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Preparation of Cyclic Peptide Libraries Using Intramolecular Oxime Formation

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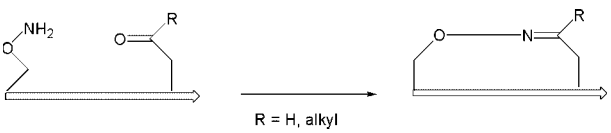
Abstract: A new method for the synthesis of cyclic head-to-side chain peptide libraries has been developed in which the key cyclization step involves reaction between a *C*-terminal ketone and an *N*-terminal hydroxylamine to form a macrocyclic oxime. This methodology efficiently delivers cyclic products that consist of mixtures of *syn* and *anti* isomers. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclic peptides; solid phase peptide synthesis; peptide libraries; oxime formation

INTRODUCTION

In connection with our studies on cyclic peptides with the potential both to interact with DNA [1] and to coordinate certain metal ions, an efficient method was required for the synthesis of cyclic peptide libraries [2]. Previously reported methods for the synthesis of cyclic peptides have been reviewed in recent years and include a diverse array of methodologies [3–5]. Macrocyclizations in the presence of reactive functional groups, such as those that are found in peptides, require the use of highly chemoselective cyclization chemistry to deliver efficient cyclization yields and purities. One appropriately chemoselective reaction is the formation of oximes by the condensation of hydroxylamine ethers with suitably electrophilic carbonyl groups. This chemistry has previously been employed for the synthesis of cyclic peptides [6–9] as well as chemoselective peptide ligation [9–17] and our aim was to determine whether intramolecular oxime formation was sufficiently reliable and robust for the generation of cyclic peptide libraries. The design of cyclization precursors essentially involved the partnering of a hydroxylamine ether nucleophile with a sufficiently electrophilic carbonyl group (Scheme 1).

In order to facilitate high throughput synthesis of the cyclic oximes, an approach was chosen in which the key cyclization reaction was performed on the solid phase and to this end, the condensation of an aldehyde was initially investigated with a hydroxylamine ether. Others have shown that peptide glyoxaldehydes can be generated by oxidative cleavage of terminal serine residues with sodium periodate [6–8,15]. In this case, oxidative cleavage



Scheme 1

Abbreviations: Abbreviations listed in the guide published in *J. Pept. Sci.* 1999: **5**: 465 are used without further explanation. Additional abbreviations are listed in the text where first cited.

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was performed on support-bound terminal serines and while initial results with our own systems showed promise, repetition of these experiments provided variable results and therefore this method was assessed to be too unreliable for the consistent formation of the glyoxaldehyde electrophile and thence the cyclic peptides. After investigation of numerous methods for the introduction of a suitable carbonyl group, it was found that the best results in terms of ease, reproducibility and product quality were obtained using a levulinic acid residue, a residue commonly used as the source of an electrophilic carbonyl group for chemoselective peptide ligation [10,11,14].

METHODS AND MATERIALS

SynPhase™ crowns and Multipin™ components; inert plastic stems, (8 × 12) polypropylene microtitre plate stem holders and Beckman chemical resistant 1 ml polypropylene microtitre plates were supplied by Mimotopes (Melbourne, Australia). 1 ml Titertube® micro tubes were purchased from Bio-Rad (Sydney, Australia).

Unless stated otherwise, all reagents used were AR grade and used as supplied without further purification. N^{α}-Fmoc-L-α-amino acids were peptide synthesis grade and purchased from Calbiochem-Novabiochem (San Diego, USA), Auspep (Melbourne, Australia) or supplied by Mimotopes (Melbourne, Australia). *N*-Trityl-aminooxyacetic acid was prepared using established methodology [18]. HBTU, DIPEA and piperidine were peptide synthesis grade and purchased from Auspep (Melbourne, Australia). HOBt was purchased from Fluka (Sydney, Australia). DCM, diethyl ether, DMF and methanol were obtained from BDH Laboratory Supply (Melbourne, Australia). Acetonitrile was HPLC grade and obtained from BDH Laboratory Supply (Melbourne, Australia). Water was Milli-Q grade. Inorganic salts were obtained from APS Ajax Finechem or BDH Laboratory Supply (Melbourne, Australia). TFA, triethylsilane (TES) and trisopropylsilane (TIPS) were obtained from Aldrich (Sydney, Australia).

