Combinatorial Library Screening Coupled to Mass Spectrometry to Identify Valuable Cyclic Peptides

Silvia A. Camperi,[1,2] Silvana L. Giudicessi,[1,2] María C. Martínez-Ceron,[1,2] Juan M. Gurevich-Messina,[1,2] Soledad L. Saavedra,[1] Gerardo Acosta,[4,5] Osvaldo Cascone,[1,2] Rosa Erra-Balsells,[2,3] and Fernando Albericio[4,5,6]

[1]NANOBIOTEC Institute, UBA-CONICET, Cathedra of Biotechnology, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina
[2]National Scientific and Technological Research Council (CONICET), Buenos Aires, Argentina
[3]CIHDECAR-CONICET, Department of Organic Chemistry, School of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina
[4]Department of Organic Chemistry, Barcelona Science Park, University of Barcelona, Barcelona, Spain
[5]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Barcelona, Spain
[6]School of Chemistry & Physics, University of Kwazulu-Natal, Durban, South Africa

Combinatorial library screening coupled to mass spectrometry (MS) analysis is a practical approach to identify useful peptides. Cyclic peptides can have high biological activity, selectivity, and affinity for target proteins, and high stability against proteolytic degradation. Here we describe two strategies to prepare combinatorial libraries suitable for MS analysis to accelerate the discovery of cyclic peptide structures. Both approaches use ChemMatrix resin and the linker 4-hydroxymethylbenzoic acid. One strategy involves the synthesis of a one-bead–two-peptides library in which each bead contains both the cyclic peptide and its linear counterpart to facilitate MS analysis. The other protocol is based on the synthesis of a cyclic depsipeptide library in which a glycolamidic ester group is incorporated by adding glycolic acid. After library screening, the ring is opened and the peptide is released simultaneously for subsequent MS analysis.

Keywords: affinity • ChemMatrix • combinatorial • depsipeptides • MALDI MS

How to cite this article:
Camperi, S.A., Giudicessi, S.L., Martínez-Ceron, M.C., Gurevich-Messina, J.M., Saavedra, S.L., Acosta, G., Cascone, O., Erra-Balsells, R. and Albericio, F. 2016. Combinatorial library screening coupled to mass spectrometry to identify valuable cyclic peptides. *Curr. Protoc. Chem. Biol.* 8:109-130.
doi: 10.1002/cpch.2

INTRODUCTION

Nowadays short peptides play a crucial role in many aspects of health research and development. Peptides are widely used as ligands for affinity chromatography to reduce the high cost of producing and purifying recombinant therapeutic proteins (Camperi et al., 2014). Biologically active peptides are increasingly used as the active ingredient in therapeutic drugs (Albericio and Kruger, 2012) and in many diagnostic kits based on their high affinity for key molecular targets (Carmona et al., 2012). Cyclic peptides have structural constraints compared to linear peptides. The reduced conformational

Current Protocols in Chemical Biology 8:109-130, June 2016
Published online June 2016 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpch.2

flexibility of cyclic peptides usually confers high biological activity, selectivity, and affinity for target proteins, and strong resistance to proteolytic degradation. Based on these critical points, the use of peptides as therapeutic drugs or as ligands for affinity chromatography is promising (Joo, 2012). Although functional cyclic peptides may be found in natural sources, screening of combinatorial peptide libraries synthesized using natural and unnatural amino acids facilitates the discovery of a more diverse array of functional peptides for any given purpose (Martínez-Ceron et al., 2016).

In the solid phase strategy known as split-and-mix or divide-couple-recombine, a peptide library is synthesized on a solid support of amphiphilic beads. The support is divided into equal portions, each coupled to a different building block (amino acid). The portions are then mixed and again divided to couple the next amino acid. This method allows the synthesis of an equimolar, combinatorial peptide library, in which each bead displays only one peptide entity (Lam et al., 1991).

Combinatorial peptide libraries are synthesized in organic solvents and screened in aqueous solution, so the beads to which the peptides are attached must be compatible with both environments. Screening is performed by mixing an aliquot of a combinatorial peptide library with a target molecule in a buffer solution. A reporter group such as a fluorescent dye or biotin can be conjugated to targets that cannot be detected directly. Affinity between a labeled protein and peptides in a one-bead–one-peptide library can be visualized by adsorption of the target by the beads containing those peptides, and positive beads can be isolated manually with needles. When targets are labeled with fluorescent dyes, the isolation of positive beads can be facilitated by flow sorting using the Complex Object Parametric Analyzer and Sorter (COPAS) BIOBEAD flow-sorting equipment (Union Biometrica). COPAS is capable of sorting large objects (e.g., 120 to 300 μm) based on size, density, and fluorescence (Marani et al., 2009). Otherwise, beads can be detected using a fluorescence stereoscopic microscope and isolated manually with needles. To avoid false-positive beads selection, here we describe a two-stage screening approach in which positive beads from the first screening are subjected to a different screening process (Martínez-Ceron et al., 2015).

After library screening, peptides present on positive beads can be identified by peptide sequencing. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) are the techniques of choice for peptide sequencing. Fragmentation of linear peptides using tandem mass spectrometry (MS/MS) simplifies sequence identification. However, the complex fragmentation patterns of cyclic-peptides impair sequence identification (Redman et al., 2003).

Here we describe two strategies to prepare combinatorial libraries. Both are suitable for MS analysis to accelerate the discovery of cyclic-peptide structures that could be useful as bioactive peptides or as ligands for affinity chromatography.

These strategies do not require hazardous reagents such as palladium (Pd) or cyanogen bromide (CNBr), and therefore they can be applied in a broad range of laboratories without special expertise in organic synthesis.

BASIC PROTOCOL 1

SYNTHESIS OF CYCLO-(1,8)-Ala-$X_1X_2X_3X_4X_5$-Val-Asp-Gly-HMBA-CM LIBRARY WITH THE CORRESPONDING LINEAR CODE Ala-$X_1X_2X_3X_4X_5$-Val-Ala-Gly-HMBA-CM

This protocol describes how to synthesize a cyclic combinatorial library suitable for MS analysis. This strategy involves the synthesis of a one-bead–two-peptides library in which each bead contains a cyclic peptide and its linear version, which serves as a coding tag to facilitate MS identification of the functional cyclic peptide (Fig. 1; Giudicessi et al., 2013).

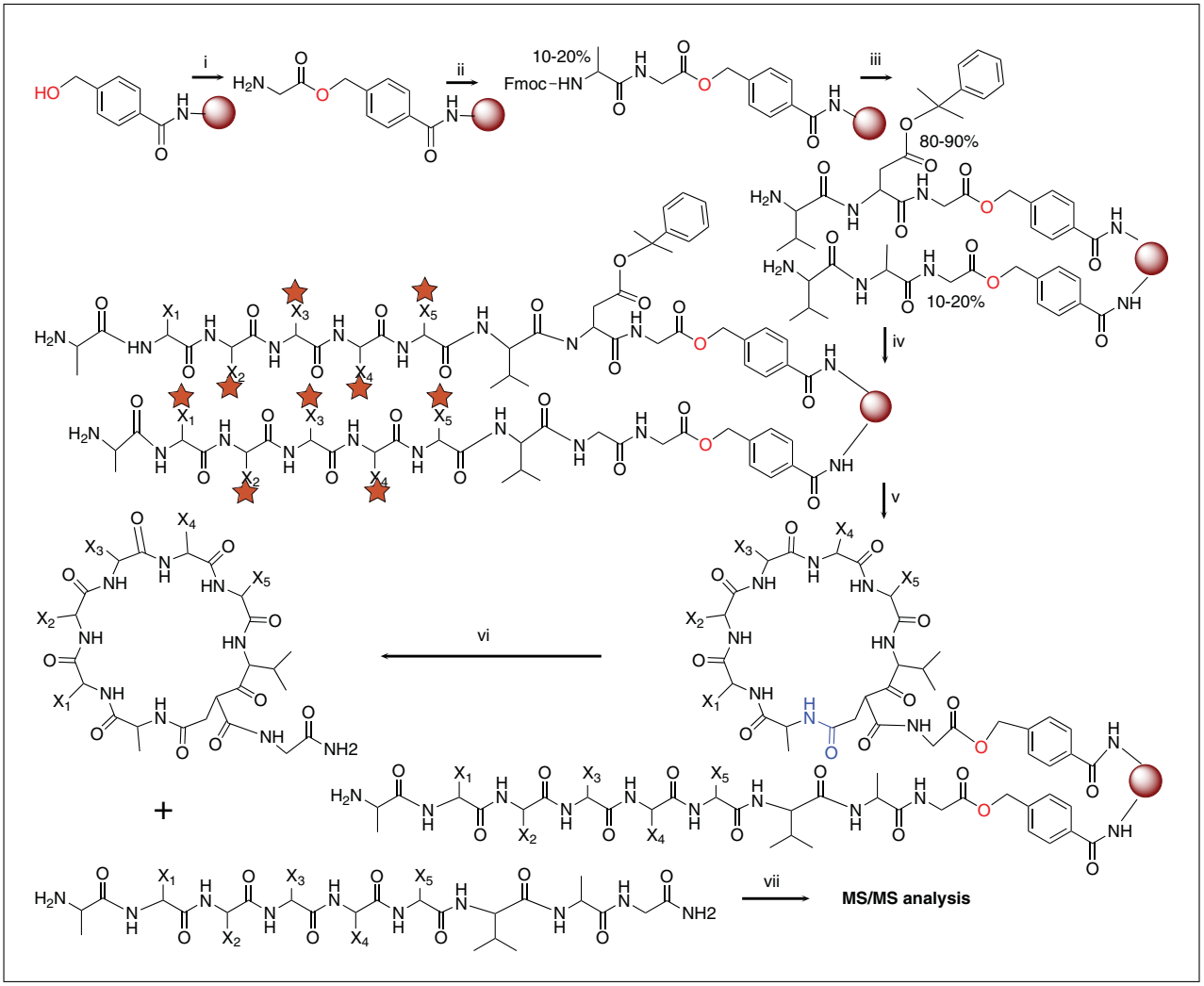

Figure 1 Strategy for the preparation of the cyclo-(1,8)-Ala-$X_1X_2X_3X_4X_5$-Val-Asp-Gly-HMBA-CM library with the corresponding linear code Ala-$X_1X_2X_3X_4X_5$-Val-Ala-Gly-HMBA-CM. (i) Attach Fmoc-Gly-OH to beads and remove Fmoc group. (ii) Attach Fmoc-Ala-OH. (iii) Attach Fmoc-Asp(OPp)-OH and remove Fmoc groups. (iv) Divide, couple, recombine to add 5 combinatorial amino acid positions (X_n, stars), attach N-terminal Fmoc-Ala-OH, and remove Fmoc groups. (v) Selectively cleave the OPp protection group, cyclize the peptide, and deprotect amino acid side chains. (vi) Screen the library with conjugated target, sort and isolate positive beads, cleave linear peptides from beads with NH_4OH, and elute. (vii) Perform MS/MS analysis.

