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Examination of methodology for the synthesis of cyclic thioether peptide libraries derived from linear tripeptides

KADE D. ROBERTS* and NICHOLAS J. EDE
Mimotopes Pty Ltd, 11 Dueridin Street, Clayton, Victoria 3168, Australia

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Abstract: Work was undertaken to examine methodology for the cyclization of linear tripeptides on the solid phase via intramolecular *S*-alkylation using the Multipin™ Solid-Phase Peptide Synthesis platform. While previous work had shown that this chemistry could be used to efficiently cyclize linear tetrapeptide libraries, application of this synthetic strategy to the model linear tripeptide sequence Leu-Ser-Lys resulted in significant cyclic dimer formation. Ultimately, it was found that the addition of a large excess of lithium in the form of LiCl to the cyclization solution, significantly reduced cyclic dimer formation affording highly pure crude cyclic monomer. The application of this modified cyclization protocol to the preparation of cyclic peptide libraries was successfully demonstrated with the synthesis of a 20-membered library **4**{***1–20***} based on the linear tripeptide sequence Leu-**Xxx**-Lys in which the position **Xxx** was varied with the standard 20 proteogenic residues. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclization of linear peptides; cyclic thioether peptides: cyclic peptides; peptide libraries; lithium chloride; SynPhase; lanterns; Multipin

INTRODUCTION

The ability to simultaneously generate large libraries of diverse peptides has allowed for rapid advancements in the exploration of protein–protein interactions (e.g. epitope mapping, enzyme substrate mapping, structure–activity relationship (SAR) studies, analogue development) and as such, has made peptide libraries valuable tools in the drug discovery process. Whilst to date the literature on the synthesis of cyclic peptides has been numerous and diverse as highlighted in several recent reviews on the topic [1–6], there have been few reports detailing the parallel synthesis of large libraries of discrete cyclic peptides containing three or more amino acid residues [7–11]. This is largely due to the fact, that in order for the high throughput synthesis and screening of large cyclic peptide libraries to be an efficient process, the synthetic platform utilized must be able to generate large numbers of crude products in parallel without the need for extensive work up, a feat that is not easily achieved for cyclic peptides [12–14].

Recently, we have reported efficient methodology for the cyclization of linear tetrapeptides on the solid phase using intramolecular *S*-alkylation and its successful application to the parallel synthesis and cyclization of a 72-membered library of linear tetrapeptides using the Multipin™ Solid-Phase Peptide Synthesis (SPPS) platform [11]. This synthetic approach involves the incorporation of an orthogonally protected cysteine residue at the *C*-terminus of the linear sequence and capping of the *N*-terminus of the sequence with bromoacetic acid. The side chain thiol of the cysteine is selectively deprotected and reacts exclusively with the *N*-terminus to form a cyclic thioether link (Scheme 1). From the perspective of preparing large libraries, this synthetic approach is quite attractive in that it makes use of commercially available reagents, the chemical reactions are robust, the cyclization reaction appears to be tolerant to sequence variation and when used in combination with the Multipin™ SPPS platform has the potential to provide 10s to 1000s of discrete cyclic peptides in parallel [5,11,15,16]. Furthermore, *in vitro* studies have shown the cyclic thioether end products obtained from this synthetic approach to be very stable towards proteolytic degradation [17,18], which makes them attractive targets with respect to the development of peptide therapeutics.

In our quest to establish generic methodology for the synthesis of cyclic peptide libraries, we have continued to explore the use of the intramolecular *S*-alkylation cyclization strategy and in this paper describe work undertaken to extend the application of this synthetic approach to the preparation of cyclic peptides libraries derived from diverse linear tripeptide sequences. Cyclization of a linear tripeptide sequence using the traditional approach of head-to-tail intramolecular lactam formation is a formidable task as demonstrated by the fact there are no examples in the literature of homodetic cyclic trimers containing only L-α-amino acid residues throughout the sequence [4,6]. Attempting to cyclize small linear peptides containing

Abbreviations: Abbreviations listed in the guide *J. Pept. Sci.* 2006; **12**: 1 are used without further explanation. Additional references are listed in the text where first cited.

*Correspondence to: Kade D. Roberts, Department of Pharmacology, University of Melbourne, Level 8 Medical Building, Grattan Street, Parkville, Victoria 3010, Australia; e-mail: kader@unimelb.edu.au

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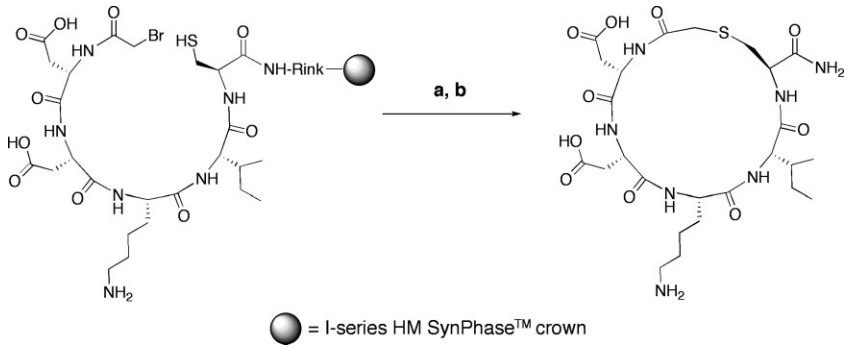

Scheme 1 Reagents and conditions: (a) 1% DIPEA/DMF; (b) 5% TES/TFA.

3–6 residues via intramolecular lactam formation is inherently difficult as the *trans* conformation of the amide bonds makes the linear sequence a highly rigid system. As a result, the structural strain placed on such a highly rigid system when trying to align the reactive termini in a conformation conducive to cyclization often makes intermolecular reactions more favourable. The consequence of this is significant linear and cyclic oligomerization severely compromising the purity of the resulting product [19–26]. This is the case whether the cyclization is carried out in solution under conditions of high dilution or on the solid phase under pseudo-dilution conditions. Therefore, cyclization strategies other than lactam formation need to be examined and to this end it has previously been observed that efficient cyclization can been achieved using intramolecular *S*-alkylation where cyclic lactam formation has been unsuccessful [27].

