Research Article

Journal of PeptideScience

Received: 15 July 2014 Revised: 17 October 2014 Accepted: 22 October 2014 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/psc.2716

A simple protocol for combinatorial cyclic depsipeptide libraries sequencing by matrix-assisted laser desorption/ionisation mass spectrometry

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Short cyclic peptides have a great interest in therapeutic, diagnostic and affinity chromatography applications. The screening of 'one-bead-one-peptide' combinatorial libraries combined with mass spectrometry (MS) is an excellent tool to find peptides with affinity for any target protein. The fragmentation patterns of cyclic peptides are quite more complex than those of their linear counterparts, and the elucidation of the resulting tandem mass spectra is rather more difficult. Here, we propose a simple protocol for combinatorial cyclic libraries synthesis and ring opening before MS analysis. In this strategy, 4-hydroxymethylbenzoic acid, which forms a benzyl ester with the first amino acid, was used as the linker. A glycolamidic ester group was incorporated after the combinatorial positions by adding glycolic acid. The library synthesis protocol consisted in the following: (i) incorporation of Fmoc-Asp[2-phenylisopropyl (OPp)]-OH to Ala-Gly-oxymethylbenzamide-ChemMatrix, (ii) synthesis of the combinatorial library, (iii) assembly of a glycolic acid, (iv) couple of an Ala residue in the *N*-terminal, (v) removal of OPp, (vi) peptide cyclisation through side chain Asp and *N*-Ala amino terminus and (vii) removal of side chain protecting groups. In order to simultaneously open the ring and release each peptide, benzyl and glycolamidic esters were cleaved with ammonia. Peptide sequences could be deduced from the tandem mass spectra of each single bead evaluated. The strategy herein proposed is suitable for the preparation of one-bead-one-cyclic depsipeptide libraries that can be easily open for its sequencing by matrix-assisted laser desorption/ionisation MS. It employs techniques and reagents frequently used in a broad range of laboratories without special expertise in organic synthesis. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: glycolic acid; 4-hydroxymethylbenzoic acid; 2-phenylisopropyl; ChemMatrix resin; MALDITOF MS/MS

Introduction

Short peptides with affinity for target proteins are widely used in therapeutic and diagnostic applications and as ligands in affinity chromatography [1–6]. Particularly, cyclic peptides have a great interest because of their resistance to enzymatic degradation and higher selectivity than their linear counterparts [7,8].

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Abbreviations: *AcOH, acetic acid; Boc, N-tert-butoxycarbonyl; CHCA, α-cyano-4-hydroxycinnamic acid; CM, ChemMatrix; DIPCDI, 1,3-diisopropylcarbodiimide; DIPEA, N,N-diisopropylethylamine; DMAP, 4-(N,N-dimethylamino)pyridine; DMF, N,N-dimethylformamide; ESI, electrospray ionisation; Fmoc, fluorenylmethoxycarbonyl; Gly-HMBA-CM, gly-oxymethylbenzamide-ChemMatrix; HMBA, 4-hydroxymethylbenzoic acid; HMBA-CM, hydroxymethylbenzamide-ChemMatrix resin; HOAt, hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MALDI, matrix-assisted laser desorption/ionisation; MS, mass spectrometry; ODamb, 4-[N-(L-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)amino]-benzyl; OPp, 2-phenylisopropyl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; tBu, O-tert-butyl; TBTU, O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl.*

The screening of 'one-bead-one-peptide' combinatorial libraries is an excellent tool to find peptides with affinity for any given protein [9]. In these libraries, each bead displays only one peptide entity. After library screening, the structure of the peptide contained on each positive bead is determined.