Materials

Hydroxymethylbenzamide-ChemMatrix resin (HMBA-CM; PCAS BioMatrix Inc., cat. no. 4040)

Dichloromethane (DCM; Sigma-Aldrich, cat. no. 270997)

N,N-dimethylformamide (DMF; Sigma-Aldrich, cat. no. 227056)

4-(*N,N*-dimethylamino)pyridine (DMAP; Sigma-Aldrich, cat. no. 522805)

Fluorenylmethoxycarbonyl (Fmoc) amino acids with side chain-protecting groups (Peptides International Inc or Bachem Americas Inc)

N,N′-Diisopropylcarbodiimide (DIPCDI; Sigma-Aldrich, cat. no. 38370)

Acetic anhydride

20% (v/v) piperidine in DMF (see recipe)

1-[bis(dimethylamino)methylene]-1 H-benzotriazolium 3-oxide tetrafluoroborate (TBTU; Peptides International Inc., cat. no. KTB-1066-PI)

N,N-diisopropylethylamine (DIPEA; Sigma-Aldrich, cat. no. D125806)

OPp cleavage cocktail (see recipe), prepare just before use

(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP; Sigma-Aldrich, cat. no. 377848)
Hydroxy-7-azabenzotriazole (HOAt; Sigma-Aldrich, cat. no. 41996)
1-[bis(dimethylamino)methylene]-1 H-benzotriazolium 3-oxide hexafluorophosphate (HBTU; Sigma-Aldrich, cat. no. 12804)
Protecting group cleavage cocktail (see recipe), prepare just before use
Acetonitrile
Acetic acid

Fume hood
Empty solid phase extraction cartridges, 1- to 60-ml (Agilent Technologies, cat. no. 12131007 to 12131016).
Polypropylene frits for solid phase extraction cartridges, 20-μm, 6.4- to 67-mm (Agilent Technologies, cat. no. 12131019 to 12131024)
Manual pipette, 100- to 1,000-μl (Eppendorf, cat. no. 492000904)
Pipette tips, 50- to 1,000-μl, 71 mm (Eppendorf, cat. no. 0030 000.919)
Vacuum manifolds to evacuate the fluid from each syringe by filtration (e.g., Promega, Supelco).
Orbital shaker

NOTE: Perform synthesis inside a fume hood, and wear gloves, eye protection, and a lab coat. Follow all local, state, and federal safety regulations when disposing of chemical waste.

Couple the first amino acid to HMBA-ChemMatrix resin

1. Calculate the amount of resin required for the synthesis of the combinatorial library, considering that for statistical reasons the number of beads must exceed the number of peptides by a factor of at least 10, and that 1 g of HMBA-ChemMatrix resin contains 10^6 beads.

   For example, a one-bead–one-peptide library in which 10 amino acids (building blocks) are varied in five positions has 10^5 (100,000) peptide sequences. So 1 g of resin (10^6 beads) is required for library synthesis.

2. Place the HMBA-ChemMatrix resin (0.63 mmol/g = 1 eq) in an empty solid phase extraction cartridge fitted with a polypropylene frit, and wash the resin 5 times with two resin volumes of DCM and 5 times with two resin volumes of DMF for 1 min each wash. Suspend washed beads in sufficient DMF to swell them.

3. Dissolve 0.1 eq of DMAP in a volume of DMF necessary to prepare a 50 mmol/L solution.

   All reagent equivalents with respect to the HMBA on the beads (0.63 mmol/g beads = 1 eq).

4. Mix vigorously Fmoc-Gly-OH (3 eq) with DIPCDI (1.5 eq) in DMF for 5 min to generate the symmetrical anhydride.

5. Add the solution to the HMBA-ChemMatrix (1 eq).

6. Add 0.1 eq of DMAP (50 mmol/L solution from step 3) drop wise to the HMBA-ChemMatrix mixture.

7. Place the reaction vessel on an orbital shaker table at room temperature, and shake for 2 hr.

8. Pour off or drain the reaction solution, and wash the resin twice with two resin volumes of DMF for 2 min each.

9. Re-couple the Fmoc-Gly-OH as in steps 3 to 7.

   First amino acid coupling yield is critical to assure maximum functionalization of the resin and therefore an optimum peptide yield.

10. Pour off or drain the reaction solution and wash the resin twice with two resin volumes of DMF for 2 min each.
11. Wash the resin 5 times with two resin volumes of DCM for 1 min each.
12. Acetylate any remaining hydroxyl groups on the resin by adding acetic anhydride (6 eq), 50 mmol/L DMAP solution (0.1 eq), and sufficient DCM to allow good homogenization.
13. Place the reaction vessel on an orbital shaker at room temperature, and shake for 1 h.
14. Pour off or drain the reaction solution, and wash the resin 5 times with two resin volumes of DMF for 1 min each.
15. Wash the resin in two resin volumes of 20% (v/v) piperidine in DMF (1 × 1 min and 2 × 5 min) to remove the Fmoc group.
16. Wash the resin 5 times with two resin volumes of DMF for 1 min each.

Add library tag

17. Add Fmoc-Ala-OH (0.2 eq) and TBTU (0.2 eq) in DMF to the resin.
18. Mix to assure a good homogenization.
19. When the suspension is well mixed, add DIPEA (0.4 eq) in DMF drop wise, while stirring the suspension.

    To assure a good homogenization, the DIPEA must be added after mixing the resin and drop wise while stirring the suspension.

20. Incubate the reaction vessel at room temperature on an orbital shaker table for 30 min.
21. Wash the resin 5 times with two resin volumes of DMF for 1 min each.
22. Add Fmoc-Asp(OPp)-OH (3 eq) in the presence of TBTU (3 eq) and DIPEA (3.5 eq) in DMF.

    Prepare a solution of these reagents before adding them to the resin.

23. Place the reaction vessel on an orbital shaker at room temperature, and shake for 2 h.
24. Pour off or drain the reaction solution and wash the resin 5 times with two resin volumes of DMF for 1 min each.
25. Add 20% (v/v) piperidine in DMF (1 ×1 min and 2 × 5 min) to remove Fmoc group.
26. Wash the resin 5 times with two resin volumes of DMF for 1 min each.
27. Add Fmoc-Val-OH (3 eq) in the presence of TBTU (3 eq) and DIPEA (3.5 eq) in DMF.
28. Place the reaction vessel on an orbital shaker at room temperature, and shake for 2 hr.
29. Pour off or drain the reaction solution and wash the resin 5 times with two resin volumes of DMF and 5 times with DCM for 1 min each.

30. Take a small sample of resin beads (10 to 20 beads) for analysis. Submit the sample to the Kaiser or Chloranil test (Support Protocols 1 and 2). A positive result indicates that the reaction is incomplete.

    a. If positive, wash resin with 5 times with two resin volumes of DMF for 1 min each, and repeat the coupling reaction (steps 27 to 29) with fresh reagents.
    b. If negative, remove Fmoc group as indicated in step 31.

31. Wash the resin 5 times with two resin volumes of DMF for 1 min each, and then wash with two resin volumes of 20% (v/v) piperidine in DMF (1 × 1 min and 2 × 5 min) to remove the Fmoc protecting group.

32. Wash the resin 5 times with two resin volumes of DMF for 1 min each.

Divide-Couple-Recombine

33. Prepare a cartridge fitted with a polypropylene frit for each building block to be used in variable positions. Engrave each cartridge with its corresponding amino acid letter to ensure no mix-up during the synthesis.

34. Suspend the resin beads in one volume of DMF, homogenize the mixture, and using a manual pipette divide the resin beads into equal volumes in the engraved cartridges.

    If using resin beads of a large diameter, the automatic pipette tip must be cut at the end to prevent it from clogging.

35. Remove most of the DMF by vacuum filtration with a vacuum manifold.

36. Add the appropriate Fmoc-protected amino acid (3 eq) in the presence of TBTU (3 eq) and DIPEA (3.5 eq) in DMF to its corresponding cartridge.

37. Agitate gently for 2 h at room temperature to allow coupling.

38. Wash the resin in each vessel 5 times with two resin volumes of DMF for 1 min each.

39. Combine the resin from each cartridge in a larger cartridge. Wash the beads with two resin volumes of DMF (5 × 2 min).

40. Add two resin volumes of 20% (v/v) piperidine in DMF (1 × 1 min and 2 × 5 min) to the cartridge to remove the Fmoc protecting group.

41. Wash the resin 5 times with two resin volumes of DMF for 1 min each.

42. Repeat steps 34 to 41 for as many times as the number of random positions. If there are five random positions, for example, repeat steps 34 to 41 five times.

Couple the N-terminal alanine

43. Once the final random position has been synthesized and all of the resin has been combined in one large cartridge, couple alanine to the N-terminus of each peptide by adding Fmoc-Ala-OH (3 eq), TBTU (3 eq), and DIPEA (3.5 eq) in DMF and gently agitating for 2 h at room temperature.

    Alanine will facilitate the cyclization reaction.

