Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 525–532
Published online 19 May 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.761

JOURNAL OF PEPTIDE SCIENCE

Efficient methodology for the cyclization of linear peptide libraries via intramolecular *S*-alkylation using Multipin™ solid phase peptide synthesis

KADE D. ROBERTS,[a]* JOHN N. LAMBERT,[a‡] NICHOLAS J. EDE[b] and ANDREW M. BRAY[b§]

[a] School of Chemistry, The University of Melbourne, Grattan Street, Parkville, Victoria 3010, Australia
[b] Mimotopes Pty Ltd, 11 Dueridin Street, Clayton, Victoria 3168, Australia

Received 27 February 2006; Revised 14 March 2006; Accepted 14 March 2006

Abstract: Methodology is described here for the efficient parallel synthesis and cyclization of linear peptide libraries using intramolecular *S*-alkylation chemistry in combination with Multipin™ solid phase peptide synthesis (Multipin™ SPPS). The effective use of this methodology was demonstrated with the synthesis of a 72-member combinatorial library of cyclic thioether peptide derivatives of the conserved four-residue structural motif **DD/EXK** found in the active sites of the five crystallographically defined orthodox type II restriction endonucleases, *Eco*RV, *Eco*RI, *Pvu*II, *Bam*HI and *Bgl*I. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclization of linear peptides; cyclic thioethers; *S*-alkylation; cyclic peptides; peptide libraries; Multipin™ solid phase peptide synthesis; SynPhase™

INTRODUCTION

Conformational restriction of a linear peptide through cyclization will give the sequence more favourable physico-chemical properties, such as increased permeability, selectivity and resistance to *in vivo* enzymatic degradation, ultimately increasing its bioavailability and making it more attractive than its linear counterpart as a potential drug candidate. In connection with our studies on peptides that interact with DNA [1–3] we were interested in generating combinatorial libraries of cyclic derivatives of the conserved four-residue structural motif **DD/EXK** found in the active sites of the five crystallographically defined orthodox type II restriction endonucleases, *Eco*RV [4–10], *Eco*RI [7,11,12], *Pvu*II [13–15] *Bam*HI [16,17] and *Bgl*I [18].

The cyclization of linear peptides requires the transcension of a considerable entropic barrier that may prevent the successful alignment of chain termini in a reactive conformation. To increase the probability of intramolecular reactions between reactive termini, these reactions have been carried out in solution under conditions of high dilution. However, this approach can lead to reduced reaction rates and poor yields. An alternative approach to limit intermolecular reactions and favour intramolecular reactions is to perform cyclization on immobilized linear precursors where resin loadings are deliberately kept low. This strategy is sometimes referred to as 'pseudo dilution' [19] and offers a more efficient approach to the parallel synthesis of large libraries of cyclic peptides for high-throughput screening. Traditionally the cyclization of linear peptides has focused on intramolecular head-to-tail lactam formation, which not only provides a uniform native backbone but also provides a molecule with a high degree of conformational restriction and stability towards proteolytic digestion. While the literature is abound with protocols and powerful coupling reagents for cyclic lactam formation [20–22] the efficient translation of these methodologies to the solid phase cyclization of large libraries of peptides with sequence flexibility is yet to be achieved [23,24]. Moreover lactam cyclization can be problematic when trying to prepare small highly rigid cyclic systems of four residues or less [25].

As documented in a number of recent reviews, there is a vast array of methods available for the synthesis of cyclic peptides in which the key cyclization steps proceed via the formation of functionalities other than amide bond formation [20–22]. Moreover many of these chemistries have been successfully adapted to the solid phase and have potential for application to library synthesis. In this regard, intramolecular *S*-alkylation reactions offer a facile and versatile approach to the cyclization of linear peptides. Their use in this context was first established by Robey and Fields in 1989, while investigating the polymerization of *N*-bromoacetylated modified peptides [26]. It was observed that at concentrations of 1 mg/ml and neutral pH at room temperature, certain *N*-bromoacetyl *C*-cysteinyl containing peptides underwent cyclization

* Correspondence to: Kade D. Roberts, Mimotopes Pty Ltd, 11 Dueridin Street, Clayton, Victoria 3168, Australia;
e-mail: kade_roberts@mimotopes.com
‡ Current address: Chemistry Group, Biota Holdings Limited, 10/585 Blackburn Rd, Notting Hill, Victoria 3168, Australia.
§ Current address: PharmBank, Level 10, 52 Collins St, Melbourne, Victoria 3000, Australia.

rather than polymerization, where the thiol group of the *C*-terminal cysteine displaced the bromine of the *N*-terminal bromoacetyl group to form a cyclic thioether peptide (Scheme 1).

In the light of these results Robey went on to establish a general protocol for the synthesis and evaluation of cyclic thioether peptides using intramolecular *S*-alkylation chemistry [27]. Since then, a number of reports have been published describing syntheses adopting similar methodology to prepare a large variety of cyclic thioether peptides in solution [28–41] and in the solid phase [42–44] demonstrating the flexibility of this approach. The resulting thioether cyclic link has been shown to be resistant to enzymatic degradation [40,45] and has been used to prepare more stable analogues of naturally occurring cyclic disulfides [31,33–35,37,42,44]. Another attractive feature of this chemistry is the potential for efficient cyclization of short linear sequences that would otherwise be difficult to obtain using conventional intramolecular lactam formation [22,26,28]. Here we demonstrate the efficient use of intramolecular *S*-alkylation cyclization chemistry in combination with Multipin™ solid phase peptide synthesis (Multipin™ SPPS) to generate a library of cyclic thioether peptides that display permutations of the conserved four-residue structural motif **DD/EXK** found in the active sites of the five crystallographically defined orthodox type II restriction endonucleases, *Eco*RV, *Eco*RI, *Pvu*II, and *Bgl*I. *Bam*HI.