General Experimental Procedure for the Preparation of Oxime Cyclic Peptides 7a-l/10a-d

Synthesis was carried out on I-series HM SynPhase™ crowns (loading: 7.0 μmol) functionalized with a *p*-{(R,S)-α-[1-(9*H*-fluoren-9-yl)-methoxyformamido]-2,4-dimethyl benzyl} phenoxyacetamide (Rink) linker. The crowns were attached to inert plastic stems mounted on a 8 × 12 polypropylene microtitre plate stem holder. Unless stated otherwise, all reactions were carried out at room temperature in individual 1 ml reaction wells of a Beckman chemical resistant 1 ml polypropylene microtitre plate. Washing of the crowns was carried out in chemically resistant polypropylene wash baths containing 200 ml of the appropriate solvent.

Qualitative trinitrobenzenesulfonic acid (TNBS) test for primary amines. A solution of 0.5% (w/v) TNBS/DMF (100 μl) was added to a solution of 5% DIPEA/DMF (100 μl) and mixed thoroughly. A small shaving of one of the crowns was obtained using a razor blade and placed into the solution for 10 min after which the colour of the shaving was checked. A change in colour of the shaving to red/orange indicated that the crown had tested positive for the presence of primary amines. When there was no change in colour of the shaving, the crown had tested negative for the presence of primary amines.

Synthesis of the linear precursor. The crowns were Fmoc deprotected by immersion in a solution of 20% piperidine/DMF (500 μl) for 20 min. The crowns were removed from the solution washed with DMF (2 × 5 min), MeOH (1 × 10 min) and air-dried (TNBS test: positive). The crowns were then placed in a solution of Fmoc-Lys(Dde)-OH (31 mg, 0.05 mmol), HBTU (19 mg, 0.05 mmol) and HOBt (8 mg, 0.05 mmol) in DMF, (500 μl), which had been pre-activated with DIPEA (9 μl, 0.05 mmol) for 5 min. After 2 h, the crowns were washed [DMF (2 × 5 min), MeOH (1 × 5 min)] and air-dried (TNBS test: negative). This cycle of deprotection/coupling was sequentially repeated for the core residues AA_2–AA_n (where $n = 5$–9) using Fmoc-Asp(OtBu)-OH (21 mg, 0.05 mmol), Fmoc-Lys(Boc)-OH (23 mg, 0.05 mmol), Fmoc-Ile-OH (18 mg, 0.05 mmol). The crowns were Fmoc deprotected (as described above, TNBS test: positive) and immersed in a solution of *N*-Trityl-aminooxyacetic acid (20 mg, 0.05 mmol) and HOBt (8 mg, 0.05 mmol) in 50% DCM/DMF (500 μl), which had been pre-activated with DIC (8 μl, 0.05 mmol) for 10 min. After 2 h the crowns were washed (as described above) and air-dried (TNBS test: negative). The crowns were then treated with a solution (500 μl) of 2% N_2H_4/DMF for 10 min, washed [DMF (2 × 5 min), MeOH (1 × 10 min)], air-dried (TNBS test: positive) and placed into a solution of levulinic acid (0.1 mmol) in 50% DCM/DMF (500 μl), which had been pre-activated with DIC (8 μl, 0.05 mmol) for 5 min. After 2 h the crowns

were washed [DMF (1 × 5 min), MeOH (1 × 5 min)] and air-dried (TNBS test: negative).

Cyclization of the linear precursor. The crowns were subjected to successive treatments (4 × 5 min) with solutions of 2% TFA/5% TIPS/DCM (500 µl). The crowns were then washed [DMF (2 × 5 min), MeOH (1 × 5 min)] and air-dried.

Cleavage and side chain deprotection. Each crown was placed in a solution (in a 1 ml Titertube® micro tube) of 5% TES/TFA (1 ml) for 2 h. The crowns were removed from the cleavage mixture, allowing any excess liquid to drain back into the tube, then discarded. The TFA was evaporated under a gentle stream on nitrogen and the remaining residue taken up in diethyl ether (1 ml). The tubes were capped, gently shaken and left to stand for 5 min. The resulting white precipitate was centrifuged and the supernatant carefully removed using vacuum suction. The addition of ether and subsequent steps were repeated and the precipitate air-dried in a fume cupboard to remove any traces of ether. The precipitate was dissolved in 50% CH_3CN/H_2O (1 ml) and lyophilized to give crude peptide as a white solid.