Electrospray ionisation (ESI) [10] and matrix-assisted laser desorption/ionisation (MALDI) [11,12] mass spectrometry (MS) are the techniques of choice for peptide and protein sequencing [13]. For multiple sample analysis, less time is needed for MALDI MS than for ESI MS, so MALDI MS is more suitable for high throughput analysis of combinatorial peptide libraries. In addition, when a sample has been analysed by ESI, it cannot be reanalysed, a limitation not shared by MALDI, an offline technique in which samples are spotted to (and archived on) a plate prior to ionisation. After one-bead-one-peptide libraries screening, peptides have to be detached from each bead before MS analysis. Hence, a cleavage site to release the peptide from each bead has to be introduced. The photolabile linker reported by Fitzgerald *et al*. [14] is not of general applicability, and peptides could suffer early photolytic cleavage by room light during the screening process. Other linker such as *N*-[(9-hydroxymethyl)-2-fluorenyl] succinamic acid [15] is not applicable because it is based on the same principle than Fmoc chemistry. On the other hand, the linker 4-hydroxymethylbenzoic acid (HMBA), described by Atherton *et al*. [16], is suitable for Fmoc chemistry, and peptides can be released with ammonia, which is easily evaporated without leaving contaminants that could interfere in the MS analysis [17,18]. The fragmentation patterns of cyclic peptides are quite more complex than those of their linear counterparts, and the elucidation of the resulting tandem mass spectra is rather more difficult [19]. 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. In these cases, both entities are released and analysed together by MS [20,21]. To facilitate cyclic peptide library sequencing without using a coding tag, two different approaches were recently developed. Both are based on ring opening before MS analysis. Liang *et al*. [22] proposed a practical strategy that uses CNBr for ring opening. However, it has the disadvantage of using a very hazardous reagent, especially when working with hundreds of individual beads selected from the screening process. On the other hand, Menegatti *et al*. [23] synthesised a depsipeptide combinatorial library that employs lactic acid and the dipeptide ester (*N*α-Ac)-Ser(Ala)- as linkers for dilactonisation. In this strategy, allyl is used as orthogonal protecting group, which is removed by a hazardous and air sensitive cleavage mixture containing Pd (0), excluding the use of this method in those laboratories that are not strictly organic oriented. Furthermore, in this procedure, the combinatorial region of the peptide is released from the whole molecule, generating low molecular weight peptides. That makes MALDI MS/MS analysis difficult because of the interference of matrix-related peaks in the low *m/z* region.

Here, we propose an alternative protocol for the synthesis of combinatorial cyclic depsipeptide libraries and ring opening before MS analysis. The present strategy combines the use of ChemMatrix (CM) resin, the HMBA linker, the glycolic acid and the Asp[2-phenylisopropyl (OPp)]-OH for cyclic depsipeptide synthesis. Vapour phase aminolysis is used to simultaneously open the ring and release the peptide for MS/MS.

Materials and Methods

Chemicals, Consumables and Equipment

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

HMBA-ChemMatrix™ resin was kindly donated by Matrix Innovation Inc. (Montreal, Québec, Canada). Fluorenylmethoxycarbonyl (Fmoc)-Ala-OH, Fmoc-Arg[2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf)]-OH, Fmoc-gly-OH, Fmoc-leu-OH, Fmoc-thr[*O*-tert-butyl (tBu)]-OH, Fmoc-tyr(tBu)-OH, O-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBt) were from Peptides International Inc. (Louisville, KY, USA). Fmoc-His[*N*-tert-butoxycarbonyl (Boc)]-OH and Fmoc-Asp[2-phenylisopropyl (OPp)]-OH were from Bachem Americas, Inc. (CA; USA). 4-(*N*,*N*-Dimethylamino)pyridine (DMAP), hydroxy-7-azabenzotriazole (HOAt), α-cyano-4-hydroxycinnamic acid (CHCA), serine and glycolic acid were from Sigma-Aldrich (St Louis, MO, USA). 1,3-Diisopropylcarbodiimide (DIPCDI), *N*,*N*-diisopropylethylamine (DIPEA), triisopropyl silane (TIS) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were from Fluka Chemie AG (Buchs, Switzerland). All other reagents were AR grade.