44. Pour off or drain the reaction solution and wash the resin 5 times with two resin volumes of DMF for 1 min each.

45. Wash with two resin volumes of 20% (v/v) piperidine in DMF (1 × 1 min and 2 × 5 min) to remove Fmoc groups.

46. Wash the resin 5 times with two resin volumes of DMF for 1 min each.

Cleave the OPp group

47. Prepare the OPp cleavage cocktail (see recipe).

    CAUTION: The OPp cleavage cocktail contains the corrosive trifluoroacetic acid (TFA).

48. Cleave the OPp group by washing the resin in OPp cleavage cocktail (3 × 15 min).

    To ensure a good yield of cyclization, the OPp group on the Asp residue must be cleaved without eliminating the permanent protecting groups on the remaining amino acid side chains.

    To monitor the efficiency of the OPp cleavage, cleave a few resin beads with ammonia vapor and evaluate the peptides from each bead by mass spectrometry. Incomplete cleavage of OPp will result in two signals with a difference of 119 u corresponding to the OPp group.

Cyclize the peptide

49. Wash the peptide library on the resin 5 times with two resin volumes of DMF for 1 min each, suspend the resin in DMF, and allow it to swell for 1 h.

50. Add PyBOP (4 eq), HOAt (4 eq), and DIPEA (5 eq) in DMF, and agitate gently for 2 h at room temperature.

51. Wash the resin twice with two resin volumes of DMF for 2 min each.

52. Remove a small sample and perform a Kaiser test (see Support Protocol 1) to monitor the progress of cyclization.

    Blue coloration of the solution and/or resin beads (a positive result) indicates that the reaction is incomplete.

53. Repeat steps 50 and 51, if necessary.

Deprotect side chains

54. Prepare protecting group cleavage cocktail (see recipe).

    CAUTION: This solution contains the corrosive TFA.

55. Wash the resin 5 times with two resin volumes of DCM for 1 min each.

56. Add 15 ml of the protecting group cleavage cocktail to the resin, and incubate 2 h at room temperature to cleave protecting groups from side chains.

57. Wash the resin 5 times with two resin volumes of DMF and 5 times with DCM for 1 min each.

58. Store the bead library in DCM at 4°C.

    The libraries can be stored in DCM at 4°C for up to a year.

SYNTHESIS OF CYCLO-(1,8)-Ala-OCH$_2$CO-X$_1$X$_2$X$_3$X$_4$X$_5$-Asp-Ala-Gly-HMBA-CM LIBRARY

ALTERNATE PROTOCOL 1

This alternative protocol is based on the synthesis of a combinatorial cyclic depsipeptide library, in which glycolic acid is used to add a glycolamidic ester group at the end of peptide elongation. After library screening, the ring is opened and the peptide released simultaneously with ammonia vapor (Fig. 2; Gurevich-Messina et al., 2015). Peptide linearization facilitates its MS analysis.

Additional Materials *(also see Basic Protocol 1)*

Glycolic acid (Sigma-Aldrich, cat. no. 124737).
1-hydroxybenzotriazole (HOBt; Peptides International, cat. no. KHB-1065-PI).
N-methyl-2-pyrrolidone (NMP; Sigma-Aldrich, cat. no. 328634).

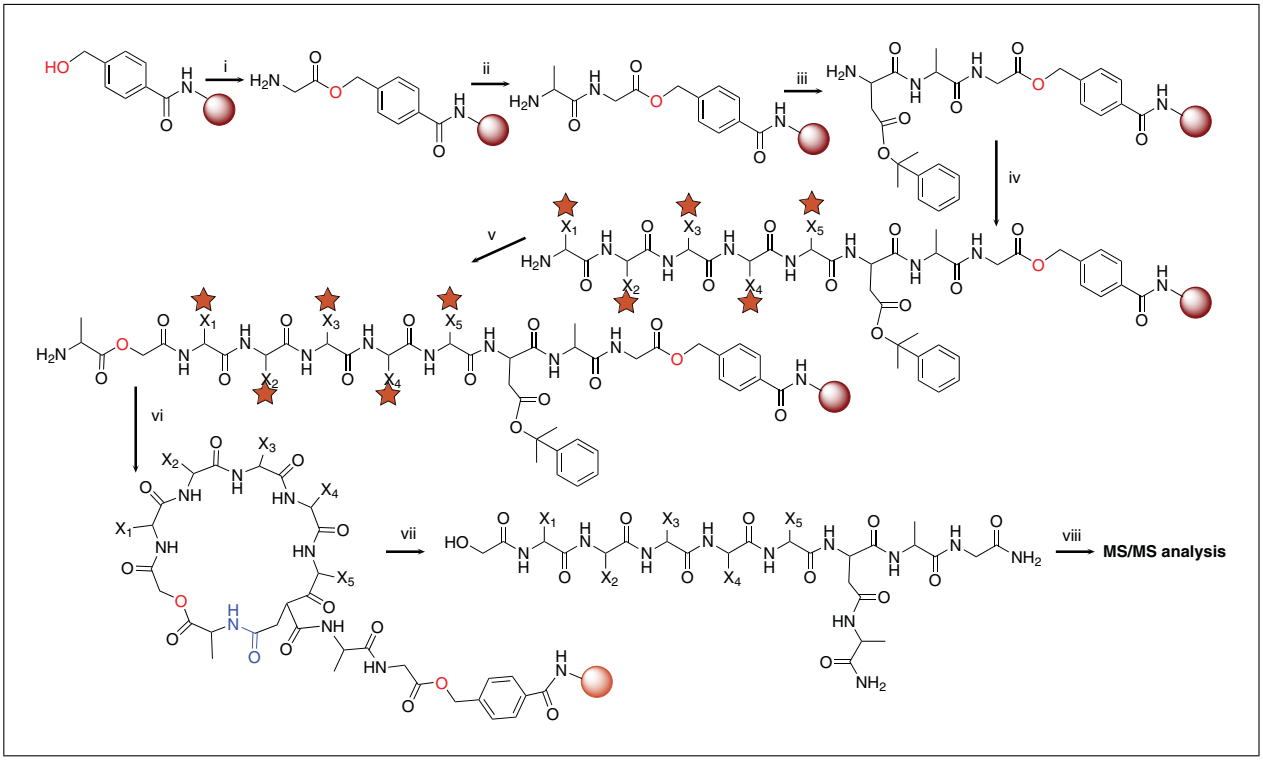

Figure 2 Strategy for the preparation of the cyclo-(1,8)-Ala-OCH_2CO-$X_1X_2X_3X_4X_5$-Asp-Ala-Gly-HMBA-CM library. (i) Attach Fmoc-Gly-OH to beads and remove Fmoc group. (ii) Attach Fmoc-Ala-OH and remove Fmoc group. (iii) Attach Fmoc-Asp(OPp)-OH and remove Fmoc group. (iv) Divide, couple, recombine to add 5 combinatorial amino acid positions (X_n, stars). (v) Add a glycolamidic ester group, attach N-terminal Fmoc-Ala-OH, and remove Fmoc group. (vi) Selectively cleave the OPp protection group, cyclize the peptide, and deprotect amino acid side chains. (vii) Screen the library with conjugated target, sort and isolate positive beads, open the cyclic peptide ring (blue amide bond), cleave linear peptides from beads with NH_4OH, and elute. (viii) Perform MALDI MS/MS analysis.

NOTE: Perform synthesis inside a fume hood, and wear gloves, eye protection, and a lab coat. Follow all local, state, and federal safety regulations when disposing of chemical waste.

Couple the first amino acid to HMBA-ChemMatrix resin

1. Perform steps 1 to 16 of Basic Protocol 1.

Add library tag

2. Add Fmoc-Ala-OH (3 eq), TBTU (3 eq), and DIPEA (3.5 eq) in DMF to the resin.
3. Place the reaction vessel on an orbital shaker at room temperature, and shake for 1 h.
4. Pour off the reaction solution, and wash the resin 5 times with two resin volumes of DMF for 1 min each.
5. Wash the resin with two resin volumes of 20% (v/v) piperidine in DMF (1 × 1 min and 2 × 5 min) to remove Fmoc groups.
6. Wash the resin 5 times with two resin volumes of DMF for 1 min each.
7. Add Fmoc-Asp(OPp)-OH (3 eq) in the presence of TBTU (3 eq) and DIPEA (3.5 eq) in DMF.
8. Place the reaction vessel on a shaker table at room temperature, and shake for 2 h.
9. Pour off or drain the reaction solution and wash the resin 5 times with two resin volumes of DMF for 1 min each.

10. Wash the resin with two resin volumes of 20% (v/v) piperidine in DMF (1 × 1 min and 2 × 5 min) to remove Fmoc groups.

11. Wash the resin 5 times with two resin volumes of DMF for 1 min each.

Divide-Couple-Recombine

12. Perform steps 33 to 42 of Basic Protocol 1.

Incorporate the glycolamidic ester group

13. Once the final random position has been synthesized and all of the resin has been combined in one large cartridge, incorporate a glycolamidic ester group by adding glycolic acid (2 eq), HOBt (2 eq) and DIPCDI (2.5 eq) and gently agitating for 2 h at room temperature.

    The risk of incorporating multiple glycolic acid moieties is low because ester bond formation needs harsher conditions than amide bond. Nevertheless, to avoid multiple incorporation of glycolic acid only 2 eq. are added in the reaction mixture.

14. Pour off or drain the reaction solution and wash the resin 5 times with two resin volumes of DMF for 1 min each.

Couple the N-terminal alanine

15. Vigorously mix anhydrous Fmoc-Ala-OH (20 eq) with DIPCDI (10 eq) in DCM for 5 min to generate the symmetrical anhydride. NMP can be added to the mixture to facilitate dissolution.

    To generate the symmetrical anhydride, use anhydrous Fmoc-Ala-OH instead of hydrous Fmoc-Ala-OH.

16. Add the mixture to the peptidyl-resin, and then add (drop wise) a solution of 50 mmol/L DMAP (0.1 eq) dissolved in DMF.

