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Efficient Synthesis of Thioether-Based Cyclic Peptide Libraries

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Abstract: A new method for the synthesis of cyclic peptide libraries has been developed where the key cyclisation step involves reaction between a C-terminal cysteine side chain and an N-terminal bromoacyl group. We report conditions whereby liberation of peptides from the solid support and cyclisation occur concurrently to form thioether-linked cyclic peptides in generally >95% yield.

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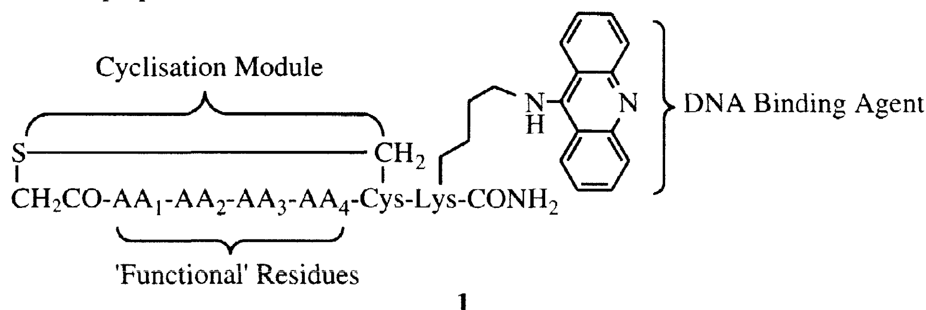
In connection with our studies on cyclic peptides that interact with DNA, we were faced with the need to develop an efficient method for the synthesis of cyclic peptide libraries.[1,2] Previously reported methods for the synthesis of cyclic peptides have involved the formation of lactam,[3] oxime,[4] thioether[5] and disulfide[6] bonds but we were interested in developing a new method that showed a high tolerance to variations in peptide sequence and could be readily performed on large numbers of compounds simultaneously. Here we report the synthesis of a library of cyclic thioether peptides where diversity is generated by the scrambling of functional residues relative to a pendant DNA-binding agent and a thioether cyclisation module. By the employment of orthogonal protection strategies, we have equipped each member of our library with a 9-aminoacridine DNA binding agent. We also report conditions whereby cleavage of the linear peptides from the solid support and cyclisation to form thioether-linked cyclic peptides can be performed simultaneously. The generic cyclic peptide **1** is representative of the peptides we have prepared.



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The synthetic strategy for the preparation of the linear peptides is shown in Scheme 1. Synthesis of these linear peptides was carried out using simultaneous multiple synthesis[7] with Multipin™ methodology and standard Fmoc chemistry[8] on SynPhase™ crowns functionalised with a Rink amide handle. The crowns were first deprotected using 20% piperidine/DMF, then an orthogonally protected lysine (Fmoc-Lys(Mtt)-OH) was coupled to the crowns in the presence of HOBt, HBTU and DIPEA to give **2** (Scheme 1). The hyperacid-labile methyltrityl side chain protecting group of the lysine was then removed with 1% TFA in CH_2Cl_2 to expose the ε-amino group of the lysine side chain and attachment of the 9-aminoacridine DNA binding agent was accomplished by condensation of the exposed primary amine with 9-phenoxyacridine in the presence of catalytic amounts of acetic acid. The lysine α-amino group was then deprotected and an orthogonally protected cysteine (Fmoc-Cys(Mmt)-OH) was coupled to the lysine N-terminus to give **3**. At this point multiple simultaneous synthesis allowed us to generate a sub-library of twelve linear peptides using various sequences consisting of isoleucine, lysine and two aspartate residues (**4a-l**). These amino acids were coupled as their pentafluorophenyl (Pfp) esters in the presence of HOBt. Following installation of the amino acids and α-bromoacetylation of the peptide N-termini,[9] exposure of the orthogonally protected cysteine side chain was accomplished using 1% TFA