Combinatorial Cyclic Depsipeptide Library Synthesis

One-bead-one-peptide combinatorial library of the depsipeptide cyclo-(1,8)-Ala-OCH_2CO-$X_1X_2X_3X_4X_5$-Asp-Ala-Gly-oxymethylbenzamide-ChemMatrix, where X = Arg, His, Leu, Pro, Thr and Tyr, was synthesised on HMBA-CM resin (35–100 mesh-wet sieved-, 100-200 mesh-dry-, 0.63 mmol/g) by using the divide–couple–recombine method according to Lam *et al*. [9] with Fmoc chemistry. The library had 7776 different peptides (6^5). Gly was incorporated at the C-terminal, by adding Fmoc-Gly-OH (3 eq.) with DIPCDI (4 eq.), followed by dropwise addition of a solution of DMAP dissolved in DMF (0.1 eq., ~50 mmol/l) as described by Mellor *et al*. [24]. After 1 h, the resin was washed with DMF, and the steps for Gly incorporation were repeated to recouple the amino acid. After washing the resin with DMF, the remaining hydroxyl functions were acetylated with Ac_2O (6 eq.) and DMAP (0.1 eq.) in a minimum amount of DMF during 1 h. Then, Fmoc-Ala-OH and Fmoc-Asp(OPp)-OH were assembled with 3 eq. of each Fmoc-AA-OH, TBTU (3 eq.) and DIPEA (6 eq.) in DMF during 2 h. Random positions were synthesised by repeated cycles of dividing, coupling and recombining as previously described [9]. Each portion was coupled with 3 eq. of a different Fmoc-amino acid, TBTU (3 eq.) and DIPEA (6 eq.) in DMF during 2 h. After the five combinatorial positions, a glycolamidic ester group was incorporated by adding glycolic acid (2 eq.), HOBt (4 eq.) and DIPCDI (2.5 eq.), and the reaction was monitored by the Kaiser test. To facilitate the further library cyclisation reaction, Ala was assembled at the *N*-terminal by mixing vigorously Fmoc-Ala-OH (not hydrated) (20 eq.) with DIPCDI (10 eq.) in CH_2Cl_2 during 5 min to generate the symmetrical anhydride and adding the mixture to the peptidyl resin followed by dropwise addition of a solution of DMAP dissolved in DMF (0.1 eq., ~50 mmol/l). After 1 h, the resin was washed with DMF (2 × 2 min), and the steps for Ala incorporation were repeated to recouple the amino acid. To determine the extent of resin substitution a known quantity of Fmoc-loaded peptidyl resin was treated with piperidine in DMF, and the amount of the

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dibenzofulvene-piperidine adduct released was measured spectrophotometrically at 290 nm [25]. In case of incomplete loading, Ala coupling steps were repeated. 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 Ala *Nα*-terminus was removed with piperidine/DMF (1/4), and OPp cleavage was conducted with TFA/CH_2Cl_2 (4/96) (4 × 15 min). Peptide cyclisation was performed on the solid-phase support with PyBOP (4 eq.), HOAt (4 eq.) and DIPEA (8 eq.) in DMF/CH_2Cl_2 (3/1) (3 × 1 h). The progress of the cyclisation was monitored by the Kaiser test and also by MALDI MS by treating a small amount of resin with TFA/TIS (triisopropyl silane)/H_2O (95/2.5/2.5) during 4 h and analysing peptides from single beads as is explained in the succeeding texts. In case of incomplete cyclisation, the reaction was repeated. 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 (Scheme 1).