MATERIALS AND METHODS

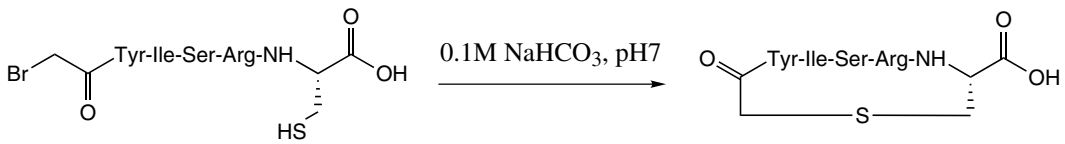
SynPhase™ crowns and Multipin™ components, inert plastic stems (8 × 12), polypropylene microtitre-plate stem holders and Beckman chemical resistant 1 ml/0.2 ml polypropylene microtitre plates were supplied by Mimotopes (Melbourne, Australia). One-millilitre Titertube microtubes were purchased from BIO-RAD (Hercules, USA). 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). 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 MeOH were obtained from BDH Laboratory Supply (Melbourne, Australia). Acetonitile 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). DIC, TFA, triethylsilane (TES) and triisopropylsilane (TIPS) were obtained from Aldrich (Sydney, Australia).

General Experimental Procedure for the Preparation and Cyclization of Linear Peptide Libraries

Synthesis was carried out on an I-series hydroxyethyl-methacrylate (**HM**) SynPhase™ crowns (loading: 5.4 μmol) functionalized with a Rink amide linker. The crowns were attached to inert plastic stems mounted onto an 8 × 12 polypropylene microtitre-plate stem holder. Unless stated otherwise, all reactions were carried out in parallel at room temperature in individual 1 ml reaction wells of a Beckman chemical resistant 96 well polypropylene microtitre plate. Washing of the crowns was carried out in chemically resistant polypropylene wash baths containing 200 ml of the appropriate solvent. Deprotection and coupling steps were monitored qualitatively using a trinitrobenzenesulfonic acid (TNBS) test as outlined below.

Qualitative 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 the crown 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 precursors. 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 (DMF (2 × 5 min), MeOH (10 min)) and air-dried (TNBS test: positive). The crowns were then immersed in a solution of Fmoc-Cys(Mmt)-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 (13 μl, 0.075 mmol) for 10 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 residues AA_2–AA_5 using Fmoc-Asp(OtBu)-OH (21 mg, 0.05 mmol), Fmoc-Glu(OtBu)-OH (22 mg, 0.05 mmol), Fmoc-Ile-OH (18 mg, 0.05 mmol), Fmoc-Leu-OH (18 mg, 0.05 mmol), Fmoc-Lys(Boc)-OH (23 mg, 0.05 mmol) and Fmoc-Phe-OH (19 mg, 0.05 mmol) using the same conditions. The crowns were then Fmoc deprotected (as described above, TNBS test: positive) and immersed into a solution of bromoacetic acid (14 mg, 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 (as described above) and air-dried (TNBS test: negative).



Scheme 1 Cyclization of *N*-bromoacetyl *C*-cysteinyl containing peptides.

Cyclization of the linear precursors. The crowns were then subjected to successive treatments (4 × 10 min) with solutions of 2% TFA/5% TIPS/DCM (500 μl). After washing (DMF (2 × 5 min), MeOH (1 × 5 min)) and air-drying, the crowns were placed into a solution (500 μl) of 1% DIPEA/DMF and incubated for 24 h. The crowns were removed from the solution, washed (DMF (1 × 5 min), MeOH (1 × 5 min)) and air-dried.

Cleavage and side chain deprotection. Each crown was placed into a solution (in a 1 ml Titertube microtube) 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 tubes and then discarded. The TFA was removed using a vacuum centrifuge and the remaining residues were taken up in diethyl ether (1 ml). The tubes were capped, gently shaken and left to stand for 5 min. The resulting white precipitates were centrifuged, and the supernatant carefully removed using vacuum suction. The addition of ether and the subsequent steps were repeated and the precipitates air-dried in a fume cupboard to remove any traces of ether. The precipitates were individually dissolved in 50% CH_3CN/H_2O (1 ml) and lyophilized to give crude cyclic peptides as a white solid.

Quantitative Ellman test for free thiols. 0.1 μmol of the appropriate peptide was dissolved in 0.1 M sodium phosphate, pH 8.0/25 °C (3 ml), and 0.4% (w/v) 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)/0.1 M sodium phosphate, pH 8.0/25 °C (100 μl) added. After 10 min the absorbance of the solution was measured at 410 nm (reference solution: 0.4% (w/v) DNTB/0.1 M sodium phosphate; pH 8.0/25 °C (100 μl) in 0.1 M sodium phosphate; pH 8/25 °C (3 ml)). The concentration of free thiols (SH) present in the sample was calculated as $(SH) = [Abs_{410}(\text{peptide}) - Abs_{410}(\text{reference})]/13\,650\ M^{-1}$.