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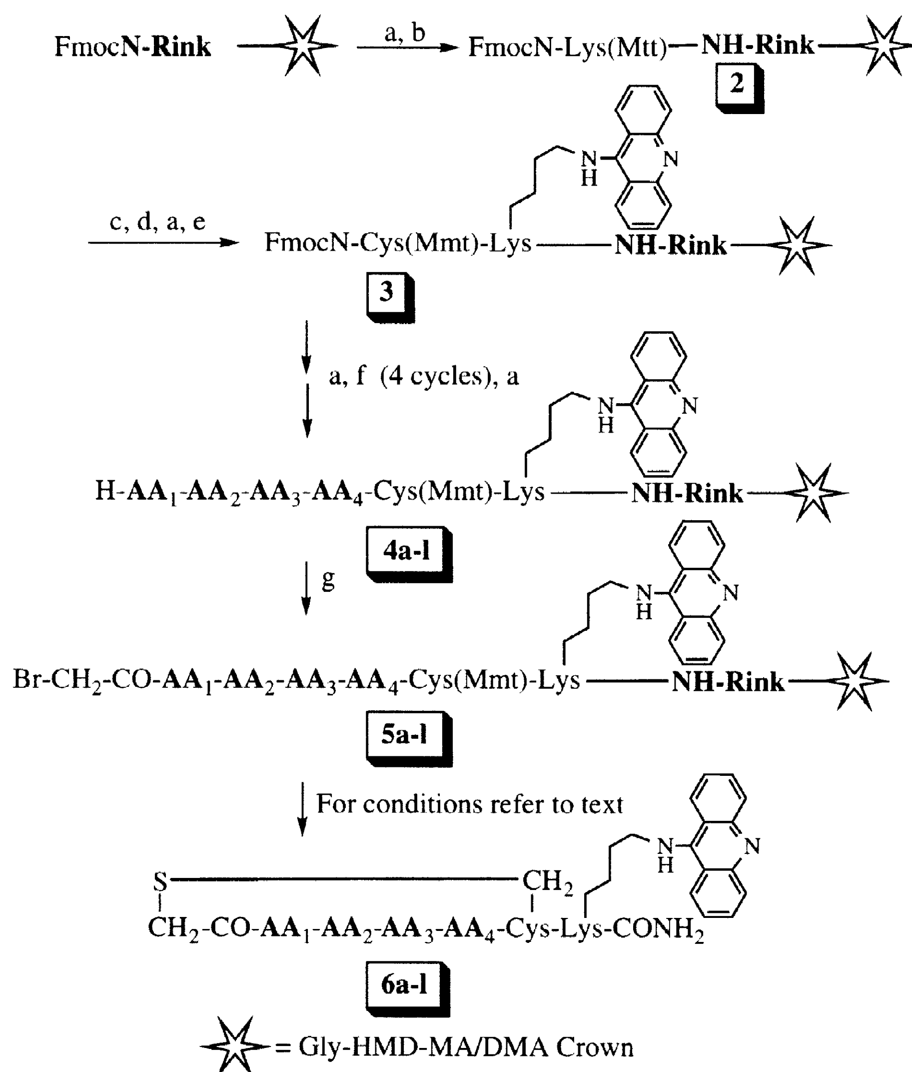
in CH_2Cl_2. Optimisation of the cyclisation conditions was initially performed on the support-bound peptides using different cyclisation conditions (1%AcOH/DMF, 1%AcOH/MeOH, 1%DIPEA/DMF, 1%DIPEA/MeOH) with linear peptide sequence **a**. The crowns were agitated in the appropriate solutions for 24 hours and peptide products were cleaved from the support under standard conditions.[10]

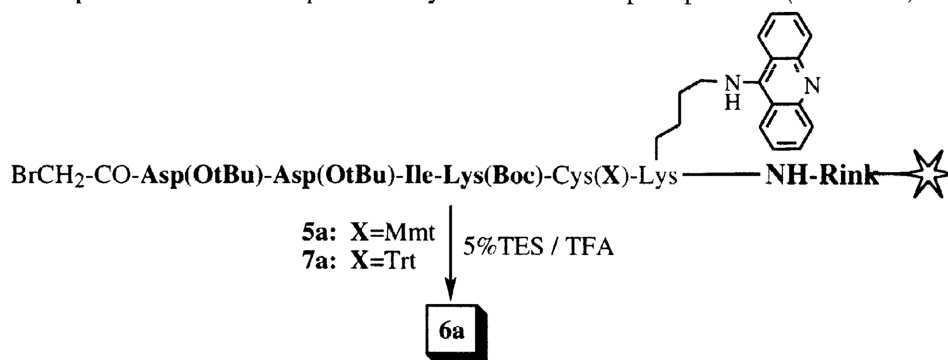


Scheme 1. (a) 20% piperidine/DMF; (b) Fmoc-Lys(Mtt)-OH, HBTU/HOBt/DIEPA; (c) 1%TFA/5%TIPS/CH_2Cl_2; (d) 9-phenoxyacridine, MeOH, 1% HOAc; (e) Fmoc-Cys(Mmt)-OH, HBTU/HOBt/DIEPA; (f) Fmoc-AA-OPfp, HOBt; (g) $BrCH_2CO_2H$/DIC/25%CH_2Cl_2/DMF