Depsipeptide Ring Opening and Peptide Release

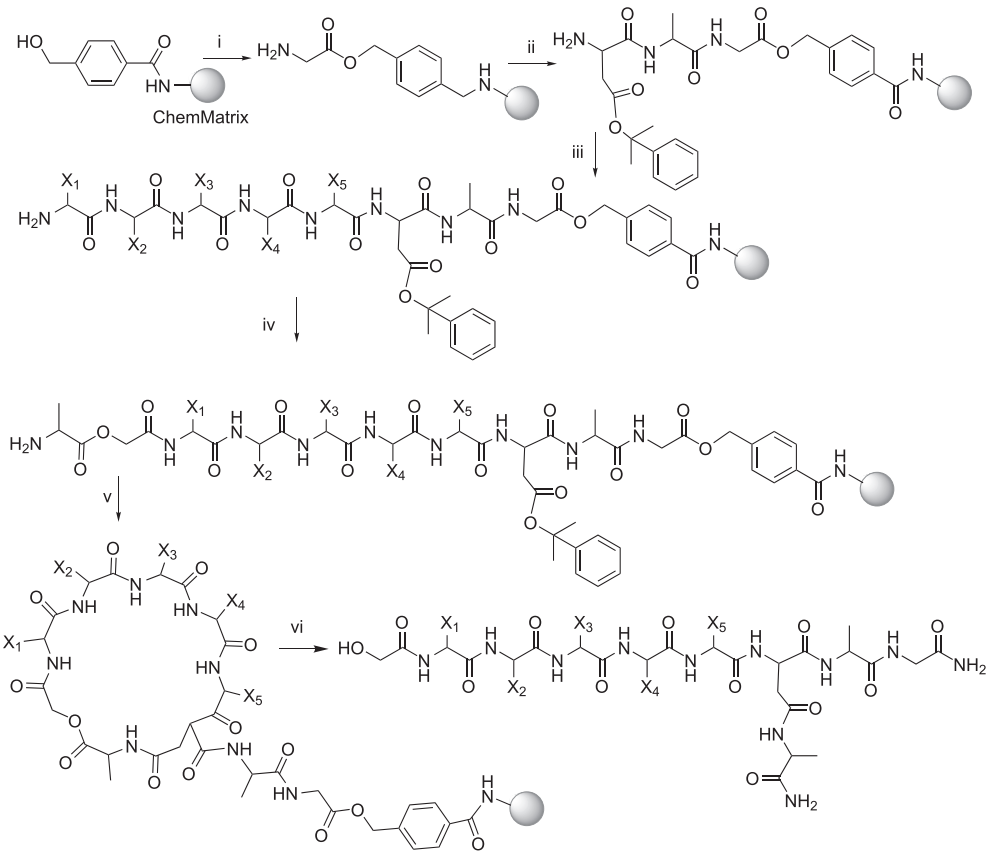
Single beads were placed into separate micro tubes, which were placed in a drying chamber together with a flask containing 30% NH_4OH during 15 h. Ring opening and peptide release from each bead was performed simultaneously by vapour phase aminolysis. Released peptides were eluted from each bead by adding 10 µl acetic acid (AcOH)/acetonitrile (CH_3CN)/H_2O (3/4/3) to each micro tube [1] (Scheme 1).

MALDI MS Analysis

Matrix-assisted laser desorption/ionisation MS were recorded in an Ultraflex II TOF/TOF (Bruker Daltonics GmbH, Leipzig, Germany). The analysis was performed as per Martínez-Ceron *et al.* [26]. An amount of 1 µl aliquot of eluted peptide from a single bead was loaded onto the sample plate, air dried at room temperature, and then, 1 µl CHCA 4 mg/ml in CH_3CN/H_2O (1/1) with 0.1% TFA and doped with serine 20 mM [27] 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 ion mode.