LC-MS Analysis. LC-MS analysis was carried out using Perkin-Elmer Sciex API-100 single-quadrupole mass spectrometer equipped with an ESI source coupled to a Shimadzu chromatography system (equipped with duel LC-10AD pumps and an LC-10A UV/vis detector set at 214 and 254 nm). HPLC analysis was conducted on a reversed-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 100–2000.

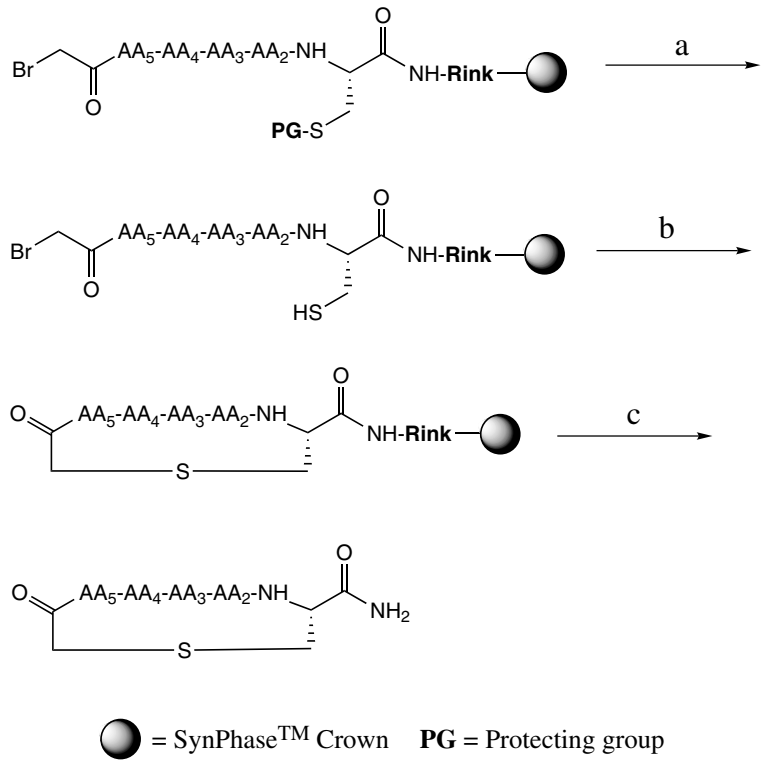
RESULTS AND DISCUSSION

Development and application of the synthetic methodology was carried out using Multipin™ SPPS [46–49]. This methodological tool allows for the multiple parallel solid phase synthesis of libraries of discrete, spatially arrayed peptides using Fmoc chemistry. The key component of Multipin™ SPPS is the (**HM**) SynPhase™ crown, a polyethylene pin, the surface of which has been grafted with a polymeric coating of **HM**, which is the reaction medium in which synthesis takes place. The crowns are attached to inert plastic stems fixed in a 8 × 12 microtitre-plate format that allows for multiple parallel synthesis of sets of up to 96 discrete peptides. Multipin™ SPPS has previously been used in combination with intramolecular *S*-alkylation chemistry to successfully generate libraries of cyclic thioether β-turn mimetics [50,51]. It should be noted that since the completion of this work a new generation of Multipin™ solid support called SynPhase™ Lanterns has been released [47–49].

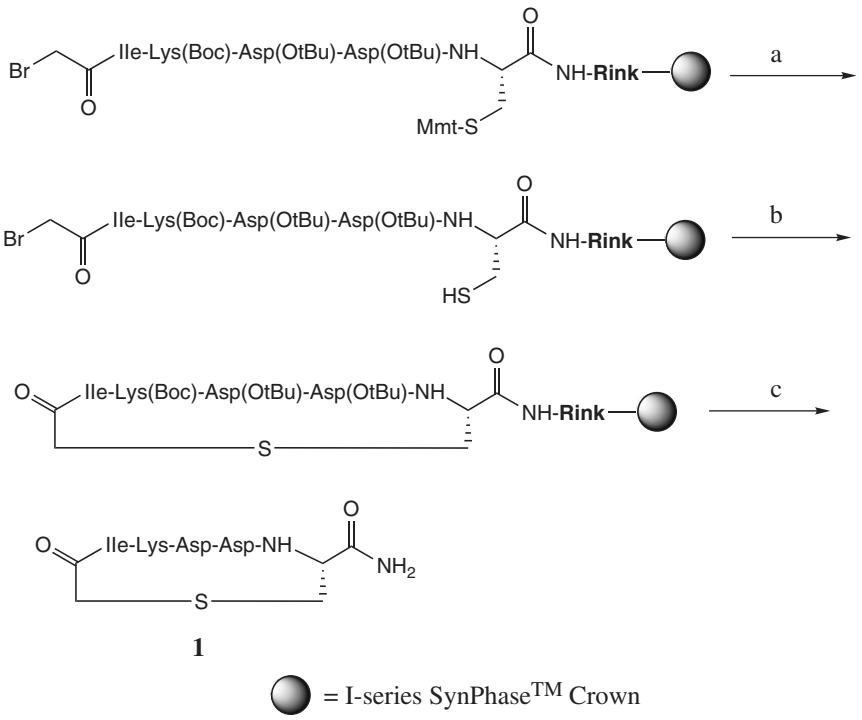
The proposed synthetic strategy is outlined in Scheme 2 and involves the synthesis of solid phase-bound linear peptides bearing a *C*-terminal cysteine and an *N*-terminal bromoacetyl group using standard Fmoc SPPS. Selective deprotection of the cysteine thiol group using mildly acidic conditions is followed by based-induced cyclization and TFA-mediated cleavage to deliver the target cyclic thioethers.