17. Agitate gently for 1 h at room temperature.

18. Wash the resin twice with two resin volumes of DMF for 2 min each.

19. Repeat the steps 15 to 17 to ensure complete incorporation of alanine.

    To ensure the glycolamidic ester bond formation, alanine is assembled using the symmetrical anhydride method with 20 eq. of Fmoc-Ala-OH. To determine the extent of resin substitution, treat a known quantity of Fmoc-loaded peptidyl-resin with piperidine in DMF and measure the release of the dibenzofulvene-piperidine adduct by spectrophotometry at 290 nm ($\varepsilon = 5800\ M^{-1}cm^{-1}$). In case of incomplete loading, the coupling of alanine must be repeated.

20. Leave the reaction vessel at room temperature on an orbital shaker overnight.

21. Pour off or drain the reaction solution and wash the resin 5 times with two resin volumes of DMF for 1 min each.

22. Wash the resin with two resin volumes of 20% (v/v) piperidine in DMF (1 × 1 min and 2 × 5 min) to remove Fmoc groups.

23. Wash the resin 5 times with two resin volumes of DMF for 1 min each.

Cleave the OPp group, cyclize the peptide, and deprotect side chains

24. Continue with steps 47 to 58 of Basic Protocol 1.

SUPPORT PROTOCOL 1

NINHYDRIN (KAISER) TEST

After each coupling step, the Kaiser test should be performed to confirm completion of the coupling reaction. The Kaiser test is used as a qualitative test for primary amines; hence, it cannot reliably evaluate Fmoc-AA-OH coupling to Pro or other *N*-substituted amino acids. In those cases, the chloranil test should be used (see Support Protocol 2). The Kaiser test can also be used to evaluate the progress of cyclization in the depsipeptide library. In the library with a linear code, the test cannot be used. Instead, mass spectrometry can be used to evaluate cyclization of peptides from a fraction of the beads.

Materials

Peptidyl-resin from Basic Protocol 1 or Alternate Protocol 1
DCM
Solution A (see recipe)
Solution B (see recipe)
Drying oven

1. Put a small quantity of resin beads (10 to 20 beads) previously washed with DCM in a small glass tube (6 × 50 mm).
2. Under a fume hood, add 60 μl of solution A and 20 μl of solution B to the tube.
3. Heat at 110°C for 3 min.
4. Observe the color of the beads. Blue resin beads indicate the presence of resin-bound free amine, suggesting that the coupling reaction is incomplete.

   Some amino acids can produce unusual colorations, ranging from red to blue (Asn, Cys, Ser, and Thr in particular).

SUPPORT PROTOCOL 2

CHLORANIL TEST

This test is recommended to evaluate Fmoc-AA-OH coupling to Pro or other *N*-substituted amino acids.

Materials

Peptidyl-resin from Basic Protocol 1 or Alternate Protocol 1
Acetone
Saturated chloranil solution in toluene: In a conical microcentrifuge tube mix 25 mg of chloranil and 1 ml of toluene. Centrifuge and recover the supernatant. Store protected from light at 2° to 8°C.

1. Place a small amount of resin beads (10 to 20 beads) in a small test tube.
2. Add 5 μl of chloranil solution and 15 μl of acetone.
3. Mix and incubate 5 min at room temperature.
4. Observe the color of the beads. Green staining of the resin beads indicates the presence of secondary amines, suggesting that the coupling reaction is incomplete.

BASIC PROTOCOL 2

TWO-STAGE COMBINATORIAL CYCLIC-PEPTIDE LIBRARY SCREENING

Combinatorial library screening is performed by mixing an aliquot of the cyclic-peptide beads with target molecule in an aqueous buffer. Targets that cannot be detected directly can be conjugated to a reporter group, such as a fluorescent dye or biotin. Non-ionic detergents and blocking agents (e.g., gelatin or powdered milk) are used to avoid nonspecific interactions during the screening. False positives, however, are commonly selected.

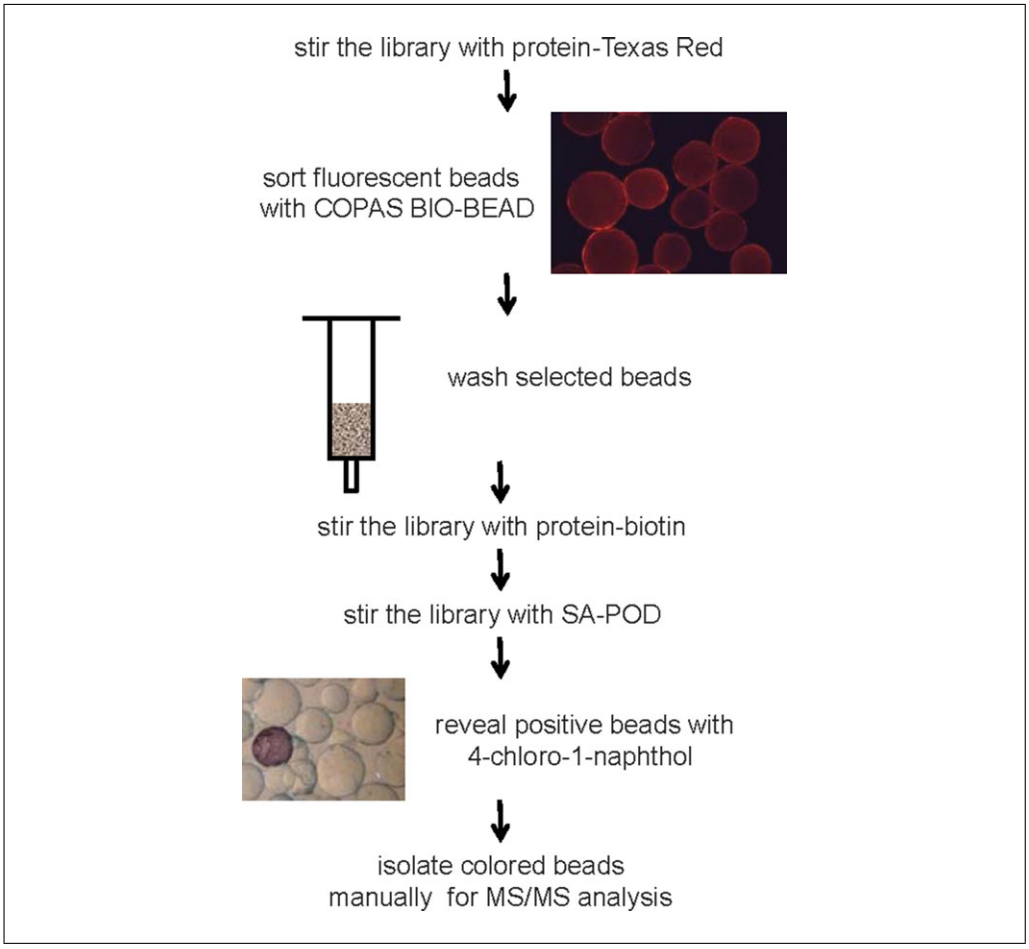

Figure 3 Combinatorial library screening protocol.

Peptides with His-Pro-Gln and His-Pro-Met motifs have high affinity for streptavidin (Lam and Lebl, 1992), and can be directly selected when using streptavidin-conjugated peroxidase as a reporter. These motifs should be avoided in library design to minimize the selection of false-positive beads when using biotin-streptavidin-based reporters. On the other hand, when using the target protein coupled with fluorescent dyes, peptides with high content of hydrophobic amino acids may interact non-specifically with fluorescent dyes (Rozinov and Nolan, 1998). We have noticed however that false-positive beads show a homogeneous bright fluorescence pattern, whereas positive beads exhibit a heterogeneous halo-like fluorescence pattern, with a high fluorescence intensity on the bead surface and a low intensity in the core (Marani et al., 2009). Nevertheless, the difference between them is not always obvious, and depends on the target and the fluorescent dye used. Thus, many false-positive beads are usually isolated and their peptides sequenced. To minimize the selection of false-positive beads, we describe a two-stage screening in which the positive beads from the first screening are subjected to a different screening process (Fig. 3; Martínez-Ceron et al., 2015).

Materials

Combinatorial cyclic-peptide library (see Basic Protocol 1 and Alternate Protocol 1)
DCM
DMF
Phosphate buffered saline (PBS; see recipe)
Skim milk
Bovine serum albumin, fraction V (BSA; Sigma-Aldrich, cat. no. A8531).

Tween 20 (Sigma-Aldrich, cat. no. P9416).
Target protein conjugated to Texas Red (see Support Protocol 3).
Target protein conjugated to biotin (see Support Protocol 3).
COPAS GP Sheath (Union Biometrica, cat. no. 300-5070-000).
Acetic acid
Acetonitrile
Streptavidin-conjugated peroxidase (SA-POD; Sigma-Aldrich, cat. no. S5512)
4-chloro-1-naphthol (Sigma-Aldrich, cat. no. C8890)
Methanol
3,3′-diaminobenzidine (DAB) tablets (Sigma-Aldrich, cat. no. D4293)
30% H_2O_2 (Sigma-Aldrich, cat. no. 216763).

Solid-phase extraction cartridge fitted with a polypropylene frit
COPAS BIOBEAD flow sorting equipment (Union Biometrica).
Leica MZ FLIII stereoscopic microscope (Leica Microsystems GmbH, Wetzlar, Germany).
Orbital Shaker

Hydrate the library and block nonspecific binding sites

1. Deposit an aliquot of the library in an empty solid-phase extraction cartridge fitted with a polypropylene frit.
2. Hydrate the combinatorial peptide library by washing successively as follows:

   5 times in DCM, 1 min each
   5 times in DMF, 1 min each
   5 times in 70% DMF in water, 1 min each
   5 times in 50% DMF in water, 1 min each
   5 times in 30% DMF in water, 1 min each
   5 times in water, 1 min each

3. Suspend the library beads with PBS containing 10% (w/v) skim milk and 2% (w/v) BSA, and incubate at room temperature for 1 h on an orbital shaker to block nonspecific binding sites.
4. Pour off or drain the solution by filtration.
5. Wash 5 times with two resin volumes of PBS containing 0.05% (v/v) Tween 20 for 1 min each wash.