METHODS AND MATERIALS

L-Series PA SynPhase™ Lanterns (loading: 2.3 μmol/lantern) functionalized with a Rink amide linker and Multipin™ components; inert plastic stems, (8 × 12) polypropylene microtiter plate stem holders and Beckman chemical resistant 1 ml polypropylene microtiter plates were supplied in-house by Mimotopes (Melbourne, Australia). Terumo® 5 and 10 ml plastic disposable syringes were obtained from Terumo (Laguna, Philippines). 10 ml centrifuge tubes were obtained from TECHNO-PLAS (St Mary's, Australia). Unless stated otherwise, all the reagents used were AR grade and used as supplied without further purification. N^{α}-Fmoc-L-α-amino acids and N^{α}-Fmoc-D-α-amino acids were peptide synthesis grade and purchased from Calbiochem-Novabiochem (San Diego, USA). HOBt was purchased from GL Biochem (Shanghai, China). Diethyl ether was GR (general reagent) grade and was obtained from Merck (Melbourne, Australia). It was dried over $CaCl_2$ and filtered through Al_2O_3 before further use. DMF was GR grade and was obtained from Merck (Melbourne, Australia). It was distilled over Ninhydrin before further use. DCM, MeOH and piperidine were GR grade and were obtained from Merck (Melbourne, Australia). Acetonitile was HPLC grade and was obtained from Merck (Melbourne, Australia). Water was Milli-Q grade. Lithium chloride (LiCl) was obtained from BDH Laboratory Supply (Melbourne, Australia). TFA was Biograde™ and was obtained from Halocarbon (New Jersey, USA). DIPEA was obtained from Fluka (Sydney, Australia). DIC, triethylsilane (TES) and triisopropylsilane (TIPS) were obtained from Sigma-Aldrich (Sydney, Australia).

LC-MS analysis was carried out using an Applied Biosystems MDS Sciex API-2000 triple quadropole mass spectrometer equipped with an electrospray ionisation source coupled to an Agilent 1100 series chromatography system equipped with a Gilson 215 Autosampler. The UV/VIS detector was set at 214 nm. RP-HPLC analysis was conducted on a reverse phase Monitor, 5 micron C18 50 × 4.6 mm column using a linear gradient of 0–100% B over 12.0 min at a flow rate of 1.5 ml/min (Solvent A: 0.05% v/v TFA/H_2O, Solvent B: 0.05% v/v TFA/90% v/v 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 20 min with a declustering potential (DP) of +60 V and a scan range of m/z 100–1800.

Optimized Experimental Procedure for the Preparation of Cyclic Thioether Peptide 1

This protocol applies to the synthesis of one copy of the peptide, i.e. use of one lantern or multiple copies of the peptide, i.e. use of multiple lanterns. Unless stated otherwise, all reactions were carried out at room temperature in 5 or 10 ml Terumo® plastic disposable syringes fitted with a filter plug and a teflon tap. Synthesis was carried out on L-series PA SynPhase™ lanterns with a loading of 2.3 μmol per lantern.

Synthesis of the linear precursor. The lantern(s) was Fmoc deprotected by immersion in a solution of 25% piperidine/DMF for 30 min. The lantern(s) was filtered and washed with DMF (3 × 3 min), MeOH (1 × 3 min) and air-dried under vacuum suction. The lantern(s) was then placed in a solution of Fmoc-Cys(Mmt)-OH (0.0161 mmol/lantern) and HOBt (0.0161 mmol/lantern) in DMF, which had been pre-activated with DIC (0.0161 mmol/lantern) for 20 min. The volume of DMF used was enough to fully cover the lantern(s). The lantern(s) was left to stand for 2 h, then filtered and washed with DMF (2 × 3 min), MeOH (1 × 3 min) and air-dried under vacuum suction. This cycle of deprotection/coupling was sequentially repeated for the residues AA_2-AA_4 using Fmoc-Lys(Boc)-OH (0.0161 mmol/lantern), Fmoc-Ser(tBu)-OH (0.0161 mmol/lantern) and Fmoc-Leu-OH (0.0161 mmol/lantern) under the same conditions.

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The lantern(s) was then Fmoc deprotected (as described above) and immersed into a solution of bromoacetic acid (0.0322 mmol/lantern) in 50% DCM/DMF, which had been pre-activated with DIC (0.0161 mmol/lantern) for 10 min. The coupling reaction was left to proceed overnight. The lantern(s) was washed with DMF (2 × 3 min), MeOH (1 × 3 min) and air-dried under vacuum suction.

Cyclization of the linear precursor. The lantern(s) was placed into a solution of 2% TFA/5% TIPS/DCM and gently agitated for 10 min. The lantern(s) was filtered and this step was repeated until no more yellow colouration (formation of the methoxy-trityl cation) was observed. After washing with DCM (3 × 3 min) and air-drying under vacuum suction the lantern(s) was placed into a solution of LiCl (250 eq, 0.575 mmol/lantern) in 2% v/v DIPEA/DMF (500 µl/lantern) (2 × 30 sec, 1 × 2 h). The lantern(s) was filtered and washed with DMF (2 × 3 min), H_2O (2 × 3 min), MeOH (1 × 3 min) and air-dried under vacuum suction.

Cleavage and side chain deprotection. The lantern(s) was placed into a solution of 2.5% H_2O/5% TIPS/TFA (3 ml) in a 10 ml centrifuge tube for 2 h. The lantern(s) was 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 of nitrogen and the remaining residue taken up in cold diethyl ether (3 ml) and placed in the freezer for 1 h. The resulting white precipitate was centrifuged, the diethyl ether decanted and the remaining precipitate air-dried in a fume cupboard to remove any traces of ether. The precipitate was dissolved in 40% CH_3CN/H_2O and lyophilized to give crude cyclic thioether peptide **1** as a white solid. Yield: 0.8 mg, 71%. LC-MS (214 nm): [$t_R = 4.05$ min], m/z 489.4. (calcd. for $C_{20}H_{37}N_6O_6S$ $[M+H]^+$ 489.6).