This synthetic approach required an appropriate orthogonal protection strategy for the side chain thiol of the *C*-terminal cysteine, which would allow selective deprotection of this residue without removing the side chain–protecting groups of the other residues or cleaving the linear precursors from the solid phase. To this end it was proposed that the methoxytrityl (Mmt) protecting group would be better utilized than a Trt protecting group which has been used previously in this context for the orthogonal protection of cysteine [42,43]. This was based on a study carried out by Barlos and co-workers [52] who found that when comparing the acid sensitivity of *S*-Trt and *S*-Mmt, complete removal of the Mmt group from the tripeptide Fmoc-Cys(Trt)-Leu-Cys(Mmt)-OH could be achieved by using 1–2% TFA, while at best only 5% of the *S*-Trt group could be removed under the same conditions. To completely remove the *S*-Trt group >90% TFA was required. Use of such conditions would not allow selective deprotection of the cysteine thiol group, resulting in simultaneous cleavage of the linear precursor from the solid phase. This work was further supported by the optimization studies by Burgess and co-workers for the solid phase synthesis of cyclic β-turn peptidomimetics via S_NAr macro-cyclization [53]. In these studies, it was found that the optimal results for protection/selective deprotection of an *S*-nucleophile in the form of a cysteine thiol group were obtained using the Mmt group rather than the Trt or Acm protecting groups.

Optimization of coupling and cyclization conditions were carried out using the target peptide **1** (Scheme 3). Appropriately protected amino acids were coupled as their activated OBt ester pre-formed using HBTU, HOBt and DIPEA for 2 h. It was found that in order to achieve complete removal of the *S*-Mmt group the linear precursor needed to be exposed to successive treatments with freshly prepared 2% TFA/5% TIPS/DCM solutions over a period of 40 min, rather than one treatment for the same length of time. The progression of this deprotection reaction could be



Scheme 2 (a) 1–2% TFA/DCM; (b) base/DMF; (c) TFA.



Scheme 3 (a) 2% TFA/5% TIPS/DCM; (b) 1% DIPEA/DMF; (c) 5% TES/TFA.

monitored visually by the appearance and then disappearance of the bright yellow methoxytrityl cation. LC-MS analysis showed that quantitative intramolecular *S*-alkylation to form the solid phase–bound cyclic thioether could be obtained by exposure to 1% DIPEA/DMF for 2 h. For further confirmation that it was the cyclic thioether that was formed, an Ellman test for free thiols [54] was carried out, the result of which showed the presence of <0.1% free thiols in the crude product.

Having found optimal reaction conditions, attention was turned to the preparation of the peptide library. Permutations for library generation were obtained by scrambling the residues of each motif found in *Eco*RV/*Bgl*I (**DDIK**), *Bam*HI (**EEAP**), *Pvu*II (**DELK**) and *Eco*RI (**DEAK**) relative to the *C*-terminal cysteine. This allowed for the generation of 12 discrete cyclic thioethers each for the *Eco*RV/*Bgl*I (**1**–**12**) and *Bam*HI (**37**–**48**) motifs and 24 discrete cyclic thioethers each for the *Pvu*II (**13**–**36**) and *Eco*RI (**49**–**72**) motifs giving a library of a total of 72 members (Table 1). To accommodate any potentially kinetically unfavourable sequences, a longer reaction time of 24 h was used for the key cyclization step. The resulting crude products were analysed by LC-MS (Table 1), the results of which showed that for all the linear precursors quantitative cyclization had been achieved to deliver the target cyclic thioethers (Figure 1). Yields of the crude products were typically in the range of 33–42% based on the crown loadings.

There was no evidence of the presence of any of the corresponding linear peptides remaining in the crude products. There was also no evidence of any major peptide by-product contamination such as deletion sequences, polymerization or oxidation of the thioether link to the corresponding sulphoxide. The only significant peptide impurity observed, which was observed in all the crude products, was the presence of a minor component that eluted shortly after the cyclic thioether. MS analysis of this minor