Results and Discussion

The strategy herein proposed is summarised in Scheme 1. HMBA, which forms a benzyl ester with the first amino acid, was used as the linker [16]. A glycolamidic ester group was incorporated after



Scheme 1. Strategy for a combinatorial cyclic depsipeptide library synthesis and vapour phase aminolysis to simultaneously open the ring and release the peptide for tandem mass spectrometry analysis. (i) (a) Fmoc-Gly-OH (3 eq.), DIPCDI (4 eq.), DMAP (0.1 eq.) in DMF; (b) piperidine/DMF (1/4). (ii) (a) Fmoc-Ala-OH (3 eq.), TBTU (3 eq.), DIPEA (6 eq.) in DMF; (b) piperidine/DMF (1/4); (c) Fmoc-Asp(OPp)-OH (3 eq.), TBTU (3 eq.), DIPEA (6 eq.) in DMF. (iii) (a) Library preparation (divide–couple–recombine). (iv) (a) Glycolic acid (2 eq.), HOBt (4 eq.), DIPCDI (2.5 eq.); (b) Fmoc-Ala-OH (20 eq.), DIPCDI (10 eq.) in CH_2Cl_2; (c) DMAP (0.1 eq.) in DMF; (d) piperidine/DMF (1/4). (v) (a) Selective OPp cleavage with TFA/CH_2Cl_2 (4/96); (b) cyclisation: PyBOP (4 eq.), HOAt (4 eq.), DIPEA (8 eq.) in DMF/CH_2Cl_2 (3/1).; (c) side chain deprotection with TFA/TIS/H_2O (95/2.5/2.5). (vi) (a) NH_4OH.

the five combinatorial positions by adding glycolic acid [28]. To avoid multiple incorporation of glycolic acid, only 2 eq. were added in the reaction mixture. However, the risk of multiples incorporation of glycolic acid moieties was not high because ester bonds formation needs harsher conditions than that of amide bonds. Afterward, to assure the glycolamidic ester bond formation, Ala was assembled using the symmetrical anhydride method with 20 eq. of Fmoc-Ala-OH and DMAP [29].

Fmoc-Asp(OPp)-OH was added to the peptide sequence for solid-phase ring formation [30]. The semipermanent group OPp was cleaved with 4% TFA, and an amide linkage was formed between the Ala amino terminus, and the carboxylic acid function as was previously reported [20]. Although most permanent protecting groups are stable in these conditions, they are sufficient to detach the side chain trityl (Trt) protection of His residue. Then, Fmoc-His(Boc)-OH must be used instead of Fmoc-His(Trt)-OH [20,31].

The glycolamidic ester and the benzyl ester are stable to piperidine, the reagent used for Fmoc group removal, making both applicable for chemical elongation of a peptide sequence with Fmoc strategies [16,30]. The glycolamidic and HMBA inertness to TFA allows side chain deprotection without either opening the ring or releasing the peptide from the resin. The removal of protecting groups is required because screening should be carried out on fully unprotected cyclic library.

Ala was assembled at the *N*-terminus to facilitate ring formation, and Ala-Gly was introduced as a spacer arm at the *C*-terminal, to facilitate ligand–protein interaction in the screening step and to increase the molecular weight of the peptides in order to improve MALDI MS analysis by avoiding matrix interferences of low molecular weight peptides.

A sample of beads from the combinatorial library was isolated for MS analysis. Depsipeptides cleavage from each bead and ring opening of cyclic depsipeptides was performed simultaneously by vapour phase aminolysis using NH_4OH. CM, used as the solid support, is stable under nucleophilic conditions because it contains exclusively primary ether bonds [17,32]. Unlike other nucleophiles such as NaOH, the ammonia is easily removed by evaporation and hence does not interfere with the MS peptide analysis. Figure 1 shows an example of a MALDI mass spectrum and MALDI tandem mass spectrum of peptides eluted from one bead of the combinatorial library. The signal at *m/z* 1009.6 corresponds $HOCH_2CO$-Leu-Arg-His-Leu-Thr-Asp(Ala-NH_2)-Ala-Gly-NH_2 [M H] obtained after ring opening with ammonia. The small signal at *m/z* 938.6 with a difference of 71 u with the *m/z* 1009.6, corresponds to $HOCH_2CO$-Leu-Arg-His-Leu-Thr-Asp-Ala-Gly-NH_2 ([M H]-71) obtained because of incomplete cyclisation (Figure 1(a), Scheme 2). The relative abundance of the signal ([M H]-71) changed with the peptide sequence because of the different yield of cyclisation obtained with each member of the library. Hence, MALDI MS analysis will indicate the original structure or structures of the ligands bounded on the bead to which the target protein has interacted during the screening process. In the tandem mass spectrum, prominent signals corresponding to the loss of ammonia (b-17 ions) because of the presence of Arg were observed.