General Experimental Procedure for the Preparation of Cyclic Peptide Library 4{*1–20*}

Synthesis was carried out on L-series PA SynPhase™ lanterns with a loading of 2.5 µmol per lantern.

Synthesis of the linear precursors. The lanterns were placed into 10 ml Terumo® plastic disposable syringes fitted with a filter plug and a teflon tap and Fmoc deprotected by immersion in a solution of 25% piperidine/DMF for 30 min. The lanterns were filtered and washed with DMF (3 × 3 min), MeOH (1 × 3 min) and air-dried under vacuum suction. The lanterns were then placed in a solution of Fmoc-Cys(Mmt)-OH (0.0161 mmol/lantern) and HOBt (0.0161 mmol/lantern) in DMF, which had been pre-activated with DIC (0.0161 mmol/lantern) for 20 min. The volume of DMF used was enough to fully cover the lanterns. The lanterns were left to stand for 2 h, then filtered and washed with DMF (2 × 3 min), MeOH (1 × 3 min), and air-dried under vacuum suction. This cycle of deprotection/coupling was repeated for the residue AA_2 using Fmoc-Lys(Boc)-OH (0.0161 mmol/lantern). The lanterns were then attached to inert plastic stems and mounted onto a (8 × 12) polypropylene microtiter plate stem holder. Unless stated otherwise, all the proceeding reactions were carried out in parallel at room temperature in individual 1 ml reaction wells of a Beckman chemical resistant 96-well polypropylene microtiter plate in a concealed container. Washing of the lanterns was carried out in chemically resistant polypropylene wash baths containing 200 ml of the appropriate solvent with gentle agitation on a shaker table. Washing times of 5 min instead of 3 min were used. The lanterns were Fmoc deprotected (as described above) and the 20 proteogenic residues (0.0161 mmol/lantern) were coupled as their pre-activated HOBt esters overnight (as described above). This was followed by the coupling of Fmoc-Leu-OH (0.0161 mmol/lantern) coupled as its pre-activated HOBt ester overnight (as described above). The lanterns were then Fmoc deprotected (as described above) and immersed into a solution of bromoacetic acid (0.0322 mmol/lantern) in 50% DCM/DMF, which had been pre-activated with DIC(0.0161 mmol/lantern) for 10 min. The coupling reactions were left to proceed overnight. The lanterns were washed with DMF (2 × 5 min), MeOH (1 × 5 min) and air-dried in the fume hood.

Cyclization of the linear precursors. The lanterns were placed into a solution of 2% TFA/5% TIPS/DCM (200 ml) in a polypropylene wash bath and gently agitated on a shaker table for 10 min. The lanterns were then added to a fresh solution of 2% TFA/5% TIPS/DCM (200 ml) for 10 min. This was repeated until no more yellow colouration (formation of the methoxy-trityl cation) was observed. After washing with DCM (3 × 5 min) and air-drying in the fume hood, the appropriate lanterns were placed into either a solution of LiCl (400 eq, 1.15 mmol/lantern) in DMF (500 µl/lantern) (2 V 30 s, 1 × 18 h) or 2% v/v DIPEA/DMF (500 µl/lantern) (2 × 30 s, 1 × 18 h). The parallel cyclization reactions were carried out in a concealed container. The lanterns were then filtered and washed with DMF (2 × 5 min), H_2O (2 × 5 min), MeOH (1 × 5 min) and air-dried in the fume hood.

Cleavage and side chain deprotection. Each lantern, still attached to its stem, was removed individually from the (8 × 12) polypropylene microtiter plate stem holder and placed into a solution of 2.5% H_2O/5% TIPS/TFA (3 ml) in individual 10 ml centrifuge tubes for 2 h. The lanterns 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 of nitrogen and the remaining residue taken up in cold diethyl ether (3 ml). The tubes were capped and placed in the freezer for 2 h. The resulting white precipitates were centrifuged, the diethyl ether decanted and the remaining precipitate air-dried in a fume cupboard to remove any traces of ether. The individual peptide products were then dissolved in 40% CH_3CN/H_2O (200 µl) and sampled for LC-MS analysis.

RESULTS AND DISCUSSION

The initial target sequence for cyclization was the linear tripeptide Leu-Ser-Lys, a model sequence that was designed to give good HPLC and MS profiles. Synthesis and cyclization of this linear sequence was carried out using standard Fmoc chemistry on Rink functionalized PA SynPhase™ lanterns (Scheme 2). PA SynPhase™ lanterns are the latest generation of modular SynPhase™ support, which have now superseded the HM SynPhase™ crowns [28–30]. The grafted polymer surface of PA SynPhase™ lanterns is based on a polyamide co-polymer that is physically more stable

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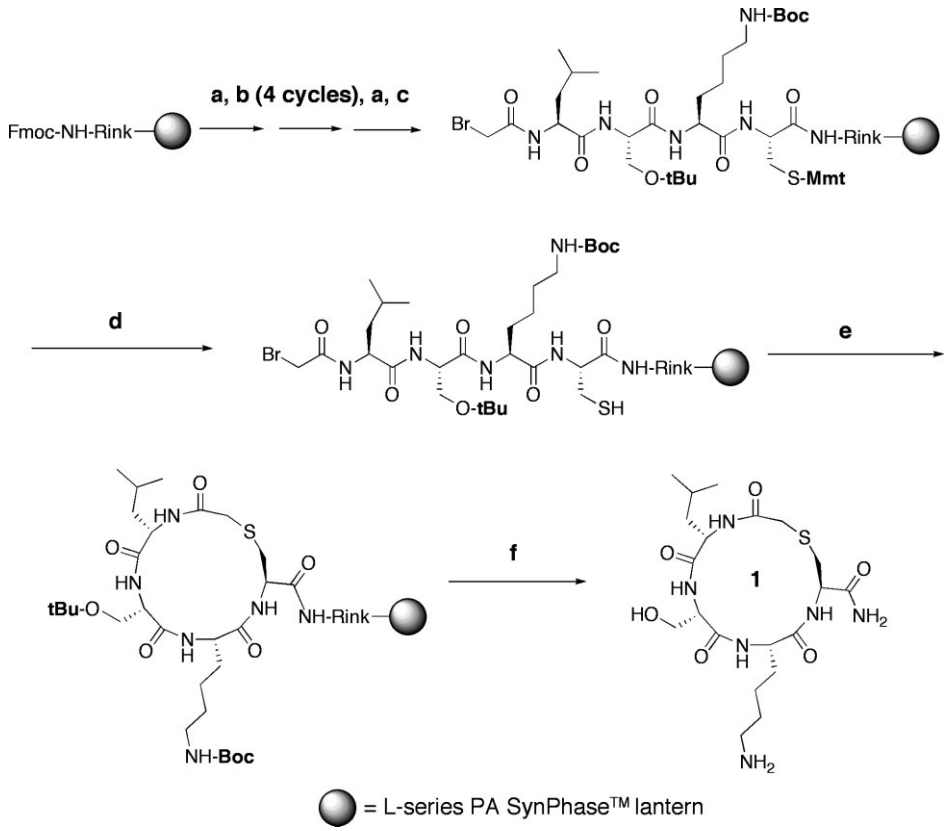