Table 1 LC-MS Data for cyclic thioether Library

Peptide	Sequence[a]	t_R min[b]	$[M+H]^{+}$[c]	Peptide	Sequence[a]	t_R min[b]	$[M+H]^{+}$[c]
1	DDKI	3.51	632.3	**37**	EEDF	4.68	681.2
2	DDIK	3.52	632.0	**38**	EEFD	4.31	681.3
3	DKDI	3.84	632.4	**39**	EDEF	4.58	681.4
4	DKID	3.41	632.2	**40**	EDFE	4.35	681.4
5	DIDK	3.66	632.2	**41**	EFED	4.48	681.2
6	DIKD	3.55	632.2	**42**	EFDE	4.50	681.4
7	KDDI	3.85	632.4	**43**	FDEE	4.59	681.2
8	KDID	3.46	632.4	**44**	FEDE	4.63	681.4
9	KIDD	3.69	632.2	**45**	FEED	4.68	681.4
10	IDKD	3.84	632.2	**46**	DEFE	4.27	681.2
11	IDDK	3.97	632.2	**47**	DEEF	4.56	681.6
12	IKDD	3.88	632.2	**48**	DFEE	4.43	681.4
13	KDLE	3.76	646.4	**49**	KDAE	4.47	604.2
14	KDEL	3.96	646.4	**50**	KDEA	4.43	604.2
15	KELD	3.84	646.4	**51**	KEAD	4.53	604.0
16	KEDL	4.04	646.4	**52**	KEDA	4.42	604.2
17	KLDE	3.86	646.4	**53**	KADE	4.49	604.2
18	KLED	3.88	646.4	**54**	KAED	4.45	604.2
19	DELK	3.55	646.4	**55**	DEAL	4.39	604.4
20	DEKL	3.76	646.2	**56**	DEKA	4.35	604.2
21	DLEK	3.87	646.2	**57**	DAEL	4.41	604.2
22	DLKE	3.76	646.4	**58**	DAKE	4.40	604.2
23	DKEL	3.99	646.4	**59**	DKEA	4.40	604.2
24	DKLE	3.54	646.2	**60**	DKAE	4.45	604.0
25	EDLK	3.47	646.4	**61**	EDAK	4.38	604.2
26	EDKL	3.82	646.2	**62**	EDKA	4.37	604.0
27	ELDK	3.98	646.2	**63**	EADK	4.44	604.4
28	ELKD	3.88	646.2	**64**	EAKD	4.41	604.2
29	EKLD	3.72	646.4	**65**	EKAD	4.51	604.2
30	EKDL	4.13	646.4	**66**	EKDA	4.38	604.2
31	LDEK	3.90	646.2	**67**	ADEK	4.36	604.2
32	LDKE	4.07	646.2	**68**	ADKE	4.38	604.0
33	LKED	4.06	646.2	**69**	AKED	4.40	604.2
34	LKDE	4.04	646.4	**70**	AKDE	4.42	604.2
35	LEKD	4.24	646.4	**71**	AEKD	4.40	604.4
36	LEDK	4.05	646.2	**72**	AEDK	4.31	604.4

[a] Sequence $AA_2 \rightarrow AA_5$.
[b] t_R at 214 nm.
[c] $[M+H]^{+}$ calculated for **1–12**: 632.2, **13–36**: 646.4, **37–48**: 681.2, **49–72**: 604.2.

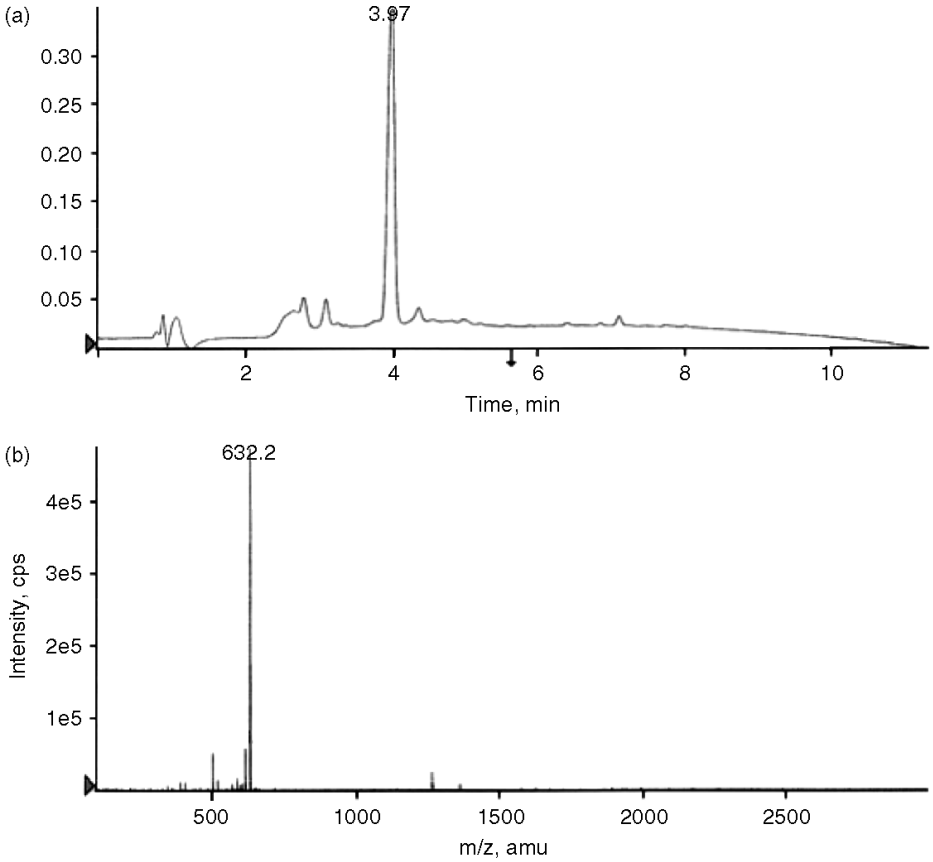


Figure 1 LC-MS data obtained for the crude cyclic thioether **11** (a) HPLC profile at 214 nm and (b) MS analysis of peak at 3.97 min. $[M + H]^+$ calculated for **11** is 632.2.

