorption/ionization (MALDI) mass spectrum and MALDI tandem mass spectrum obtained by adding 1 μL of the eluted peptides H-ATLLRHVAG-NH_2 and cyclo-(1,8)-ATLLRHVDG-NH_2 from a single bead. (Others MS spectra are showed in Supporting Information).

To prepare the linear code peptide, Fmoc-Asp(OtBu)-OH could have been used instead of Fmoc-Ala-OH but the last one was cheaper. Moreover, when preparing a cyclic peptide library of tens of thousands to millions of compounds, the yield of cyclization of some minor sequences may be incomplete. If Fmoc-Asp(OtBu)-OH is used for the code, it is not possible to be aware of incomplete cyclization of the peptide-bead analyzed because both peptides, the linear code from Asp(OtBu) and the uncyclized from Asp(OPp)-OH, will be the same peptide and it is well-known that MS is not a quantitative method. Besides, when using Fmoc-Ala-OH together with Fmoc-Asp(OPp)-OH, incomplete cyclization can be clearly visualized with MS because there will be three different signals corresponding to the cyclic peptide, its linear counterpart and its linear code clearly identified by MS (Figure 2).

In conclusion, a new strategy was developed suitable for the preparation of one-bead-one-cyclic peptide libraries encoded with a linear analogue to facilitate MS identification (Scheme 1). This strategy uses techniques and reagents frequently used for all peptide laboratories and therefore it is friendlier than other current strategies.

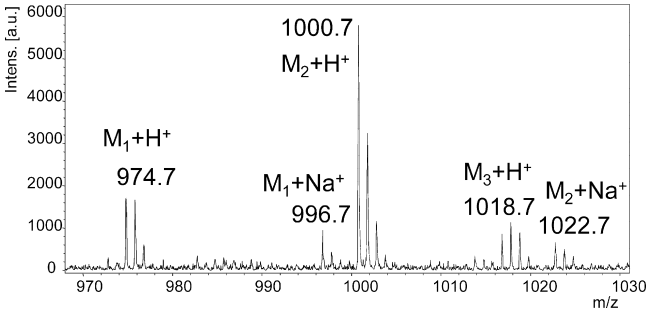

Figure 2. MALDI mass spectrum of the peptides H-AYHTTRVAG-NH_2 ($[M_1 + H]+$, m/z = 974) and cyclo-(1,8)-AYHTTRVDG-NH_2 ($[M_2 + H]+$, m/z = 1000) from the library cyclo-(1,8)-$AX_1X_2X_3X_4X_5VDG$, with the corresponding linear code $AX_1X_2X_3X_4X_5VAG$. A small signal corresponding to the linear peptide H-AYHTTRVDG-NH_2 ($[M_3 + H]+$, m/z = 1018) is also observed.

EXPERIMENTAL PROCEDURES

Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction.

Encoded Combinatorial Peptide Library Synthesis. One bead−one peptide combinatorial library of the non-apeptide cyclo-(1,8)-$AX_1X_2X_3X_4X_5VDG$, with the corresponding linear code $AX_1X_2X_3X_4X_5VAG$ where X = Arg, His, Leu, Thr, Tyr, was synthesized on the HMBA-ChemMatrix resin by using the divide-couple-recombine method according to Lam et al.[5] with Fmoc chemistry. The library had 3125 different peptides (5^5).

HMBA-CM (35−100 mesh-wet sieved, 100−200 mesh-dry, 0.63 mmol/g) was washed before use as follows: 1 N HCl (5 × 1 min), H_2O (5 × 1 min), CH_3OH (5 × 1 min), CH_2Cl_2 (5 × 1 min), *N*,*N*-dimethylformamide (DMF) (5 × 1 min). At the C-terminal, Gly was incorporated by adding fluorenylmethoxycarbonyl (Fmoc)-Gly-OH (3 equiv) with 1,3-diisopropylcar-