Analysis by RP-HPLC of the peptide samples that underwent the cyclisation reactions[11] indicated the presence of only one significant component for each of the cyclisation conditions examined and the ES-MS of each sample displayed only two significant signals corresponding to the $[M+H]^+$ and $[M+2H]^{2+}$ ions of the cyclic peptides **1**. No indication of the presence of any bromoacylated linear peptide was observed in either the HPLC or the mass spectra of these samples indicating that cyclisation had occurred in greater than 95% yield.

In order to obtain an authentic sample of a bromacetylated linear precursor to **6a**, we subjected a sample of the linear peptide **5a** to standard cleavage conditions. RP-HPLC analysis of this material again displayed a single peak with an identical retention time to that of cyclic peptide **6a** with none of the linear peptide being detected. Moreover, mass spectrometric analysis of this material displayed only ions corresponding to the $[M+H]^+$ (m/z 937) and $[M+2H]^{2+}$ (m/z 469) for the cyclic peptide **6a**, suggesting that in the acidic conditions

encountered upon cleavage from the solid support, cysteine deprotection and peptide cyclisation both occur and that it is possible to perform the final steps of this synthesis in a one-pot operation (Scheme 2).



Scheme 2

To confirm that the cyclisation and cleavage of the linear peptides **5a-l** could be combined into a single operation, we prepared the linear bromoacyl peptide (**7a**) where a more acid-stable trityl protected cysteine was employed rather than the methoxytrityl cysteine protecting group (Scheme 2). Cleavage of the linear peptide from the crown using the same conditions as above and analysis by RP-HPLC (Figure 1c) and ES-MS (Figure 1d) once again revealed cyclic peptide (**6a**) as the only significant product. Using our one-pot cyclisation/cleavage protocol, we completed the synthesis of a library of twelve cyclic peptides and in all cases, the syntheses proceeded smoothly to deliver high yields of cyclic peptide (Table).

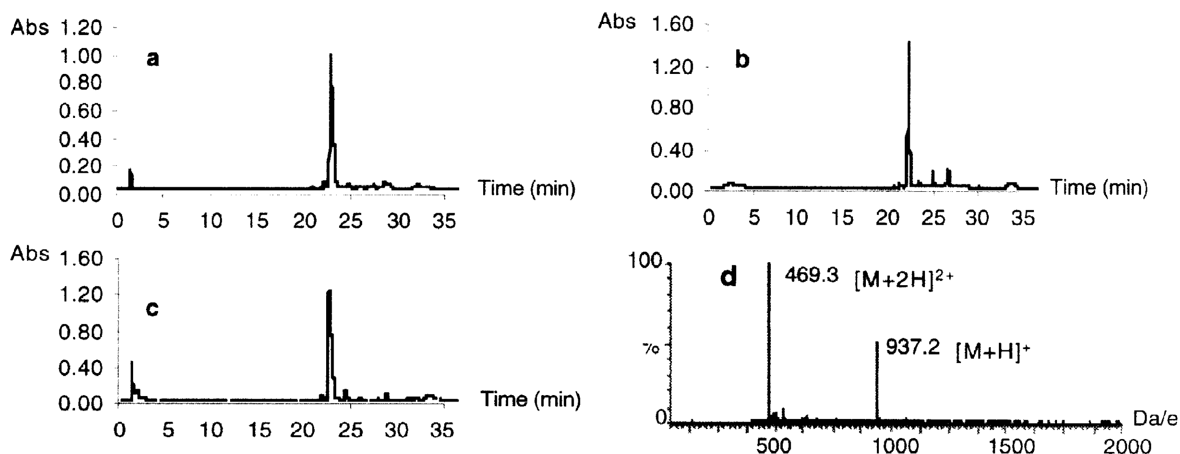


Figure 1. Analytical RP-HPLC analysis (a) Cyclic peptide **6a** obtained by cyclisation of deprotected support-bound linear peptide **5a** using 1%AcOH/DMF; (b) Cyclic peptide **6a** obtained by direct cleavage of bromoacyl peptide containing Mmt-protected cysteine (**5a**); (c) Cyclic peptide **6a** obtained by direct cleavage of bromoacyl peptide containing Trt protected cysteine (**7a**); (d) Positive ion ES-MS of crude **6a** formed by direct cleavage of trityl-protected **7a**.