Peptide sequences could be deduced from the tandem mass spectra (Figure 1(b)). The known theoretical values of y_3 ion because of D(A)AG (*m/z* 331) 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. Other MS and MS/MS spectra are shown in Supporting Information.

After library screening and MS sequencing, selected peptide ligands are resynthesised in larger amounts to study their performance for their intended use. In order to assure cyclic ligand stability, the Ala-glycolamidic ester bond can be replaced by an Ala-Gly amide bond by assembling Gly instead of glycolic acid. Although it is highly probable that the slight difference between both ligands will not interfere with peptide ligand interaction, it is advisable to synthesise first a small amount of the corresponding peptidyl resin and assess its performance with the probe protein as in the screening process.

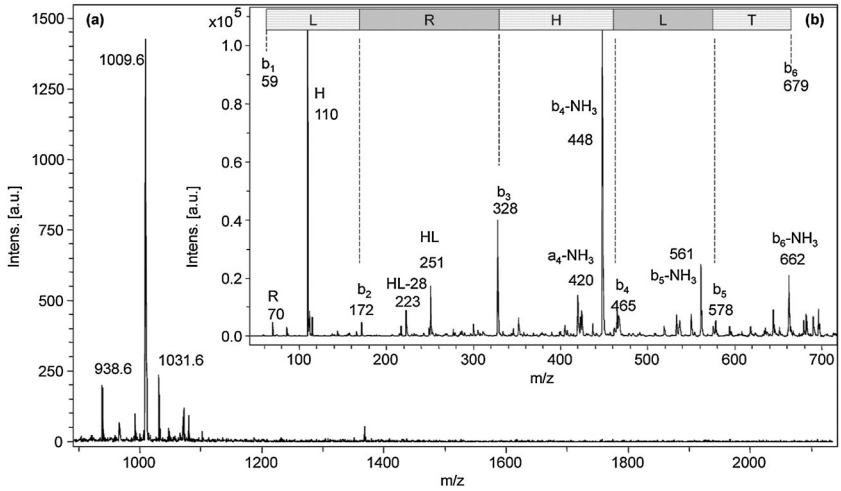


Figure 1. (a) Matrix-assisted laser desorption/ionisation mass spectrum from the sample of one bead of the depsipeptide library cyclo-(1,8)-Ala-OCH_2CO-$X_1X_2X_3X_4X_5$-Asp-Ala-Gly-NH_2. One bead was placed into a microtube, which was placed in a drying chamber together with a flask containing NH_4OH. Released peptides were eluted with 10 µl AcOH/CH_3CN/H_2O (3/4/3), and 1 µl sample was loaded onto the sample plate, air dried at room temperature, and then, 1 µl matrix solution (CHCA 4 mg/ml in CH_3CN/H_2O (1/1) with 0.1% TFA and doped with serine 20 mM) was added to the spot without mixing. 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 corresponds to $HOCH_2CO$-Leu-Arg-His-Leu-Thr-Asp-Ala-Gly-NH_2, ([M H]-71) obtained because of incomplete cyclisation. (b) Matrix-assisted laser desorption/ionisation tandem mass spectrometry spectrum of $HOCH_2CO$-Leu-Arg-His-Leu-Thr-Asp(Ala-NH_2)-Ala-Gly-NH_2 [M H]. The b_1 and b_6 fragments were calculated: b_6 ion (m/z = [M H]-y_3 + 1 = [M H]-330 = 679) and b_1 ion (m/z = [M H]-y_8 + 1 = 59).