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bodiimide (DIPCDI) (4 equiv), followed by dropwise addition of a solution of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) dissolved in DMF (0.1 equiv, ~50 mmol/L) as described by Mellor et al.[27] After Fmoc removal with piperidine/DMF (1/4), a minimum amount of Ala was coupled to the resin by adding 0.2 equiv of Fmoc-Ala-OH and *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (TBTU) (0.2 equiv) and *N*,*N*-diisopropylethylamine (DIPEA) (0.4 equiv) in DMF. To ensure a good homogenization, the active amino acid solution (mixture of Fmoc-Ala-OH and TBTU) was added dropwise and agitated for 15 min and then DIPEA was added dropwise while stirring the suspension. After mixing during 30 min, the resin was washed with DMF (5 × 1 min) and then Fmoc-Asp(OPp)-OH (3 equiv) was added in the presence of TBTU (3 equiv) and DIPEA (6 equiv) in DMF. After Fmoc removal with piperidine/DMF (1/4), Val was coupled with TBTU/DIPEA as previously described.[28] Random positions were synthesized by repeated cycles of dividing, coupling, and recombining. Each portion was coupled with 3 equiv of a different Fmoc-amino acid/TBTU/DIPEA for 2 h. After the five random positions were synthesized, an Ala was added to the N-terminus of all peptides to facilitate the cyclization reaction. Fmoc removal was achieved with piperidine/DMF (1/4). Washings between deprotection, coupling, and subsequent deprotection steps were accomplished with DMF and CH_2Cl_2. Following elongation completion, Fmoc in Nα amino terminus was removed and OPp cleavage was conducted with TFA/CH_2Cl_2 (4/96) (3 × 15 min). The resin was washed with DMF (5 × 1 min), suspended in DMF and allow to swell during 1 h. Peptide cyclization was performed on solid phase support with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (4 equiv), 1-hydroxy-7-azabenzotriazole (HOAt) (4 equiv), and DIPEA (8 equiv) in DMF (2 × 4 h). To monitor the progress of the cyclization a small amount of resin was treated with TFA/TIS (triisopropylsilane)/H_2O(95/2.5/2.5) during 4 h and peptides from single beads were cleaved and analyzed by MALDI MS. Finally, side-chain permanent protecting groups were removed by treating all the resin with TFA/TIS/H_2O (95/2.5/2.5) during 4 h, leaving the unprotected peptides anchored to the resin.

Fmoc Quantification. Fmoc was quantified to determine resin substitution after Ala addition and then after Asp addition. An aliquot of 100 mg of resin to be analyzed was suspended in 40 mL of DMF and stirred during 30 min. Then 0.8 mL of 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) were added, and this mixture was stirred for another 30 min. An aliquot of the supernatant was diluted 1/25 with CH_3CN to measure its absorbance at 304 nm (extinction coefficients = 7624 M^{-1} cm^{-1}). The reference solution was prepared in the same way but without resin.[29]

Peptide Cleavage and Elution from Each Bead for MALDI MS Analysis. Peptides were cleaved from each bead by vapor phase ammonolysis. Single peptide-beads were placed into separate microtubes, which were placed in a drying chamber together with a flask containing NH_4OH 30%. The drying chamber was clamped shut and left to stand overnight at room temperature. Released peptides were eluted from each bead by adding 10 μL acetic acid (AcOH)/ acetonitrile (CH_3CN)/H_2O (3/4/3) to each micro tube. Elution was performed overnight.[18]

MALDI-TOF MS Analysis. MALDI-TOF MS were recorded in an Ultraflex II TOF/TOF (Bruker Daltonics GmbH, Leipzig, Germany). The analysis was performed as per Martínez-Ceron et al.[18] A 1 μL portion of eluted peptide from a single bead was loaded onto the sample plate, air-dried at room temperature, and then 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) 4 mg/mL in CH_3CN/H_2O (1/1) with 0.1% TFA was added on the sample dry layer (successive-dry-layers deposit method). Mass spectra were acquired in the MS reflector positive-ion mode. Tandem mass spectra were obtained using the MS/MS positive acquisition method.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MALDI-TOF mass spectra and MALDI-TOF/TOF MS/MS mass spectra obtained from eluted peptides from single beads. 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

AcOH, acetic acid; CHCA, α-cyano-4-hydroxycinnamic acid; CM, ChemMatrix; DBU, 1,8-diazobicyclo[5.4.0]undec-7-ene; DCR, divide-couple-recombine; DIPCDI, 1,3-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; Gly-HMBA-CM, Gly-oxymethylbenzamide-ChemMatrix; HMBA, 4-hydroxymethylbenzoic acid; HMBA-CM, hydroxymethylbenzamide-ChemMatrix resin; HOAt, hydroxy-7-azabenzotriazole; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; ODamb, 4-[*N*-(l-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)amino]-benzyl; OPp, 2-phenylisopropyl; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; TBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl

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