LC-MS analysis was carried out using a Perkin-Elmer Sciex API-100 single quadropole mass spectrometer equipped with an electrospray ionization source coupled to a Shimadzu chromatography system (equipped with duel LC-10AD pumps and a LC-10A UV/VIS detector set at 214 nm and 254 nm). HPLC analysis was conducted on a reverse phase Monitor 5 µm C18 50 × 4.6 mm column using a linear gradient of 0–100% B over 11.0 min at a flow rate of 1.5 ml/min (solvent A: 0.1% TFA/H_2O, solvent B: 0.1% TFA/90% CH_3CN/H_2O). After splitting from the column, the eluent (300 µl/min) was infused directly into the ESI source. Mass spectra were acquired in the positive ion mode (ESP+) over 12.5 min with a cone voltage of +50 V and a scan range of m/z 100–3000.

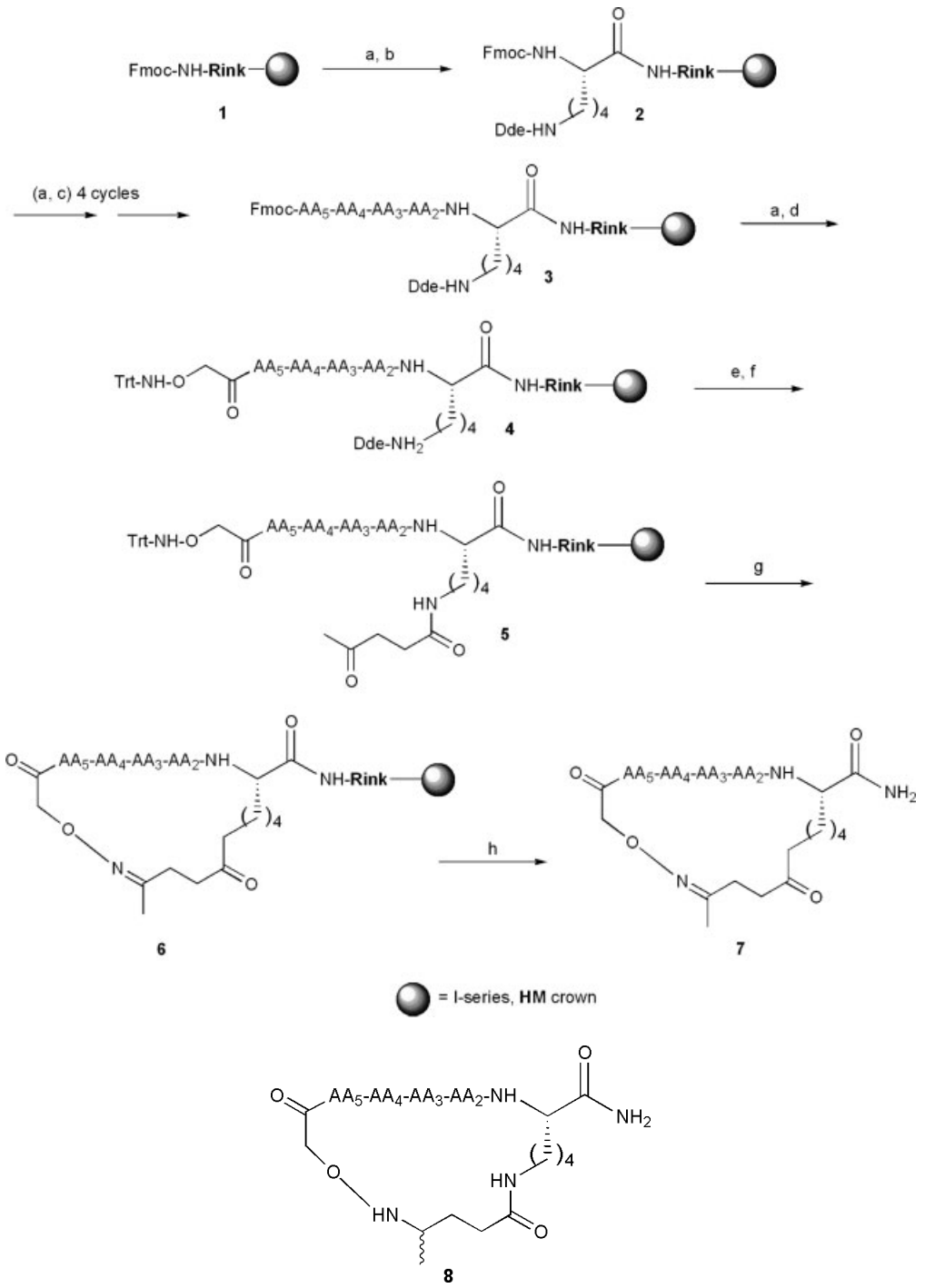
Analytical reverse phase HPLC was performed on a Waters chromatography system equipped with duel 510 pumps and a Model 440 UV detector set at 214 nm and 254 nm. Analysis was conducted on a reverse phase Rainin Microsorb-MV C18 (5 µm 100A), 50 × 4.6 mm column using a linear gradient of 0–100% B over 11.0 min at a flow rate of 1.5 ml/min (solvent A: 0.1% H_3PO_4/H_2O, solvent B: 0.1% H_3PO_4/90% CH_3CN/H_2O).