Incubate the library with target protein conjugated to Texas Red

6. Prepare 3 to 10 μg/ml target protein conjugated to Texas Red in PBS containing 10% (w/v) skim milk and 0.05% Tween 20, and add one resin volume to the beads to prepare a 1:1 suspension of the resin beads in buffer.
7. Incubate 1 h at 4°C with gentle mixing on an orbital shaker.
8. Wash the beads 5 times with two resin volumes of PBS containing 0.05% Tween 20 for 1 min each.

Sort the fluorescent beads by COPAS

9. Suspend the beads in COPAS GP Sheath reagent at a density of about 50 beads per ml, and pour the suspension into the sample cup.
10. Isolate fluorescent beads using the COPAS BIO-BEAD flow sorting equipment (Union Biometrica). Define gating and sorting regions to sort beads with COPAS based on their time-of-flight (TOF), to separate uniform-sized beads, and red fluorescence intensity.

COPAS records five parameters for each bead: axial length (time-of-flight, TOF), optical density (optical extinction, EXT), and up to three fluorescent bandwidths (e.g., green, yellow, and red). To define a region for analysis and sorting, first evaluate the axial length of a sample of beads to define the TOF range. Using the defined TOF range, set the red fluorescent range by selecting beads and inspecting them with a fluorescence microscopy.

11. Deposit sorted beads in an empty solid phase extraction cartridge fitted with a polypropylene frit.

Wash, hydrate, and block isolated beads

12. Wash selected beads twice with 10 μl acetic acid/acetonitrile/water (3/4/3) for 15 min each to eliminate the adsorbed protein.

13. Soak the beads sequentially as follows:

    5 times in acetonitrile, 1 min each
    5 times in DCM, 1 min each
    5 times in DMF, 1 min each
    5 times in 70% DMF in water, 1 min each
    5 times in 50% DMF in water, 1 min each
    5 times in 30% DMF in water, 1 min each
    5 times in water, 1 min each

14. Block the sorted beads in PBS (pH 7.4) containing 10% (w/v) skim milk, and 2% (w/v) BSA.

15. Incubate 1 h on an orbital shaker at room temperature.

16. Pour off or drain the solution by filtration (using the corresponding frit).

17. Wash the beads 5 times with PBS containing 0.05% Tween 20 for 1 min each wash.

Incubate beads with target protein conjugated to biotin

18. Dilute biotinylated target protein (3 to 10 μg/ml) in PBS containing 10% (w/v) skim milk and 0.05% Tween 20, and add it to the beads.

19. Incubate 1 h at 4°C with gentle mixing on an orbital shaker.

20. Wash the beads 5 times with PBS containing 0.05% Tween 20 for 1 min each.

Incubate beads with SA-POD conjugate

21. Mix the beads with a solution of 5.8 μg/ml SA-POD in PBS-0.05% Tween.

22. Incubate 1 h with gentle mixing on an orbital shaker.

23. Wash the beads 5 times with PBS-0.05% Tween (5 × 1 min).

Perform colorimetric assay to reveal positive beads

NOTE: Prepare 4-chloro-1-napthol and hydrogen peroxide solutions just before use.

NOTE: Other protocols based on different peroxidase substrates (e.g., 3,3′-diaminobenzidine) can be used.

24. Dissolve 3 mg of 4-chloro-1-naphthol in 1 ml methanol.

    4-chloro-1-naphthol is toxic.

25. Dilute 20 μl of 30% H_2O_2 into 4 ml PBS.

26. Mix the 1-ml 4-chloro-1-naphthol solution and the 4-ml H_2O_2 solution with the beads, and incubate 5 min at room temperature. Positive beads will turn violet.

27. Wash the beads 5 times with PBS-0.05% Tween 20 for 1 min each wash.
28. Isolate violet-colored beads manually with a stereoscopic microscope and needles.

Wash positive beads after screening (Martínez-Ceron et al., 2010)

29. Prepare a solution of acetic acid/acetonitrile/water (3/4/3).
30. Soak each positive bead 5 times with 1 μl of water for 1 min each wash, then once with 10 μl acetic acid/acetonitrile/water (3/4/3) for 5 min.
31. Wash each bead 5 times with 1 μl acetonitrile, then 5 times with 1 μl DCM for 1 min each wash.
32. Air-dry the beads.

Positive beads can be stored dry at 4°C for up to a year.

SUPPORT PROTOCOL 3

TARGET PROTEIN LABELING WITH TEXAS RED OR BIOTIN

Target proteins that cannot be directly detected are typically labeled with a fluorescent dye or biotin. Many reporter groups are commercially available, and most are sold with activate groups that allow them to be conjugated to primary amines (e.g., lysines) on target proteins.

If using a fluorescent dye, choose a maximum emission in the red region so that positive signals will be well separated from intrinsic green fluorescence exhibited by beads. This protocol details how to label the target protein with biotin or Texas Red.

Materials

N-hydroxysuccinimide (NHS)-Biotin (Sigma-Aldrich, cat. no. H1759).
Texas Red sulfonyl chloride, 10 × 1 mg (Thermo Fisher Scientific, cat. no. T1905).
Labeling buffer (see recipe)
PD-10 desalting column (5 kDa exclusion limit; GE Healthcare, cat. no. 17-0851-01).
Phosphate buffered saline (see recipe)

NOTE: It is highly recommended to read the instructions of the suppliers.

NOTE: Perform steps 1 and 2 on ice.

1. Dissolve 1 to 5 mg protein in 1 ml labeling buffer.
2. Prepare the labeling solution just before use:
   a. *For labeling with Texas Red*: Dissolve 6-fold molar excess of Texas Red sulfonyl chloride in the minimum volume of DMF needed to dissolve the label.

      Do not dissolve Texas Red sulfonyl chloride in dimethyl sulfoxide (DMSO) because it reacts with sulfonyl chlorides. Dyes with maximum emission in the red are selected because resin beads exhibit a significant intrinsic fluorescence with maximum emission in the green.

   b. *For labeling with biotin*: Dissolve 6-fold molar excess of NHS-Biotin in the minimum volume of cold water needed to dissolve the label.
3. Mix the labeling solution with the protein sample, and incubate the reaction mixture for 4 h on ice.
4. Desalt the reaction mixture using a PD-10 column equilibrated with PBS—or another suitable buffer—to separate the conjugated protein from the uncoupled reagents.

MALDI-TOF/TOF MS/MS ANALYSIS

After library screening, peptides from selected beads are analyzed by mass spectrometry. Peptides bound through HMBA to the resin beads can be released with rather strong peptide nucleophiles (e.g., NaOH, NH_3, NH_2NH_2). ChemMatrix contains exclusively primary ether bonds, so it is stable under conditions nucleophilic to peptide bonds. Ammonia vapor has the advantage that it is easily removed by evaporation, thus avoiding the presence of contaminants that could interfere with the MS peptide analysis. When using a cyclic peptide library with a linear code, both peptides will be released together. A small proportion of the linear code peptide (non-cycled Ala containing peptide) is enough for MS analysis, because MS is highly sensitive and HMBA-CM resin has a high capacity (0.63 mmol/g substitution; Giudicessi et al., 2013). When using the depsipeptide library, the ring is opened and the peptide is released simultaneously with ammonia vapor (Gurevich-Messina et al., 2015).

The peptide sequence is then determined by MS/MS. Although both ESI MS/MS and MALDI MS/MS can be used for peptide analysis, the latter is more suitable for multiple sample analyses because less time is needed to acquire each spectrum.

Materials

Positive peptide-beads (see Basic Protocol 3)
30% (v/v) ammonium hydroxide
Acetic acid
Acetonitrile
Matrix solution (see recipe), prepared just before use

Drying chamber
Target plate
MALDI TOF/TOF MS

Cleave the peptide and elute from the bead (Fig. 4)

NOTE: This process must be performed in an efficient fume hood.

1. Place each peptide-bead into its own microcentrifuge tube.

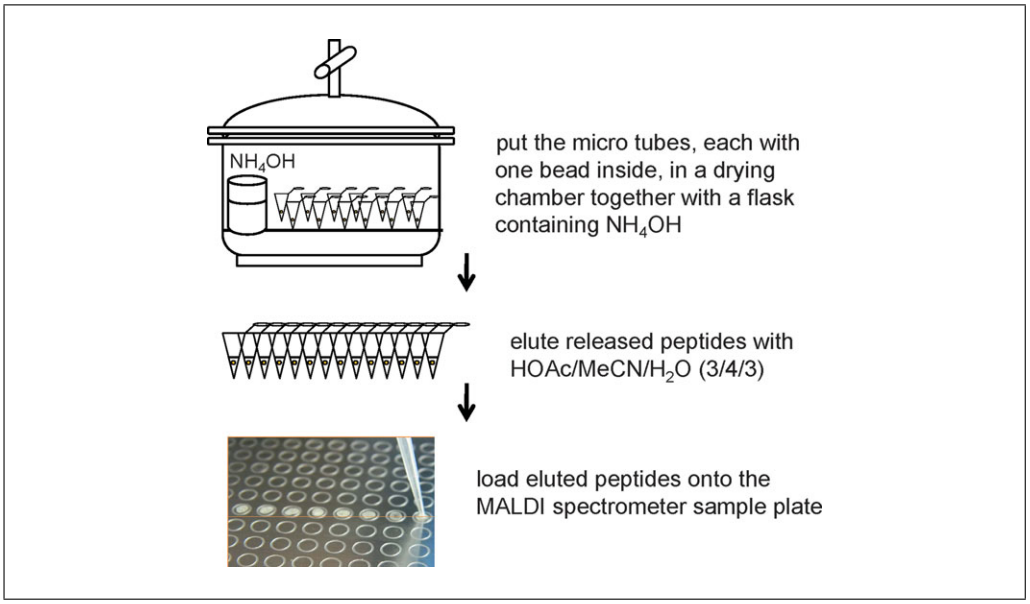

Figure 4 Peptide cleavage with ammonia vapor, elution from beads, and peptide loading onto MALDI spectrometer sample plate.