Scheme 2 Reagents and conditions: (a) 25% piperidine/DMF; (b) Fmoc-Cys(Mmt)-OH/Fmoc-Lys(Boc)-OH/Fmoc-Ser(tBu)-OH /Fmoc-Leu-OH, DIC, HOBt (1 : 1 : 1); (c) $BrCH_2COOH$, DIC (2 : 1); (d) 2% TFA/5%TIPS/DCM; (e) 2% DIPEA/DMF; (f) 2.5% H_2O/5% TIPS/TFA.

than the polymer surface of the HM SynPhase™ crowns and is also efficiently wetted in both polar and non-polar solvents including aqueous solutions [29,30]. However attempts to synthesize the target cyclic thioether **1**, as outlined in Scheme 2, consistently resulted in formation of two significant products in approximately 1 : 1 ratio, which were identified through MS analysis as being the desired cyclic monomer **1** and the corresponding homogenic cyclic dimer **2** (Figure 1). The structure of the homogenic cyclic dimer **2** is illustrated in Figure 2. In previous work examining the cyclization of linear tetrapeptides via intramolecular *S*-alkylation [11], homogenic cyclic dimer formation was also observed in a large proportion of the compounds prepared, although to a significantly lesser degree (<5%) than was observed here. This strongly suggested that the increase in cyclic dimer formation observed here was a consequence of going to a smaller, more rigid linear sequence [19–26]. In light of this result, work was carried out to identify a strategy that would decrease the amount of cyclic dimer formation. In this regard, Burgess and Feng have observed that for the synthesis of small 13-membered cyclic peptidomimetics via intramolecular *S*-alkylation on the solid phase, cyclic dimer formation could be reduced by decreasing the loading of the solid support being used [31]. In this comparative study, it was found that cyclic dimer formation was decreased by a factor that was approximately proportional to the reduction in the loading (e.g. a two-fold decrease in the loading of the solid support resulted in a two-fold decrease in the amount of cyclic dimer that was formed). Based on the current loading of the SynPhase™ lanterns and the amount of cyclic dimer formation observed in the synthesis of the target cyclic thioether **1**, the loading of the lanterns could not be reduced to the level needed to reduce the amount of cyclic dimer formation to an acceptable level (<20%). Moreover, consideration needs to be given to the possibility that in the preparation of libraries, sequences maybe encountered that result in greater cyclic-dimer formation than observed in the synthesis of the target cyclic thioether **1**. In order to suppress cyclic dimer formation, ideally what was needed was a strategy that would make intramolecular cyclization of the linear sequence more energetically favourable. To this end, it has been well established that for the synthesis of small cyclic peptides (3–7 residues) via intramolecular lactam formation, incorporation of 'turn inducing' residues into the linear sequence, that is residues that can readily adopt a *cis* conformation such as proline [32,33], pseudoprolines [23,26,34], D-residues [24,35], *N*-methyl [32] and *N*-benzyl [20–22,25] secondary amino acids can help

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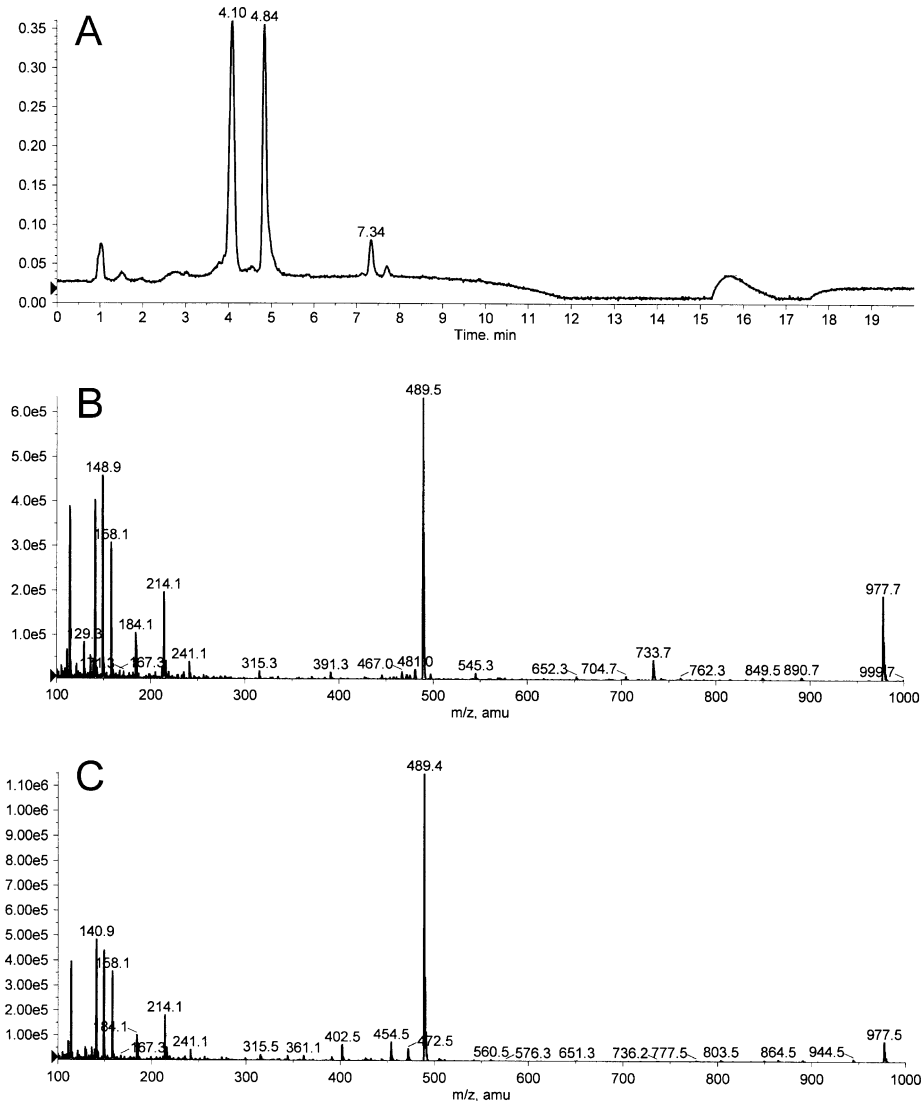