RESULTS AND DISCUSSION

A number of optimization studies were carried out in which a general protocol for the synthesis of head-to-side chain cyclic oxime peptides was established outlined in (Scheme 2). Synthesis of the linear precursors was carried out using standard Fmoc chemistry on SynPhase™ crowns functionalized with a Rink amide handle (**1**). The crowns were firstly Fmoc-deprotected using 20% piperidine/DMF, then an orthogonally protected lysine residue (Fmoc-Lys(Dde)-OH) was coupled as its activated HOBt ester by the use of HOBt, HBTU and DIPEA to give **2**. The lysine α-amino groups were then deprotected and the core of the linear sequences established using the same coupling conditions to give **3**. The *N*-terminal Fmoc groups were then removed under standard conditions and the exposed *N*-terminal amines acylated using the HOBt ester of trityl-protected aminooxyacetic acid to give peptide **4**. The *C*-terminal Dde groups were then removed using 2% hydrazine in DMF and the exposed amines were acylated with levulinic anhydride to give the keto-peptide **5**. Finally, deprotection of the masked hydroxylamines with mild acid revealed the nucleophilic hydroxylamine ethers, which condensed with the levulinamide ketone carbonyl groups to give the solid phase-bound target macrocyclic oximes (**6**). These macrocyclic peptides were then cleaved from the solid support under standard conditions to give the target cyclic oxime peptides (**7**).

To investigate the suitability of this methodology for the synthesis of cyclic peptide libraries a small library of 12 peptides was prepared using simultaneous multiple synthesis with Multipin methodology [19,20] on SynPhase™ crowns, as described above. Variation in the peptide sequences was accomplished by scrambling the four core residues AA_2–AA_5, relative to the *C*-terminal lysine residue (Table 1). Residues AA_2–AA_5 were representative of the residues displayed in the DD/EXK motif found in the active site of the type II restriction endonucleases *Eco*RV/*Bgl*I [21,22]. After cleavage from the solid phase the peptide products were subjected to LC-MS and analytical RP-HPLC analysis (Table 2).

LC-MS analysis revealed that, for each peptide, quantitative cyclization had been achieved. An interesting feature of the HPLC profiles was the resolution of the cyclic oxime peptide into two major isomeric components (Figure 1). It was reasoned that these isomeric components



Scheme 2 Reagents and conditions: (a) piperidine, r.t, 20 min; (b) Fmoc-Lys(Dde)-OH, HBTU, HOBt, DIPEA, r.t, 2 h; (c) Fmoc-AA_{2-5}-OH, HBTU, HOBt, DIPEA, r.t, 2 h; (d) Trt-$NHOCH_2COOH$, DIC, HOBt, r.t, 2 h; (e) N_2H_2, r.t, 10 min; (f) $CH_3COC_2H_4COOH$, DIC, r.t, 2 h; (g) TFA, TIPS, r.t, 4 × 5 min; (h) TES, TFA, r.t, 2 h.

were, respectively, the *syn* and *anti* isomers of the macrocyclic oximes. While oximes have been employed previously for peptide cyclization [6–9], to our knowledge, this is the first occasion on which the expected *syn* and *anti* isomers have been successfully resolved. However, this phenomenon has been observed in the reaction of hydroxylamines with carbohydrates resulting

in similar HPLC profiles [23–25]. The relative proportions of these isomers (Table 2) is approximately equal in all cases and presumably under thermodynamic control as oximes and hydrazones are known to equilibrate under acidic conditions [26].