Table. Results of the synthesis of thioether cyclic peptide library (**6a-l**).

Peptide Library (6)					**RP HPLC Data**[11]	**ES-MS Data**
Peptide 6	**AA_1**	**AA_2**	**AA_3**	**AA_4**	**R_t(purity at 214nm)**	**$[M+H]^+$**
a	Asp	Asp	Ile	Lys	19.7(>95)	937.2
b	Asp	Asp	Lys	Ile	21.5(>95)	937.2
c	Asp	Lys	Asp	Ile	22.2(>95)	937.2
d	Asp	Lys	Ile	Asp	21.8(>95)	937.2
e	Asp	Ile	Asp	Lys	20.0(>95)	937.2
f	Asp	Ile	Lys	Asp	21.3(>95)	937.2
g	Lys	Asp	Asp	Ile	20.7(61)	937.2
h	Lys	Asp	Ile	Asp	22.1(>95)	937.2
i	Lys	Ile	Asp	Asp	20.1(>95)	937.2
j	Ile	Lys	Asp	Asp	21.2(>95)	937.2
k	Ile	Asp	Lys	Asp	21.5(>95)	937.2
l	Ile	Asp	Asp	Lys	22.7(>95)	937.2

In summary we have shown that a thioether strategy provides a viable route to libraries of cyclic peptides and that cleavage from the solid support and cyclisation can be performed simultaneously to deliver cyclic peptides in high yields and excellent purity. We anticipate that the efficiency of cyclisation displayed here and the capacity to integrate the cleavage and cyclisation steps into a single operation will greatly facilitate the generation of libraries of cyclic peptides for a diverse array of applications. In the future we will report on the synthesis of other libraries and the biological activity our cyclic peptide products.

Acknowledgments
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List of Abbreviations
AA, L-amino acid fragment; DIC, diisopropylcarbodiimide; DIPEA, diisoproplethylamine; ES-MS, electrospray mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; Mmt, methoxytrityl; Mtt, methyltrityl; Pfp, pentafluorophenyl; TES, triethylsilane; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; Trt, trityl.

References and Notes
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8. Peptide synthses were performed on Rink-Amide-Handle-Gly-HMD-MA/DMA crowns with a loading of approximately 6.5μmole crown^{-1}. Details of the chemical nature of these crowns is available at http://www.chirontechnologies.com.au/techn/pub_pdf.htm. Unless otherwise specified, Asp and Lys residues were protected as Asp(OtBu) and Lys(Boc). For Fmoc-AA-OH amino acid couplings, DMF solutions that were 0.1M with respect to Fmoc-AA-OH, HBTU, HOBt and DIPEA were prepared and allowed to stand for 5 minutes prior to addition to the crown supports. The crowns were then agitated gently in the solution for 2 hours at room temperature. For Fmoc-AA-OPfp amino acid couplings, the crowns were rotated in a solution of DMF that was 0.1M with respect to Fmoc-AA-OPfp and HOBt for 2 hours.
9. Crowns were agitated in a solution of 25%DCM/DMF, 0.1M with respect to DIC and 0.2M with respect to α-bromoacetic acid, for 2 hours at room temperature. The solution was activated for 5 minutes before the crowns were added.
10. Crowns were placed in a solution of 5% TES/95%TFA acid for 1.5 hours. The crowns were then removed from the solution and the TFA evaporated under a stream of nitrogen. A 1:2 solution of diethyl ether/40-60 petroleum spirit was added to the remaining residue and the tubes containing the solution were agitated. At this point the peptide precipitated out of solution. The suspension was then centrifuged and the ether/pet.spirit decanted. The remaining pellet was then dissolved in 25%H_2O/MeCN and then freeze dried to give the crude target peptide.
11. Analytical HPLCs were performed using a 100 x 4.6mm Phenomenex Ultremex C18 reverse phase column connected to Waters 510 HPLC pumps and a Waters 996 PDA detector. Gradient elution was from A (0.1%TFA in water) to B(acetonitrile) over 40 minutes. HPLC samples were prepared by dissolving the peptides in 50/50 water/acetonitrile.