Figure 1 LC-MS data obtained for the crude cyclic thioether peptide **1**. (A) RP-HPLC profile at 214 nm. (B) MS analysis of LC peak at 4.84 min, m/z 489.5 and 977.7 are the $[M+2H]^{2+}$ and $[M+H]^{+}$ signals respectively for the homogenic cyclic dimer **2**. (C) MS analysis of LC peak at 4.10 min, m/z 489.5 and 977.7 are the $[M+H]^{+}$ and $[2M+H]^{+}$ signals, respectively for the cyclic monomer **1**.

to prevent or minimize intermolecular side reactions significantly improving cyclic monomer formation. As illustrated in Figure 3, the incorporation of the turn inducing residues, D-Leucine or *N*-methyl leucine, into the linear precursor of the target cyclic thioether **1** significantly improved the efficiency of cyclization on the solid phase, with incorporation of D-leucine giving quantitative cyclic monomer formation.

Whilst these results were encouraging, unfortunately the incorporation of traditional 'turn inducing' residues into a linear sequence imposes restrictions on the sequence diversity that can be generated. For example, the use of proline and pseudoprolines imposes restrictions on the amino acid residues that can be incorporated into the sequence, where as the incorporation of D-residues places restrictions on the chiral configuration of the sequence. Whilst *N*-methyl derivatives of both the L- and D-residues can either be purchased or readily prepared, *N*-methylation of peptide amide bonds cannot be readily reversed, hence *N*-methylation can be considered a permanent modification. This is problematic as it would be necessary to prepare cyclic peptides without *N*-methylation, as *N*-methylation is not a benign modification and can have a significant effect on properties such as peptide conformation, binding affinity and permeability [36]. In the case of the secondary amino acid surrogates, incorporating the *N*-benzyl derivatives, Hmb (2-hydroxy-5-methoxybenzyl) [20], Dmb (2,5-Dimethoxybenzyl) [22] and Hnb (2-hydroxy-6-nitrobenzyl) [21,25], the *N*-substitution is reversible allowing for regeneration of the native sequence. However, cleavage of the Hmb and Dmb groups results in the formation of benzyl cations

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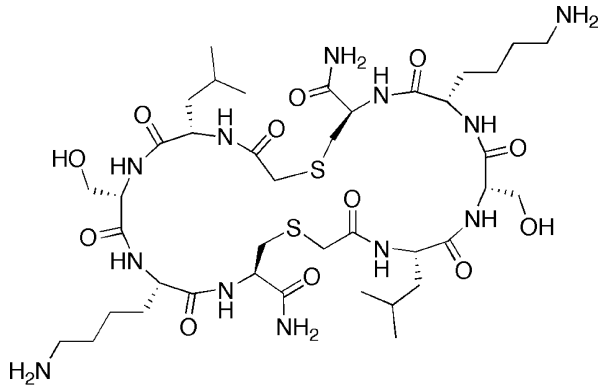

2

Figure 2 Structure of the homogenic cyclic dimer **2**.

which can irreversibly bind to adjacent thioether groups such as the thioether cyclic link to form a permanent sulfonium ion adduct [37,38], whilst removal of the Hnb group requires photolysis, a process that is not readily amenable to the Multipin SPPS™ platform [21,25]. Furthermore it has been demonstrated that photolytic removal of the Hnb group can lead to hydrolysis of the amide backbone converting the cyclic product back to its linear form [25].

A potential alternative to the incorporation of a 'turn inducing' residue into the linear sequence in order to promote intramolecular cyclization of the linear precursor is to perform the cyclization reaction in the presence of monovalent ion lithium [39–42]. Robey has observed that using lithium in the form of LiCl to help in the solubilizing of linear precursors of various C4 domain peptide analogues of the HIV-1 gp120 protein in DMF also aided in the efficient cyclization via intramolecular *S*-alkylation [39]. Similarly, Ye and co-workers have demonstrated that cyclization yields were increased for the solution phase synthesis of several homodetic cyclic hepta and pentapeptides in the presence of lithium as well as the monovalent metal ions sodium, cesium and potassium [40–42]. Based on the results of Robey and Ye, the effect of lithium on cyclization efficiency was also explored here and it was found that the addition of lithium in the form of LiCl to the cyclization reaction (step e in Scheme 2) resulted in a significant reduction in the amount of cyclic dimer formation (Figure 4). As illustrated in Figure 4, the degree of cyclic monomer formation that was observed increased as the concentration of lithium in solution was increased, with maximum cyclic monomer formation (>90%) being achieved when using a 1.25 M solution of lithium in 2% DIPEA/DMF. To explore the generality of the effect of lithium on the efficiency of cyclization and concurrently demonstrate the applicability of the synthetic methodology to the parallel preparation of libraries, a small library containing 20 members (**4**{***1–20***}) based on the tripeptide sequence Leu-**Xxx**-Lys was generated in which the second residue of this sequence (**Xxx**) was varied using all of the 20 standard proteogenic residues