The HPLC profiles of the cyclic oximes **7a-l**, in some cases also revealed the presence of a minor component that eluted before the cyclic oxime isomers (component A, Figure 2). MS analysis of this component revealed a dominant molecular ion at m/z 772.6 that corresponded to the $[M+H]^+$ molecular ion for the cyclic aminooxy-ether **8**. The $[M+2H]^{2+}$ for **8** was also observed at m/z 387.0. The formation of this reduced compound was believed to be a result of the TFA/TES conditions encountered upon cleavage of the cyclic peptide from the solid support, as such conditions have previously been used for the deliberate reduction of non-peptide oximes [27,28]. Reduction of the oxime bond was further supported by exposure of cyclic oxime **7e** to cleavage conditions for a further 24 h. HPLC analysis of the resulting product (Figure 3) showed a decrease in the relative amount of the cyclic oxime isomers (**7e**) and an increase in the corresponding cyclic aminooxy-ether **8e** (Table 3). A second aminooxy-ether cyclic peptide was also observed (component A, Figure 3), presumably the minor diastereoisomer of **8e**. These results also provided further support the formation of (*E*-) and (*Z*-) geometric isomers for the cyclic oximes **7a-l**. It was found that formation of the aminooxy-ether by-products could be minimized to an acceptable level (< 1%) by decreasing the amount of TES used in the cleavage step or by using the less reactive silane scavenger TIPS.

Experiments were also carried out to determine the extent to which the length of the linear peptide

Table 1 Sequences for Peptides **3–8(a-l)**

3–8[a]	AA_2	AA_3	AA_4	AA_5
a	Asp	Asp	Lys	Ile
b	Asp	Asp	Ile	Lys
c	Asp	Lys	Asp	Ile
d	Asp	Lys	Ile	Asp
e	Asp	Ile	Asp	Lys
f	Asp	Ile	Lys	Asp
g	Lys	Asp	Asp	Ile
h	Lys	Asp	Ile	Asp
I	Lys	Ile	Asp	Asp
j	Ile	Asp	Lys	Asp
k	Ile	Asp	Asp	Lys
l	Ile	Lys	Asp	Asp

[a] In **3–6** Asp = Asp(OtBu) and Lys = Lys(Boc)

Table 2 LC-MS and Analytical RP-HPLC Data for **7a-l**

Peptide	LC-MS data		RP-HPLC data	
7	t_R[a] (min)	$[M+H]^+$[b]	t_R[a] (min)	Ratio
a	4.50, 4.61	770.4, 770.4	4.47, 4.56	0.98
b	4.17	770.4	4.00, 4.47	0.98
c	4.46	770.4	4.38, 4.46	0.98
d	4.24, 4.39	770.4, 770.2	4.16, 4.29	0.97
e	4.68, 4.97	770.4, 770.4	4.63, 4.96	0.93
f	4.31	770.4	4.12, 4.20	0.98
g	4.61, 4.75	770.4, 770.4	4.50, 4.71	0.96
h	4.10, 4.24	770.4, 770.4	4.00, 4.12	0.97
i	4.57, 4.90	770.6, 770.6	4.62, 4.97	0.93
j	4.46, 4.61	770.6, 770.4	4.65, 4.93	0.95
k	4.53, 4.75	770.4, 770.6	4.45, 4.66	0.94
l	4.61, 4.86	770.4, 770.6	4.52, 4.80	0.94

[a] t_R at 214 nm; [b] $[M+H]^+$ calculated for **7a-l**: 770.4.

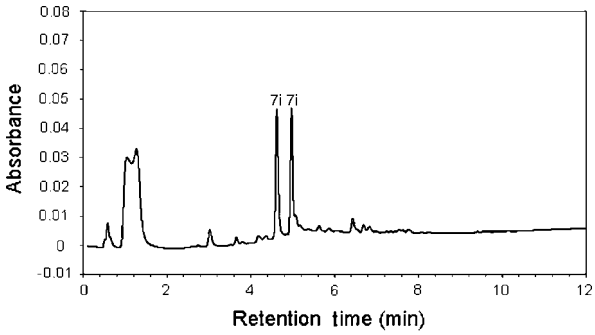


Figure 1 HPLC profile at 214 nm of crude **7i**.

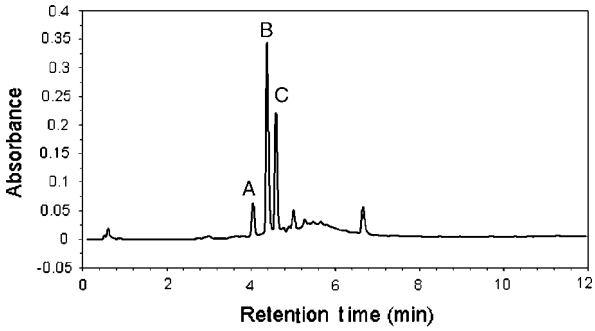


Figure 2 HPLC profile at 214 nm of crude **7e**.