component gave the same major signal at m/z 632.2 suggesting the presence of an isomeric form of the cyclic thioether. Initially it was thought that this apparent 'isomeric' component resulted from epimerization of the *C*-terminal cysteine, as this residue was common to all of the cyclic thioethers. Han and co-workers [55] have shown that *N*,*S*-protected derivatives of cysteine can undergo epimerization when coupled using protocols that involve pre-activation mediated by aminium salts such as HBTU in the presence of a tertiary amine like DIPEA. However, a closer examination of the isotopic distribution of the signal at m/z 632.2 for the minor component revealed that it was in fact a $[M + 2H]^{2+}$ signal and not a $[M + H]^+$ signal whereas for the major component, the signal at m/z 632.2 was a $[M + H]^+$ signal. This strongly suggested that the minor component was the homogenic cyclic dimer of the desired cyclic product (Figure 2). This was not surprising, as it has been well documented that the formation of cyclic dimers is a side reaction often observed with the cyclization of small, highly constrained, cyclic systems [25,53]. Reduction in resin loading or the incorporation of turn-inducing elements such as D-amino acids or proline may help decrease the degree of cyclic dimer formation [25,53].

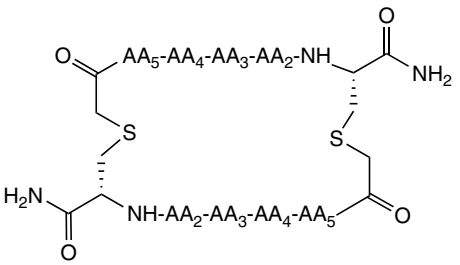


Figure 2 Structure of homogenic cyclic dimer.

CONCLUSIONS

We have shown that cyclization via intramolecular *S*-alkylation offers a facile general strategy to the cyclization of linear peptides. The results obtained showed that the efficiency of the cyclization reaction was tolerant to variation in the peptide sequence with the residues utilized in this work. The crude products that resulted from using this methodology were generally of a high purity, which would allow them to be used directly in high-throughput screening experiments for biological activity. Further studies need to be carried out to determine whether the efficiency of the *S*-alkylation cyclization reaction observed here extends to larger macrocycles as well as to sequences

containing other proteogenic and non-proteogenic residues not utilized in this work. While a cyclic thioether does not have the degree of conformational restriction exhibited by a cyclic lactam, it is much greater than its linear precursor and also gives a macrocycle of a comparable size. These properties in combination with the simplicity of the chemistry make the synthetic approach outlined here a useful tool for increasing the rigidity of linear peptide sequences. Moreover when intramolecular *S*-alkylation chemistry is used in combination with Multipin™ SPPS, it provides an efficient and robust methodology for the high-throughput parallel solid phase synthesis and cyclization of linear peptide libraries.