2. Put the microcentrifuge tubes with the beads in a drying chamber together with a flask containing 30% (v/v) NH_4OH.
3. Clamp the drying chamber shut, and incubate overnight at room temperature.
4. Remove the microcentrifuge tubes from the drying chamber and place them in a fume hood to evaporate the ammonia.
5. Add 10 μl of acetic acid/acetonitrile/water (3/4/3) and briefly centrifuge each microcentrifuge tube to ensure the beads are in the bottom of the tubes.
6. Incubate overnight to elute the peptides from each bead.

   Eluted peptides can be stored at −20°C for up to a month.

Load the peptide sample and perform MALDI MS/MS

7. Load 1 μl of eluted peptide from a single bead onto the MALDI spectrometer sample plate.
8. Air-dry at room temperature.
9. Add 1 μl of matrix solution.
10. Air-dry at room temperature.
11. Acquire positive-ion mass spectra in the modes: linear, reflector, and tandem (MS/MS).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes.

Labeling buffer

8 mM Na_2CO_3
92 mM $NaHCO_3$
pH should be 9.2

Matrix solution

Just before use, prepare 4 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.1% TFA.

Ninhydrin reactive

Solution A: Dissolve 40 g phenol in 10 ml ethanol. Dilute 2 ml 0.001 M potassium cyanide in 98 ml pyridine. Combine equal volumes of each to make Solution A. Store protected from light.
Solution B: Prepare 5% (w/v) ninhydrin in ethanol. Store protected from light.

OPp cleavage cocktail

4% (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich, cat. no. T6508)
96% (v/v) DCM
Prepare 15 ml for 1 g resin, just before use

Phosphate buffered saline (PBS)

10 mM Na_2HPO_4
2 mM KH_2PO_4
137 mM NaCl
2.7 mM KCl
Adjust pH to 7.4

Piperidine, 20% (v/v)

Prepare 100 ml of 20% (v/v) piperidine (Sigma-Aldrich, cat. no. 104094) in DMF. Store at room temperature.

Protecting group cleavage cocktail

95% (v/v) TFA
2.5% (v/v) triisopropyl silane (Sigma-Aldrich, cat. no. 233781)
2.5% (v/v) water
Prepare 15 ml for 1 g resin, just before use

COMMENTARY

Background Information

As we have recently reviewed (Martínez-Ceron et al., 2016), there has been much interest in the development of OBOC combinatorial cyclic-peptide libraries, and many protocols have been developed for synthesis of OBOC combinatorial cyclic-peptide libraries using on-resin homodetic cyclization.

Here, we described two strategies to prepare combinatorial libraries suitable to identify useful cyclic peptides. Both strategies are suitable for MS analysis to accelerate the discovery of the cyclic peptide and are based on the same chemical principles: (i) Fmoc/tBu chemistry, commonly used in many laboratories; (ii) amphiphilic ChemMatrix (CM) resin, which swells in aqueous media and is therefore suitable to carry out the biological test on solid-phase; (iii) a 4-hydroxymethylbenzoic acid (HMBA) linker, which allows peptide release by simple and safe ammonolysis (Camperi et al., 2005). In approaches that use methionine as a linker, peptides are released with cyanogen bromide—a very hazardous reactive, especially when working with the large number of beads that are usually isolated during the screening (Liang et al., 2013).

The key of our strategies is the use of the HMBA linker. The ester formed between the C-terminal amino acid and the linker can be readily cleaved with ammonia vapor, which is easily evaporated without leaving contaminants that could interfere in the MS analysis. Vapor phase ammonolysis cleaves most oxymethylbenzamide esters with high yield with exception of Ile and Val esters. To assure homogeneous peptide release from each bead, both strategies incorporate a C-terminal Gly (Martínez-Ceron et al., 2009). As previously described by Liu et al. (2009), Ala is added to the N-terminus of the random sequence to ensure efficient cyclization (i.e., high yields of cyclic peptides).

In an approach developed by Menegatti et al. (2013), allyl ester is used as the orthogonal protecting group, which must be removed using a hazardous and oxygen-sensitive cleavage mixture containing palladium(0). Furthermore, this procedure releases the combinatorial region of the peptide from the whole molecule, resulting in low molecular weight peptides that make MALDI MS/MS analysis difficult due to the interference of matrix-related peaks in the low m/z region. Although ESI MS/MS can be used in this case, MALDI MS/MS is better suited for multiple sample analysis because less time is needed to acquire each spectrum.

When preparing a cyclic peptide library, the cyclization of some sequences may be incomplete. With either strategy described here, incomplete cyclization—i.e., linear peptides—will be obvious in the resulting MS spectra, which can aid in revealing the identity of cyclic peptides that bind the target protein.

The approaches described here allow the screening of combinatorial cyclic-peptide libraries and identification of suitable ligands for diverse applications, particularly in the pharmaceutical industry. These strategies do not require the use of hazardous reagents, such as palladium or cyanogen bromide, and therefore can be used by a broad range of laboratories without specialized expertise in organic synthesis.

Critical Parameters

In both strategies, cyclic peptides are synthesized by an amide linkage between the α-amino terminus and the carboxylic acid group of an Asp residue added to the peptide sequence. To ensure chemoselectivity, Fmoc-Asp[2-phenylisopropyl ester (OPp)]-OH is used. The semi-permanent OPp group is cleaved with 4% trifluoroacetic acid in DCM, whereas most permanent protecting groups commonly used in peptide synthesis are stable. Trityl (Trt) protection of the His side-chain is sensitive to 4% trifluoroacetic acid in DCM, however, so Fmoc-His[tert-butyloxycarbonyl

Table 1 Troubleshooting Advice

Step	Problem	Possible reason	Solution
Alternate Protocol 1	Incomplete coupling of N-terminal Ala	Hydrous Fmoc-Ala-OH was used	Repeat coupling reaction using anhydrous Fmoc-Ala-OH
Basic Protocol 2	Too many positive beads	Library was not properly blocked or too much target protein was used during screening	Block the library or reduce protein concentration
	No positive beads	Low target protein concentration	Increase target protein concentration during the screening
		Target protein not properly labeled	Use fresh reagents. Check the H_2O_2, SA-POD, the DAB, and the 4-chloro-1-naphthol.
		Old reagents	Label a new batch of target protein.
Basic Protocol 3	Bad-quality mass spectra Poor peptide ionization Matrix cluster signals mask the identification of peptide signals	The pH of the sample is too basic due to residual ammonia	After peptide cleavage with ammonia vapor, leave the bead samples in a fume hood to eliminate any residual ammonia.
		Matrix solution is old or the concentration is wrong	Prepare fresh matrix just before use. CHCA concentration must be optimized for each spectrometer. Low concentrations decrease peptide peak signal intensity. High concentrations not only decrease peptide peak signal intensity but also increase matrix cluster peaks. Assay different CHCA concentrations between 2 and 10 mg/ml.
		Matrix need additives	Check the quality of the water and solvents used to prepare the CHCA solution.
			Dope the CHCA solution with 20 mM serine or 10 mM $NH_4H_2PO_4$ final concentration (Martínez Ceron et al., 2010).

Boc)]-OH must be used instead of Fmoc-His(Trt)-OH. The semi-permanent 4-[*N*-(l-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)amino]-benzyl (ODmab) protecting group cannot be used in these strategies because ODmab removal requires hydrazine, a reactive that also cleaves the peptides from the HMBA-CM resin.

Troubleshooting

Table 1 provides a troubleshooting guide for the protocols presented in this article.

Anticipated Results

Libraries constructed using the methods described here allow the screening of cyclic peptides with affinity for target proteins. When using the one-bead–two peptides library with a cyclic peptide and its corresponding linear code, both peptides will elute from an isolated bead, as shown in Figure 5, resulting in a MALDI mass spectrum with two signals—one corresponding to the cyclo-(1,8)-$AX_1X_2X_3X_4X_5$VDG NH_2 and the other (26 mass units lower) corresponding to the linear code H-$AX_1X_2X_3$X4X_5VAG NH_2. The sequence of the positive peptide can be deduced by analyzing the MALDI MS/MS mass spectrum of the linear peptide code. If cyclization is incomplete, the resulting linear peptide will produce a signal 18 mass units higher than its cyclic-peptide counterpart (Fig. 6).

Figure 7 shows an example of MALDI MS and MS/MS spectra of peptide eluted from a single bead of a combinatorial depsipeptide

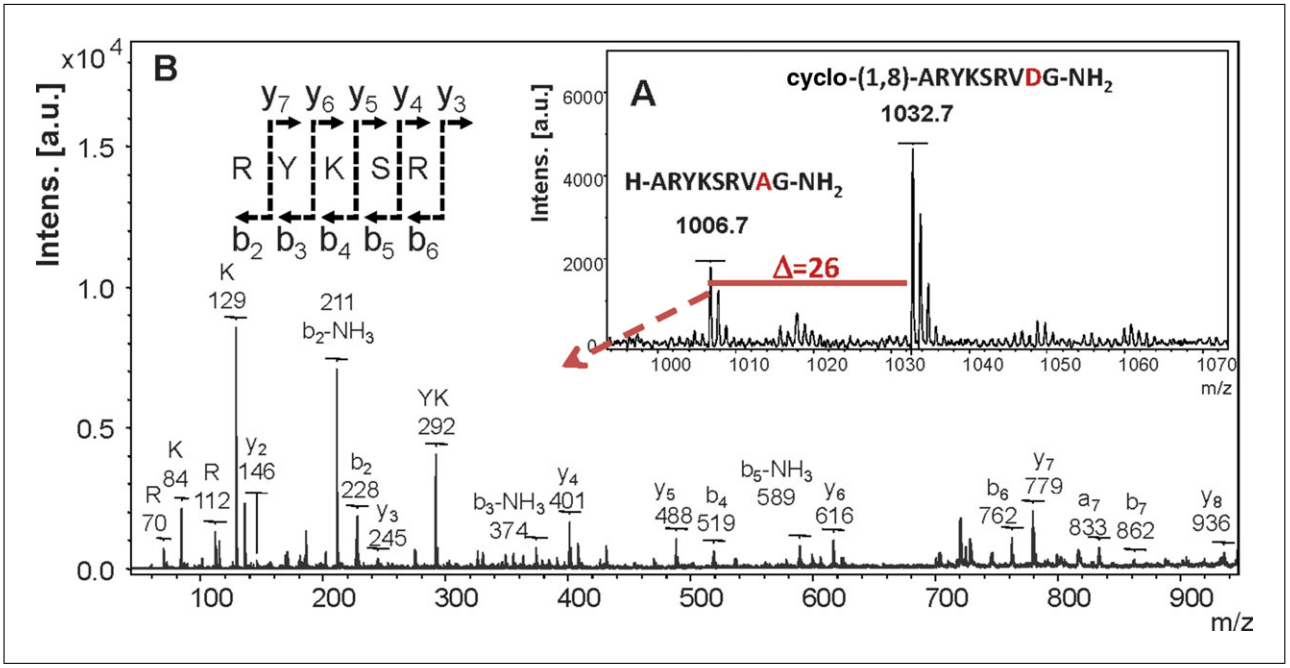

Figure 5 MALDI mass spectrum from a one-bead–two-peptides sample (see Basic Protocol 1). (**A**) The signal at m/z = 1006.7 corresponds to H-ARYKSRVAG-NH_2 $[M + H]^+$ and m/z = 1032.7 to cyclo-(1,8)-ARYKSRVAG-NH_2 $[M + H]^+$. (**B**) MALDI MS/MS mass spectrum of the linear peptide code H-ARYKSRVAG-NH_2. Matrix: CHCA.