Table 3 Results of the Analytical RP-HPLC of Crude **7e** after being Subjected to TFA-mediated Cleavage Conditions for 24 h

Component	Peptide product	% Area of peaks	$[M + H]^+$
A	Cyclic aminooxy-ether **8e**	12.52 (< 1[a])	770.4[b] (770.4)[c]
B	Cyclic aminooxy-ether **8e**	16.65 (6.44[a])	770.6[b] (770.4)[c]
C	Cyclic oxime **7e**	13.34 (35.20[a])	772.6[b] (772.4)[c]
D	Cyclic oxime **7e**	8.70 (21.52[a])	772.6[b] (772.4)[c]

[a] % Area before reduction; [b] $[M + H]^+$ observed; [c] $[M + H]^+$ calculated.

Table 4 Sequences and LC-MS Data for Peptides 10a–d

9[a]/10	*n*	$(AA_n–AA_5–AA_4–AA_3–A_2)$	t_R[b] (min)	$[M + H]^+$
a	6	Asp-Ile-Lys-Asp-Asp	4.28	885.6[c] (885.4)[d]
b	7	Asp-Asp-Ile-Lys-Asp-Asp	4.18	1000.8[c] (1000.6)[d]
c	8	Lys-Asp-Asp-Ile-Lys-Asp-Asp	3.88	1128.8[c] (1128.6)[d]
d	9	Ile-Lys-Asp-Asp-Ile-Lys-Asp-Asp	4.56	1241.8[c] (1241.6)[d]

[a] Asp = Asp(OtBu), Lys = Lys(Boc); [b] t_R at 214 nm; [c] $[M + H]^+$ observed; [d] $[M + H]^+$ calculated.

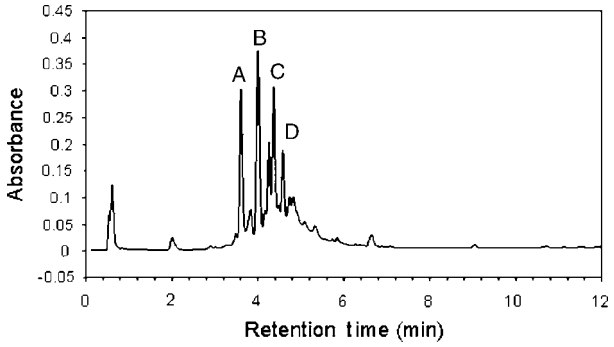


Figure 3 HPLC profile at 214 nm of crude **7e** after being subjected to TFA-mediated cleavage conditions for 24 h.

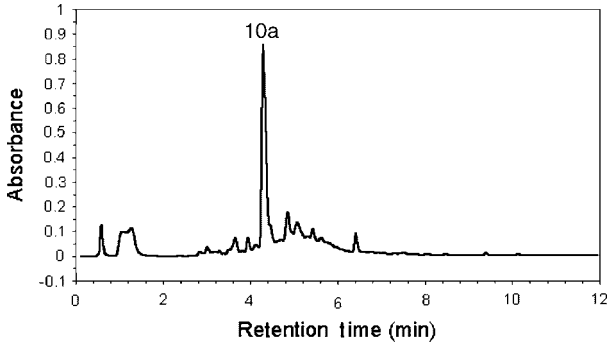


Figure 4 HPLC profile at 214 nm of crude **10a**.

precursor affected the efficiency of the cyclization reaction. To this end, four linear precursors **9a–d** were synthesized and cyclized as outlined in Scheme 2 with the core sequence varying in length from 5 to 9 residues (Table 4). LC-MS analysis of the resulting products showed quantitative cyclization of **9a–d** to the corresponding cyclic oximes **10a–d**, however, the geometrical isomers were not discernable in the HPLC profiles for **10a–d** (Figure 4). These results suggested that the methodology could be used efficiently to generate larger cyclic oximes.

CONCLUSION

In conclusion, it has been shown that the formation cyclic head-to-side chain cyclic peptides, using intramolecular oxime formation, provides a viable and robust route to libraries of cyclic peptides. It is anticipated that the efficiency of cyclization will help to facilitate the generation of libraries of cyclic peptides for a diverse array of applications.

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