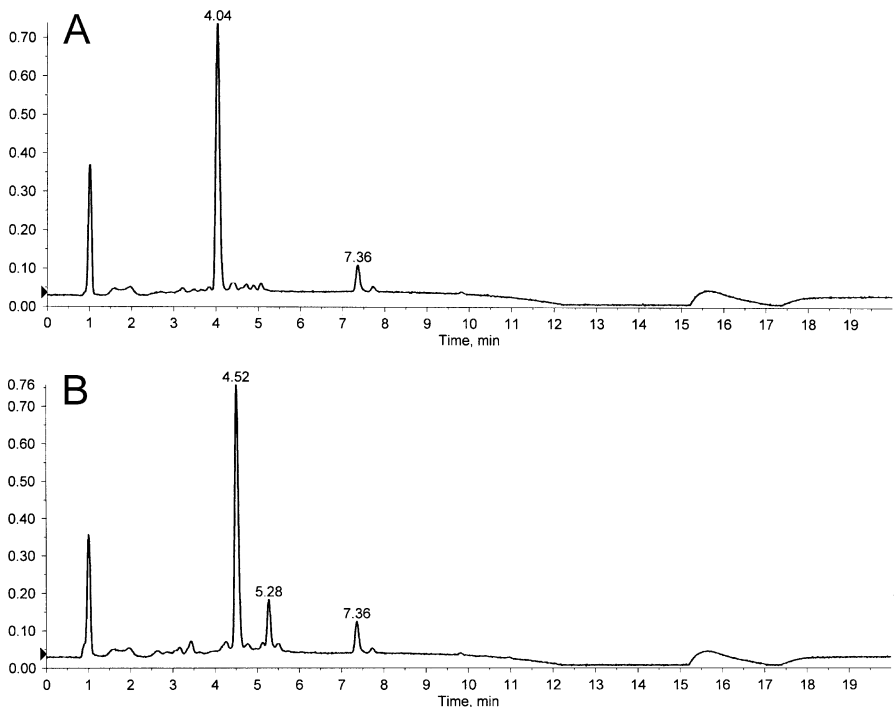

Figure 3 RP-HPLC profile at 214 nm for the crude cyclic thioether peptide **1** after the *N*-terminal Leu residue was substituted with: (A) D-Leu, peak at 4.04 min is the cyclic monomer; (B) *N*-Me-Leu, peak at 4.52 min is the cyclic monomer and the peak at 5.28 min is the cyclic dimer.

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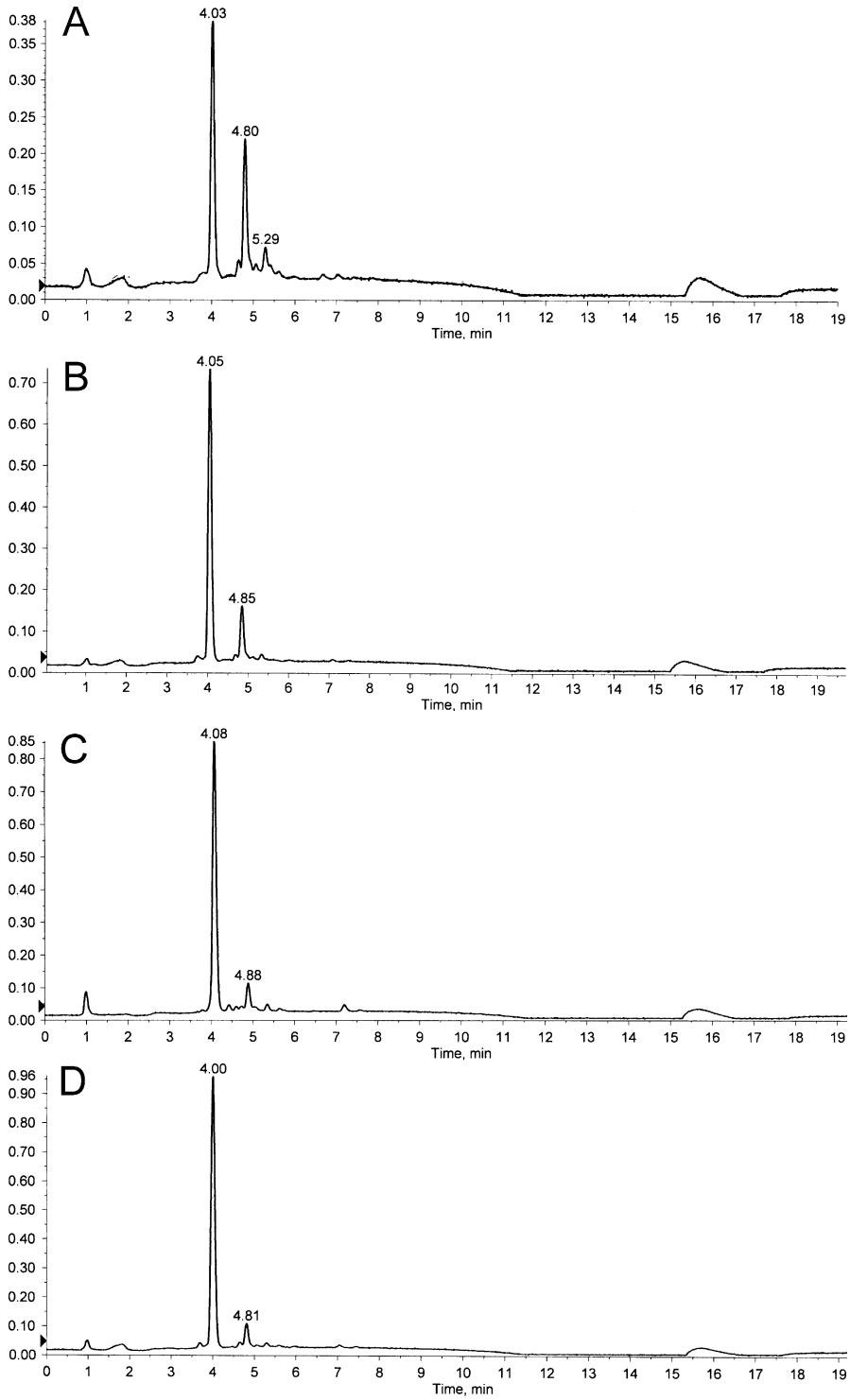

Figure 4 RP-HPLC profile at 214 nm for the crude cyclic thioether peptide **1** after cyclization using: (A) 0.25 M LiCl in 2% DIPEA/DMF; (B) 1 M LiCl in 2% DIPEA/DMF; (C) 1.25 M LiCl in 2% DIPEA/DMF; (D) 2.5 M LiCl in 2% DIPEA/DMF.