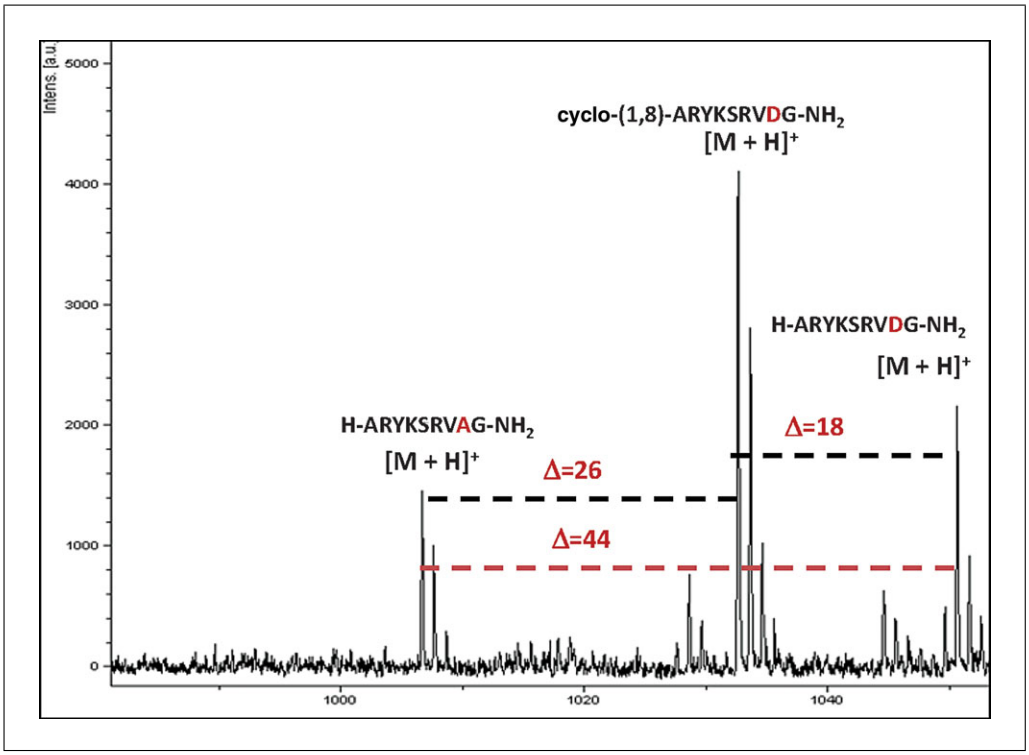

Figure 6 MALDI mass spectrum of peptides from a single one-bead–two-peptides sample with incomplete cyclization. The most abundant signal (m/z = 1032.7) corresponds to cyclo-(1,8)-ARYKSRVDG-NH_2. The signal at m/z = 1050.7 (18 u bigger) corresponds to its linear counterpart due to incomplete cyclization, and the signal at 1006.7 (26 u smaller) corresponds to the linear code H-ARYKSRVAG-NH_2. Matrix: CHCA.

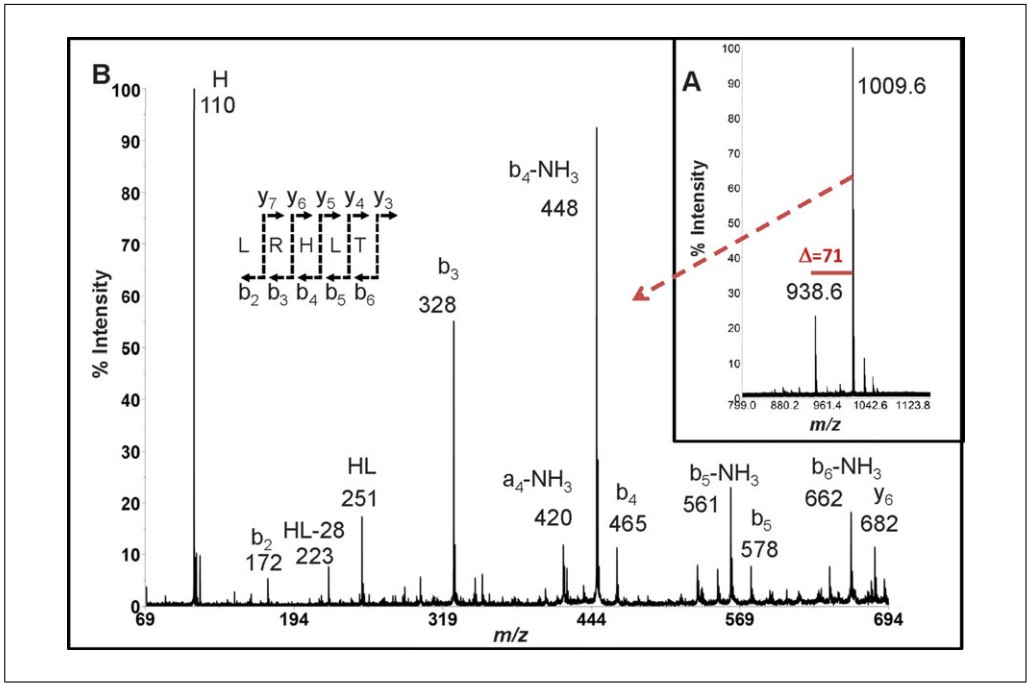

Figure 7 MALDI mass spectrum of peptides from a single depsipeptide sample (see Alternate Protocol 1). (**A**) The signal at m/z 1009.6 corresponds to $HOCH_2CO$-Leu-Arg-His-Leu-Thr-Asp(Ala-NH_2)-Ala-Gly-NH_2 $[M + H]^+$ obtained after ring opening with ammonia. The signal at m/z 938.6 (71 u smaller) corresponds to $HOCH_2CO$-Leu-Arg-His-Leu-Thr-Asp-Ala-Gly-NH_2, $[M + H]$ resulting from incomplete cyclization. (**B**) MALDI MS/MS spectrum of $HOCH_2CO$-Leu-Arg-His-Leu-Thr-Asp(Ala-NH_2)-Ala-Gly-NH_2 $[M + H]^+$. Matrix: CHCA.

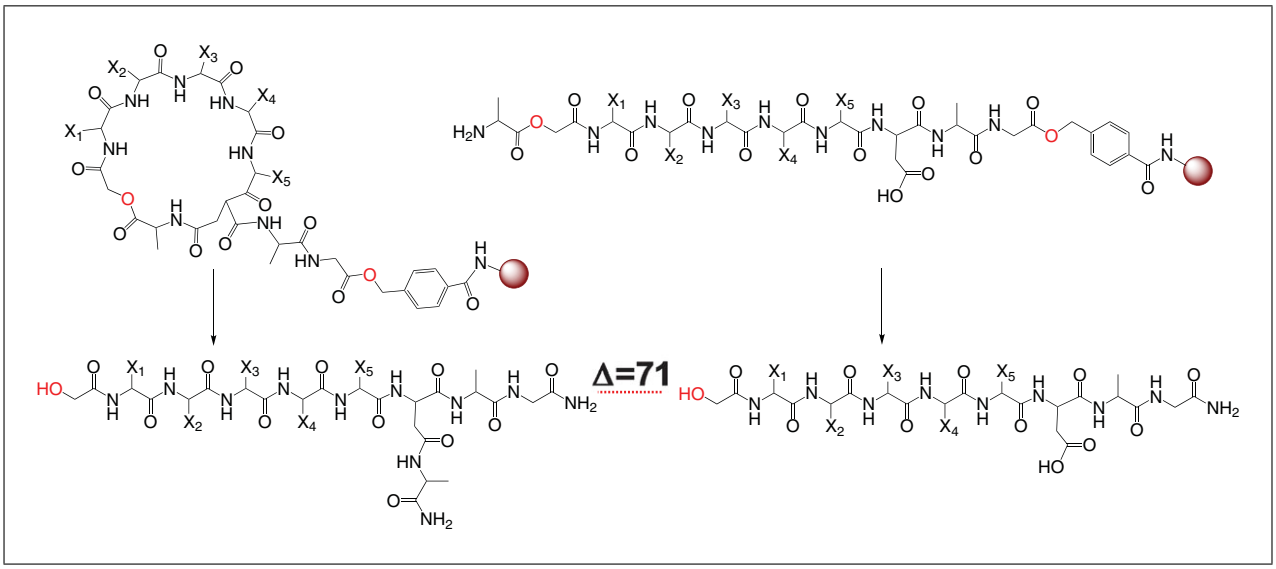

Figure 8 Structures obtained after cleaving the cyclic and linear depsipeptide with ammonia. The mass difference of 71 corresponds to the cleavage of Ala from the linear depsipeptide.

library. The signal at m/z 1009.6 corresponds to the peptide obtained after opening the ring with ammonia, $HOCH_2CO$-$X_1X_2X_3X_4X_5$-Asp(Ala-NH_2)-Ala-Gly-NH_2 $[M + H]^+$. The signal at m/z 938.6 (71 mass units lower than the cyclic peptide) corresponds to $HOCH_2CO$-$X_1X_2X_3X_4X_5$-Gly-NH_2 $[M + H]^+$ resulting from incomplete cyclization (Figs 7A and 8). Peptide sequences could be deduced from the tandem mass spectra (Fig. 7B). The known theoretical values of y_3 ion (m/z 331) due to D(A)AG and y_8 ion ($[M + H]^+ - 58$), and the corresponding b_6 ion ($m/z = [M + H]^+ - y_3 + 1 = [M + H]^+ - 330$) and b_1 ion ($m/z = [M + H]^+ - y_8 + 1 = 59$) made it easier to start sequencing.