REFERENCES

1. Roberts KD, Lambert JN, Ede NJ, Bray AM. Efficient synthesis of thioether-based cyclic peptide libraries. *Tett. Lett.* 1998; **39**: 8357.
2. Roberts KD, Development of new methodologies for the synthesis and screening of type II restriction endonucleases. Thesis (PhD), 2003; University of Melbourne.
3. Roberts KD, Lambert JN, Ede NJ, Bray AM. Preparation of cyclic peptide libraries using intramolecular oxime formation. *J. Pept. Sci.* 2004; **10**: 659.
4. Selent U, Ruter T, Kohler E, Lidetke M, Thielking V, Alves J, Oelgeschlager T, Wolfes H, Frens P, Pingoud A. A site-directed mutagenesis study to identify amino acid residues involved in the catalytic function of the restriction endonuclease, *Eco*RV. *Biochemistry* 1992; **31**: 4808.
5. Winkler FK, Banner DW, Oefner C, Tsernoglou D, Brown RS, Bryan RK, Martin PD, Petratos K, Wilson KS. The crystal structure of *Eco*RV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J.* 1993; **12**: 178.
6. Winkler FK, Kostrewa D. Mg^{2+} binding to the active site of *Eco*RV endonuclease: a crystallographic study of complexes with substrate and product DNA at 2-Ang. resolution. *Biochemistry* 1995; **34**: 683.
7. Vipond BI, Baldwin GS, Halford SE. Divalent metal ions at the active sites of the *Eco*RV and *Eco*RI restriction endonucleases. *Biochemistry* 1995; **34**: 697.
8. Sam MD, Perona JJ. Catalytic roles of divalent metal ions in phosphoryl transfer by *Eco*RV endonuclease. *Biochemistry* 1999; **38**: 6576.
9. Thomas MP, Brady RL, Halford SE, Sessions RB, Baldwin GS. Structural analysis of a mutational hot-spot in the *Eco*RV restriction endonuclease: a catalytic role for a main chain carbonyl group. *Nucleic Acids Res.* 1999; **27**: 3438.
10. Baldwin GS, Sessions RB, Halford SE. DNA cleavage by the *Eco*RV restriction endonuclease: roles of divalent metal ions in specificity and catalysis. *J. Mol. Biol.* 1999; **288**: 87.
11. Kim Y, Choi J, Grable JC, Greene PJ, Rosenberg JM. Refinement of *Eco*RI endonuclease crystal structure: a revised protein chain tracing. *Science* 1990; **249**: 1307.
12. Grabowski G, Jeltsch A, Wolfes H, Maass G, Alves J. Site-directed mutagenesis in the catalytic centre of the restriction endonuclease *Eco*RI. *Gene* 1995; **157**: 113.
13. Cheng X, Balendiran K, Schildkraut I, Anderson JE. Structure of *Pvu*II endonuclease with cognate DNA. *EMBO J.* 1994; **13**: 3927.
14. Horton JR, Cheng X. *Pvu*II endonuclease contains two calcium ions in active sites. *J. Mol. Biol.* 2000; **300**: 1049.
15. Dupureur CM, Conlan LH. A catalytically deficient active site variant of *Pvu*II endonuclease binds Mg(II) ions. *Biochemistry* 2000; **39**: 10 921.
16. Newman M, Strzelecka T, Dorner LF, Schildkraut I, Aggarawal AK. Structure of restriction endonuclease *Bam*HI and its relationship to *Eco*RI. *Nature* 1994; **368**: 660.
17. Newman M, Strezlecka T, Dorner LF, Schildkraut I, Aggarawal AK. A. Structure of *Bam*HI endonuclease bound to DNA; Partial folding and unfolding on. *Science* 1995; **269**: 656.
18. Newman M, Lunnen K, Wilson G, Greci J, Schildkraut I, Phillips SE. Crystal structure of restriction endonuclease *Bgl*I bound to its interrupted DNA recognition sequence. *EMBO J.* 1998; **17**: 5466.
19. Maclean D, Baldwin JJ, Itanov VT, Kato Y, Shaw A, Scheneider P, Gordon EM. Glossary of terms used in combinatorial chemistry. *Pure Appl. Chem.* 1999; **71**: 2349.
20. Lambert JN, Mitchell JP, Roberts KD. Synthesis of cyclic peptides. *J. Chem. Soc., Perkin Trans. 1* 2001; 471.
21. Li P, Roller PP, Xu J. Current synthetic approaches to peptide and peptidomimetic cyclization. *Curr. Org. Chem.* 2002; **6**: 411.
22. Davies JS. The cyclization of peptides and depsipeptides. *J. Pept. Sci.* 2003; **9**: 471.
23. Bourne GT, Golding SW, McGeary RP, Meutermans WDF, Jones A, Marshall GR, Alewood PF, Smythe ML. The development and application of a novel safety-catch linker for Boc-based assembly of libraries of cyclic peptides. *J. Org. Chem.* 2001; **66**: 7706.
24. Ravin J, Bourne GT, Smythe ML. A safety catch linker for Fmoc-based assembly of constrained cyclic peptides. *J. Pept. Sci.* 2005; **11**: 572.
25. Meutermans WDF, Bourne GT, Golding SW, Horton DA, Campitelli MR, Craik D, Scanlon M, Smythe ML. Difficult macrocyclizations; new strategies for synthesizing highly strained cyclic tetrapeptides. *Org. Lett.* 2003; **5**: 2711.
26. Robey FA, Fields RL. Automated synthesis of *N*-bromoacetyl-modified peptides for the preparation of synthetic peptide polymers, peptide-protein conjugates, and cyclic peptides. *Anal. Biochem.* 1989; **177**: 373.
27. Robey FA. *Peptide Synthesis Protocols*. Humana press: New Jersey, 1994.
28. Barker PL, Bullens S, Bunting S, Burdick DJ, Chan KS, Deisher T, Eigenbrot C, Gadek TR, Gantzos R, Lipari MT. Cyclic RDG peptide analogues as antiplatelet antithrombotics. *J. Med. Chem.* 1992; **35**: 2040.
29. Bitan G, Zeltser I, Byk G, Halle D, Mashriki Y, Gluhov EV, Sukhotinsky I, Hanani M, Selinger Z, Gilon C. Backbone cyclization of the C-terminal part of substance P. part 1: the important role of the sulfur in position 11. *J. Pept. Sci.* 1996; **2**: 261.
30. Akamatsu M, Roller PP, Chen L, Zhang ZY, Ye B, Burke TR. Potent inhibition of protein-tyrosine phosphatase by phosphotyrosine-mimic containing cyclic peptides. *Bioorg. Med. Chem.* 1997; **5**: 157.
31. Olingo L, Lung FT, Sastry L, Bigeolw J, Cao T, Curran M, Burke TR, Wang S, Krga D, Roller PP, Ritcher King C Jr. Nonphosphorylated peptide ligands for the Grb2 Src homology 2 domain. *J. Biol. Chem.* 1997; **46**: 29 046.
32. Yu L, Lai Y, Wade JV, Coutts SM. A simple and efficient method for the synthesis of thioether cyclic peptides. *Tet. Lett.* 1998; **39**: 6633.
33. Long Y, Voigt JH, Lung FT, Ritcher King C, Roller PP. Significant compensatory role of position Y-2 conferring high affinity to non-phosphorylated inhibitors of Grb2-Sh2 domain. *Bioorg. Med. Chem. Lett.* 1999; **9**: 2267.
34. Fotouhi N, Joshi P, Tilley JW, Rowan K, Schwinge V, Wolitzky B. Cyclic thioether peptide mimetics as VCAM-VLA-4 antagonists. *Bioorg. Med. Chem. Lett.* 2000; **10**: 1167.
35. Li Phang M, Peach ML, Liu H, Yang D, Roller PP. Concise and enantioselective synthesis of Fmoc-Pmp$(tBu)_2$-OH and design of potent Pmp-Containing Grb2-Sh2 Domain Antagonists. *Org. Lett.* 2003; **5**: 3095.
36. Demene H, Grainer S, Muller D, Guillon G, Dufour MN, Desluc MA, Hibert M, Pascal R, Mendre C. Active peptidic mimics of the second