(Table 1). The library was prepared with and without the addition of lithium to the cyclization step. For the synthesis of the two libraries, several changes were made to the synthetic methodology that had been used for the preparation of the model cyclic thioether peptide **1**. Firstly, coupling of the 20-proteogenic residues and the *N*-terminal leucine was carried out overnight to account for any slow coupling reactions. Likewise, the cyclization reactions were left to procede overnight so as to accommodate for any potentially kinetically unfavourable cyclization reactions resulting from variation in the sequences. The concentration of lithium in the cyclization reaction solution was also increased to cover any variation in cyclization efficiency

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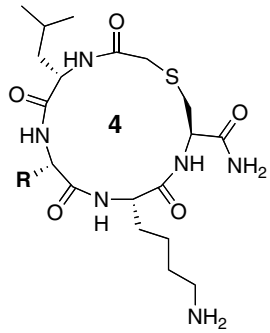
Table 1 LC-MS data for Library **4**{***1–20***} prepared with and without LiCl



		Cyclization without LiCl[a]					Cyclization with LiCl[a]				
4{}	**R**	t_R M[b]	t_R D[b]	[M + H]$^{+c}$	% M[d]	% D[d]	t_R M[b]	t_R D[b]	[M + H]$^{+c}$	% M[d]	% D[d]
1	Ser	4.11	4.90	489.4	**61**	**39**	4.09	4.90	489.4	**92**	**8**
2	Ala	4.30	5.25	473.3	**46**	**54**	4.28	5.24	473.3	**81**	**19**
3	Arg	4.02	4.60	558.3	**63**	**37**	4.00	4.59	558.2	**92**	**8**
4	Asp	4.20	4.92	517.2	**69**	**31**	4.20	4.92	517.3	**86**	**14**
5	Gly	4.20	5.10	459.5	**86**	**14**	4.16	5.09	459.5	**94**	**6**
6	Asn	4.08	4.83	516.3	**75**	**25**	4.05	4.82	516.3	**86**	**14**
7	Glu	4.32	5.03	531.3	**51**	**49**	4.27	5.03	531.3	**87**	**13**
8	Gln	4.13	4.89	530.2	**68**	**32**	4.11	4.89	530.2	**89**	**11**
9	Lys	3.87	4.45	530.2	**56**	**44**	3.82	4.45	530.2	**87**	**13**
10	Ile	5.43	6.01	515.3	**62**	**38**	5.39	6.00	515.3	**96**	**4**
11	Leu	5.53	6.31	515.3	**37**	**63**	5.50	6.33	515.3	**87**	**13**
12	Tyr	5.11	5.52	565.5	**55**	**45**	5.05	5.51	565.3	**86**	**14**
13	Thr	4.28	5.03	503.1	**53**	**47**	4.26	5.05	503.3	**96**	**4**
14	Trp	5.92	6.51	588.3	**72**	**28**	5.88	6.52	558.5	**92**	**8**
15	Val	5.01	5.58	501.4	**43**	**57**	4.96	5.59	501.5	**91**	**9**
16	His	3.83	4.30	539.3	**91**	**9**	3.83	4.30	539.3	**85**	**15**
17	Phe	5.81	6.67	549.3	**63**	**37**	5.77	6.69	549.3	**90**	**10**
18	Pro	4.57	5.30	499.4	**>99**	**<1**[e]	4.58	5.31	499.3	**>99**	**<1**[e]
19	Cys	4.73	5.01	505.1	—	—	4.74	5.01	505.3	—	—
20	Met	5.18	5.86	533.2	**51**	**49**	5.16	5.87	533.3	**86**	**14**

[a] M = cyclic-monomer D = cyclic-dimer.
[b] t_R in min at 214 nm.
[c] m/z observed for cyclic monomer.
[d] Based on the peak area at 214 nm of cyclic monomer and cyclic dimer relative to each other.
[e] No cyclic dimer was observed.

that may arise from modifying the sequence of the model system.

The analytical data obtained from these experiments is outlined in Table 1. LC-MS analysis of the resulting products showed that for the synthesis of the cyclic thioether peptides where no lithium was used in the cyclization reaction, significant cyclic dimer formation was observed except for where the residues Gly, Pro and His were used as the variable residue (Table 1). For Gly and Pro this was not unexpected, as they both have been identified as 'turn inducing' residues. However, when lithium was utilized in the cyclization reaction, cyclic dimer formation in all the cases, where it had been previously observed, was significantly reduced demonstrating that the effect of lithium on cyclization efficiency was tolerant to sequence variation (Table 1). The ability of lithium to enhance cyclic monomer formation was particularly highlighted for the cyclic thioether peptides **4**{***11***} and **4**{***15***} where the undesired cyclic dimer had been the dominant product for these two sequences prior to the addition of lithium to the cyclization reaction (Figure 5). Overall, the synthetic methodology utilized here incorporating lithium into the cyclization reaction delivered crude cyclic monomers of a satisfactory purity. The only major deviations from this were for the cyclic thioether