Time Considerations

Basic Protocol 1: Synthesis of cyclo-(1,8)-Ala-$X_1X_2X_3X_4X_5$-Val-Asp-Gly-HMBA-CM library with the corresponding linear code Ala-$X_1X_2X_3X_4X_5$-Val-Ala-Gly-HMBA-CM

Steps 1 to 16, couple first amino acid to HMBA-ChemMatrix resin: 1 h + 5 h of incubation.

Steps 17 to 32, add library tag: 1 h + 4 h, 30 min of incubation.

Steps 33 to 42, Divide-Couple-Recombine: 2 h + 2 h of incubation each step.

Steps 43 to 46, couple N-terminal amino acid: 2 h + 2 h of incubation.

Steps 47 to 48, cleave OPp group: 1 h.

Steps 49 to 53, cyclize the peptide: 30 min + 9 h incubation.

Steps 54 to 58, deprotect side chains: 30 min + 2 h incubation.

Alternate Protocol 1: Synthesis of cyclo-(1,8)-Ala-OCH_2CO-$X_1X_2X_3X_4X_5$-Asp-Ala-Gly-HMBA-CM library

Step 1, couple first amino acid to HMBA-ChemMatrix resin: 1 h + 5 h of incubation.

Steps 2 to 11, add library tag: 1 h + 4 h of incubation.

Step 12, Divide-Couple-Recombine: 2 h + 2 h of incubation each step.

Steps 13 to 14, incorporate glycolamidic ester group: 1 h + 2 h of incubation.

Steps 15 to 23, couple N-terminal amino acid: 2 h + 18 h of incubation.

Step 24, OPp cleavage, peptide cyclization and deprotection: see steps 47 to 58 of Basic Protocol 1.

Basic Protocol 2: Two-stage combinatorial cyclic library screening

Steps 1 to 5, hydrate and block library: 1 h + 1 h of incubation.

Steps 6 to 8, incubate library with target protein conjugated toTexas Red: 1 h + 1 h of incubation.

Steps 9 to 11, sort fluorescent beads: 1 h.

Steps 12 to 17, wash, hydrate, and block isolated beads: 1 h 30 min + 1 h of incubation.

Steps 18 to 20, incubate beads with target protein conjugated to biotin: 30 min + 1 h of incubation.

Steps 21 to 23, incubate beads with SA-POD conjugate: 30 min + 1 h of incubation.

Step 24 to 28, Perform colorimetric assay to reveal positive beads: 30 min.

Steps 29 to 32, wash beads: 30 min + 1 h of incubation.

Basic protocol 3: MALDI-TOF/TOF MS/MS analysis

Steps 1 to 6, cleave and elute peptide: 1 h + 12 h of incubation.

Steps 7 to 10, load sample: approximately 30 min for 50 samples.

Step 11, perform MALDI MS/MS: 2 h.

Acknowledgements

This work was partially supported by the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (PIP 0072CO and PIP 11220130100119CO), the Universidad de Buenos Aires (0055BA and 20020130100060BA); the Agencia Nacional de Promoción Científica y Tecnológica de la República Argentina (PICT 2012-1881, PICT-2014-1508, PICT 2012-0888, and Ultraflex II (Bruker) TOF/TOF mass spectrometer grant PME 125/03); the Ministerio de Ciencia y Tecnología e Innovación Productiva and Universidad de Buenos Aires (PDTS-PB03-PCTI74); the Secretaría de Estado de Cooperación Internacional (AECI); the CICYT (CTQ2012-30930); and the Generalitat de Catalunya (2014 SGR 137). S.L.G, M.C.M.C, R.E.B, O.C., and S.A.C. are researchers of the CONICET. We thank Simon Côté from Matrix Innovation Inc. for kindly donating HMBA-ChemMatrix.

Conflict of Interest

The authors declare no conflict of interest.

Literature Cited

Albericio, F. and Kruger, H.G. 2012. Therapeutic peptides. *Future Med. Chem.* 4:1527-1531. doi: 10.4155/fmc.12.94.

Camperi, S.A., Marani, M.M, Iannucci, N.B., Côté, S., Albericio, F., and Cascone, O. 2005. An efficient strategy for the preparation of one-bead-one peptide libraries on a new biocompatible solid support. *Tetrahedron Lett.* 46:1561-1564. doi: 10.1016/j.tetlet.2004.12.105.

Camperi, S.A., Martínez-Ceron, M.C., Giudicessi S.L., Marani, M.M., Albericio, F., and Cascone, O. 2014. Peptide affinity chromatography based on combinatorial strategies for protein purification. *In* Methods in Molecular Biology: Protein Downstream Processing (N.N Labrou, ed.), vol 1129, pp. 277-302. Humana Press Inc., Springer, NY, USA. doi: 10.1007/978-1-62703-977-2_22.

Carmona, S.J., Sartor, P.A., Leguizamón, M.S., Campetella, O.E., and Agüero, F. 2012. Diagnostic peptide discovery: Prioritization of pathogen diagnostic markers using multiple features. *PLoS One* 7:e50748. doi: 10.1371/journal.pone.0050748.

Giudicessi, S.L., Gurevich-Messina, J.M., Martínez-Ceron, M.C., Erra-Balsells, R.,

Albericio, F., Cascone, O., and Camperi, S.A. 2013. Friendly strategy to prepare encoded one bead-one compound cyclic peptide library. *ACS Comb. Sci*. 15:252-259. doi: 10.1021/co400039a.

Gurevich-Messina, J.M., Giudicessi, S.L., Martínez-Ceron, M.C., Acosta, G., Erra-Balsells, R., Cascone, O., Albericio, F., and Camperi, S.A. 2015. A simple protocol for combinatorial cyclic depsipeptide libraries sequencing by matrix assisted laser desorption/ionisation mass spectrometry. *J. Pept. Sci*. 21:40-45. doi: 10.1002/psc.2716.

Joo, S.H. 2012. Cyclic peptides as therapeutic agents and biochemical tools. *Biomol. Ther. (Seoul)* 20:19-26. doi: 10.4062/biomolther.2012.20.1.019.

Lam, K.S. and Lebl, M. 1992. Streptavidin and avidin recognize peptide ligands with different motifs. *Immuno. Methods* 1:11-15. doi: 10.1016/S1058-6687(05)80023-3.

Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M., and Knapp, R.J. 1991. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* 354:82-84. doi: 10.1038/354082a0.

Liang, X., Girard, A., and Biron, E. 2013. Practical ring-opening strategy for the sequence determination of cyclic peptides from one-bead-one-compound libraries. *ACS Comb. Sci.* 15:535-540. doi: 10.1021/co4000979.

Liu, T., Joo, S.H., Voorhees, J.L., Brooks, C.L., and Pei, D. 2009. Synthesis and screening of a cyclic peptide library: Discovery of small-molecule ligands against human prolactin receptor. *Bioorg. Med. Chem.* 17:1026-1033. doi: 10.1016/j.bmc.2008.01.015.

Marani, M.M., Martínez-Ceron, M.C., Giudicessi, S.L., de Oliveira, E., Côté, S., Erra-Balsells, R., Albericio, F., Cascone, O., and Camperi, S.A. 2009. Screening of one-bead-one-peptide combinatorial library using red fluorescent dyes. Presence of positive and false positive beads. *J. Comb. Chem.* 11:146-150. doi: 10.1021/cc800145c.

Martínez-Ceron, M.C., Giudicessi, S.L., Marani, M.M., Albericio, F., Cascone, O., Erra-Balsells, R., and Camperi, S.A. 2010. Sample preparation for sequencing hits from one-bead one-peptide combinatorial libraries by MALDI-TOF/TOF MS. *Anal. Biochem.* 400:295-297. doi: 10.1016/j.ab.2010.01.029.

Martínez-Ceron, M.C. Giudicessi, S.L., Kruszyn, J.N., Marani, M.M., Albericio, F. Cascone, O., and Camperi, S.A. 2015. Two-stage screening of combinatorial peptide libraries. Application to bovine serum albumin ligand selection. *Rev. Cenic Cienc. Biológicas* 46:77-86.

Martínez-Ceron, M.C, Giudicessi, S.L., Saavedra, S.L., Gurevich-Messina, J.M., Erra-Balsells, R., Albericio, F., Cascone, O., and Camperi, S.A. 2016. Latest advances in OBOC peptide libraries. Improvements in screening strategies and enlarging the family from linear to cyclic libraries. *Curr. Pharm. Biotech.* 17:449-457. doi: 10.2174/1389201017666160114095553.

Menegatti, S., Ward, K.L., Naik, A.D., Kish, W.S., Blackburn, R.K., and Carbonell, R.G. 2013. Reversible cyclic peptide libraries for the discovery of affinity ligands. *Anal. Chem.* 85:9229-9237. doi: 10.1021/ac401954k.

Redman, J.E., Wilcoxen, K.M., and Ghadiri, M.R. 2003. Automated mass spectrometric sequence determination of cyclic peptide library members. *J. Comb. Chem.* 5:33-40. doi: 10.1021/cc0200639.

Rozinov, M.N. and Nolan, G.P. 1998. Evolution of peptides that modulate the spectral qualities of bound, small-molecule fluorophores. *Chem. & Biol.* 5:713-728. doi: 10.1016/S1074-5521(98)90664-0.