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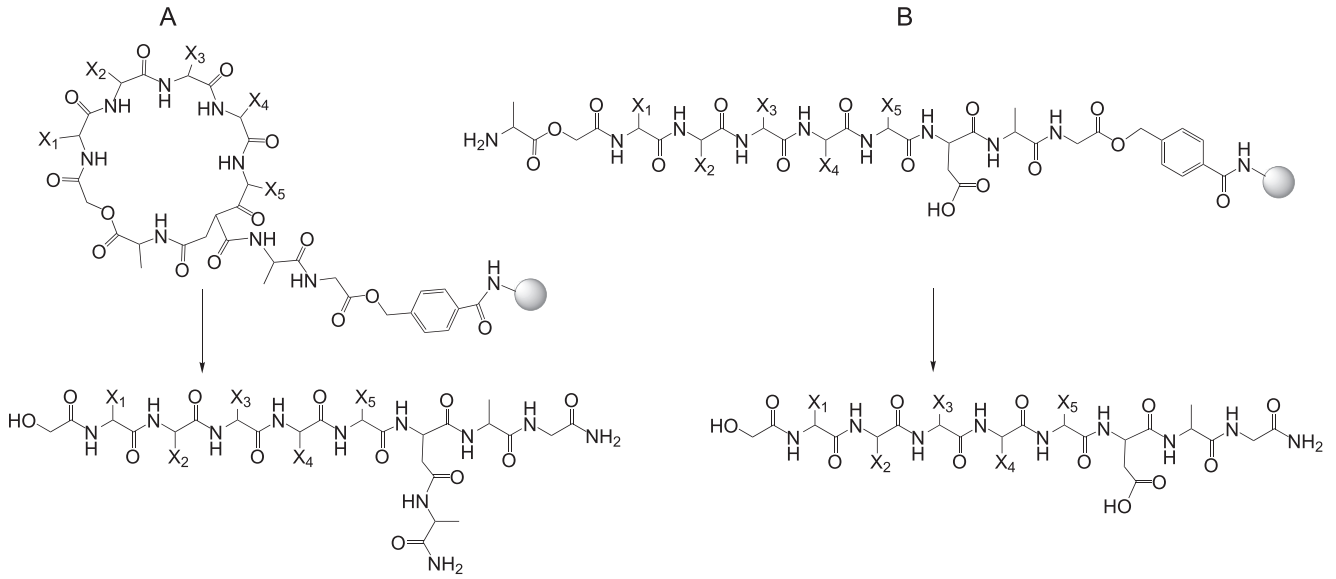

Scheme 2. Vapour phase aminolysis of (A) cyclic and (B) linear depsipeptides.

Conclusion

The strategy herein proposed is suitable for the preparation of one-bead-one-cyclic depsipeptide libraries that can be easily open for its sequence identification by MS. The molecular weights of the peptides released are suitable for MALDI MS/MS. This approach will allow the screening of these libraries with target proteins in order to find suitable cyclic ligands for diverse applications. In this strategy, hazardous reagents such as Pd or CNBr are not used, and therefore, it can be applied in a broad range of laboratories without special expertise in organic synthesis.

Acknowledgements

This work was partially supported by the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (PIP 11220090100230CO and PIP 11220090100400), the Universidad de Buenos Aires (20020100100005BA, 20020130100060BA and EX20020100100088), the Ministerio de Ciencia y Tecnología e Innovación Productiva and Universidad de Buenos Aires (PDTS-PB03- PCTI 74), the Secretaría de Estado de Cooperación Internacional (AECI), the CICYT (CTQ2012-30930) and the Generalitat de Catalunya (2009SGR 1024). 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 donation of HMBA-ChemMatrix.

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