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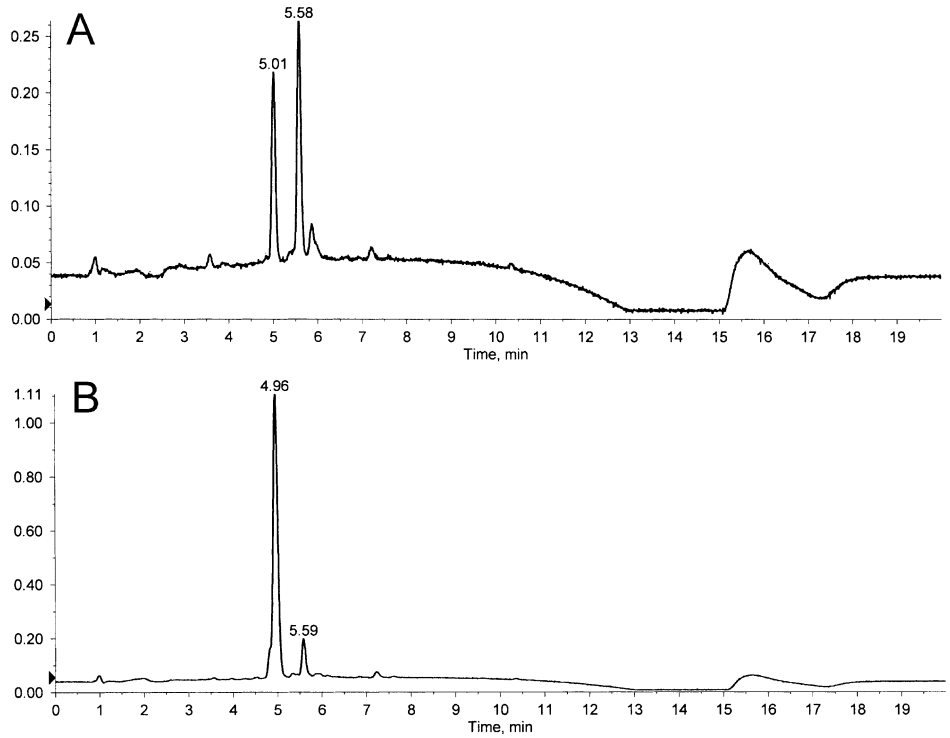

Figure 5 RP-HPLC profile at 214 nm for the crude cyclic thioether peptide **4**{***15***} after cyclization using: (A) 2% DIPEA/DMF, peak at 5.01 min is the cyclic monomer and the peak at 5.58 min is the cyclic dimer; (B) 2 M LiCl in 2% DIPEA/DMF, peak at 4.96 min is the cyclic monomer and the peak at 5.59 min is the cyclic dimer.

peptides **4**{***19***} and **4**{***20***} in which the variable residues were cysteine and methionine respectively. For the cyclic thioether peptide **4**{***20***}, there was significant oxidation of the thioether side chain of the methionine residue, which was even more pronounced when lithium was added to the cyclization reaction. However, it was found that the desired cyclic monomer, free of the methionine sulfoxide by-product, could be easily obtained by the addition of 1.4% *v*/*v* ethaneditiol (EDT) and 1.6% *v*/*v* trimethylsilylbromide (TMSBr) to a cleavage mixture of 2.5% TIPS/TFA 15 min prior to completion of the cleavage reaction [43]. In the synthesis of **4**{***19***} with and without the addition of lithium to the cyclization reaction, significant by-product formation besides the cyclic dimer formation was observed. These by-products co-eluted with the cyclic dimer, which is why no data was provided in Table 1 with regard to the amount of cyclic monomer and cyclic dimer that was formed in the synthesis of **4**{***19***}. Whilst these by-products are yet to be identified, it was found that substitution of the Trt side-chain protecting group of the variable cysteine residue with a t-Butyl group prevented these by-products from being formed. This suggested that under the cyclization conditions used, the Trt group was not an effective side-chain protecting group for the cysteine residue. However attempts to effect removal of the t-Butyl group by the addition of 10% *v*/*v* TFMSA to the cleavage solution resulted in significant trifluoroacetylation of the cyclic monomer **4**{***19***}. Therefore, at this stage further work needs to be done to identify suitable cysteine side chain protecting group/deprotection conditions before this synthetic methodology could be routinely utilized in the preparation of sequences containing an additional cysteine residue.

At present, the mechanism underlying lithium's ability to promote peptide cyclization is yet to be unequivocally deduced. Robey has suggested that the interaction of the lithium ion with the amide carbonyl groups may influence the π characteristics of the bond in such a way as to place the reactive termini in close proximity with each other such that intramolecular reactions are favoured and efficient cyclization is achieved [39]. Ye and co-workers have gone a step further and proposed that the increase in peptide cyclization that is observed with the addition of monovalent metal ions like lithium to the cyclization reaction, results from the lithium ion forming a complex with the linear sequence via the backbone amide carbonyl groups, inducing a cyclic confirmation in the linear sequence that brings the reactive termini in close proximity to each other. To this end, Ye and co-workers have described results from circular dichroism studies, which suggested that the linear precursors being examined were adopting turn conformations in the presence of the monovalent metal ions [42]. Furthermore, it has been well documented that lithium can form stable complexes with cyclic peptides of

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varying sizes in organic solvents via coordination with the amide carbonyl groups of the peptide backbone [44–48]. Enhancing the efficiency of peptide cyclization using metal ions such as lithium is an interesting phenomenon and as the results presented here suggest, could potentially be used as a general aid to improve cyclization efficiency in difficult peptide macrocyclizations. To this end, further investigation needs to be undertaken so as to get a better understanding of what is happening at the molecular level in order to fully exploit the beneficial effects of this chemistry.

CONCLUSIONS

Here, we have examined and further developed methodology for the efficient parallel synthesis and cyclization of linear tripeptide libraries. Based on the sequences examined, it was found that highly efficient cyclization via intramolecular *S*-alkylation could be achieved on the solid phase by the addition of a large excess of lithium in the form of LiCl to the cyclization reaction. The successful application of this methodology to the parallel synthesis of a small, 20-membered library of discrete cyclic thioether peptides demonstrated its potential for routinely accessing large libraries of cyclic peptides derived from diverse linear tripeptide sequences. Moreover, the results obtained from the library synthesis also serve to confirm the generality of the positive influence of lithium in promoting peptide cyclization on the solid phase via intramolecular *S*-alkylation. Further work is now being undertaken utilizing this methodology to generate larger libraries of cyclic thioether derivatives of diverse linear tripeptides of biological interest. The synthesis and screening of these libraries will be reported in due course.

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