intracellular loop of the V_{1A} vasopressin receptor are structurally related to the second intracellular Rhopdopsin Loop: A combined 1H NMR and biochemical study. *Biochemistry* 2003; **42**: 8204.
37. Long YQ, Lung FT, Roller PP. Global optimization of conformational constraints on non-phosphorylated cyclic peptide antagonists of the Grb2-SH2 domain. *Bioorg. Med. Chem.* 2003; **11**: 3929.
38. Schlosser G, Mezo G, Kiss R, Vass E, Majer Z, Feijlbreif M, Perczel A, Bosze S, Welling-Wester S, Hudecz F. Synthesis, solution structure analysis and antibody binding of cyclic epitope peptides from glycoprotein D of herpes simplex virus type I. *Biophys. Chem.* 2003; 155.
39. Baidoo KE, Knight LC, Lin KS, Gabriel JL, Romano JE. Design and synthesis of a short-chain bitistatin analogue for imaging thrombi and emboli. *Bioconjugate Chem.* 2004; **15**: 1068.
40. Tugyi R, Mezo G, Fellinger E, Andreu D, Hudecz F. The effect of cyclization on the enzymatic degradation of herpes simplex virus glycoprotein D derived epitope peptide. *J. Pept. Sci.* 2005; **11**: 642.
41. Farkas V, Vass E, Hanssens I, Majer Z, Hollosi M. Cyclic peptide models of the Ca^{2+}-binding loop of α-lactalbumin. *Bioorg. Med. Chem.* 2005; **13**: 5310.
42. Mayer JP, Heil JR, Zhang J, Munson MC. An alternative solid-phase approach to C1-oxytocin. *Tet. Lett.* 1995; **95**: 7387.
43. Gazal S, Gellerman G, Glukohov E, Gilon C. Synthesis of novel protected Nα (ω-thioalkyl) amino acid building nits and their incorporation in backbone cylic disulfide and thioetheric bridged peptides. *J. Pept. Res.* 2001; **58**: 527.
44. Bondebjerg J, Grunnet M, Jespersen T, Meldal M. Solid phase synthesis and biological activity of a thioether analogue of conotoxin G1. *ChemBioChem* 2003; **4**: 186.
45. Osapay G, Prokai L, Kim H, Medzihradszky KF, Coy DH, Liapakis G, Reisine T, Melacini G, Zhu Q, Wang SH, Mattern R, Goodman M. Lanthionine-somatostatin analogues: synthesis, characterization, biological activity, and enzymatic stability studies. *J. Med. Chem.* 1997; **40**: 2241.
46. Chan WC, White PD. *Fmoc Solid Phase Peptide Synthesis*. Oxford university Press: New York, 2000.
47. Rasoul F, Ercole F, Pham Y, Bui CT, Wu Z, James SN, Trainor RW, Wickham G, Maeji JN. Grafted supports in solid phase synthesis. *Biopolymers* 2000; **55**: 207.
48. Ede NJ. Multiple parallel synthesis of peptides on SynPhase™ grafted supports. *J. Immunol. Methods* 2002; **267**: 3.
49. Parsons JG, Sheehan GS, Zemin W, James IW, Bray AM. A review of solid-phase organic synthesis on SynPhase™ Lanterns and SynPhase™ Crowns. *Methods Enzymol.* 2003; **369**: 39.
50. Virgilio AA, Ellman JA. Simultaneous solid-phase synthesis of β-turn mimetics incorporating side-chain functionality. *J. Am. Chem. Soc.* 1994; **116**: 11 580.
51. Virgilio AA, Bray AM, Zhang W, Trinh L, Snyder M, Morrissey MM, Ellma JA. Synthesis and Evaluation of a library of peptidomimetics based upon the β-turn. *Tetrahedron* 1997; **53**: 6635.
52. Barlos K, Gatos D, Hatzi O, Koch N, Koutsogianni S. Synthesis of the very acid-sensitive Fmoc-Cys(Mmt)-OH and its application in solid-phase peptide synthesis. *Int. J. Pept. Protein Res.* 1996; **47**: 148.
53. Feng Y, Burgess K. Solid-phase S_NAr macrocyclizations to give turn-extended-turn peptidomimetics. *Chem. Eur. J.* 1999; **5**: 3261.
54. Ellman G. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 1959; **82**: 70.
55. Han Y, Albericio F, Barany G. Occurrence and minimization of cysteine racemization during stepwise solid-phase peptide synthesis. *J. Org. Chem.* 1997; **62**: 4307.

