Journal of Peptide Science
J. Peptide Sci. **4**: 335–343 (1998)

Solid Phase Synthesis of Cyclic Peptides: Model Studies Involving $i-(i+4)$ Side Chain-to-Side Chain Cyclisation

VITTORIA CAVALLARO, PHILIP THOMPSON and MILTON HEARN*

Centre for Bioprocess Technology, Department of Biochemistry and Molecular Biology, Monash University, Clayton 3168, Australia

Received 30 October 1997
Accepted 2 December 1997

Abstract: Conditions for the synthesis of $i-(i+4)$ side chain-to-side chain head-to-tail Lys→Glu and Glu→Lys linked cyclic peptides related to hypoglycaemic analogues of human growth hormone hGH [6–13] have been examined. The success of the cyclisation reaction with the corresponding resin-bound, partially protected linear peptides was found to be both reagent as well as sequence dependent, with competing inter-chain oligomerisation predominating in some cases. The results also indicated that protection with the bulky Fmoc group of the amino acid residues immediately adjacent to the side chain-deprotected Lys and Glu residues, which participate in the cyclisation reaction, enhanced the rate of lactam formation. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclic peptides; human growth hormone; lactam cyclisation; side chain effects

INTRODUCTION

Cyclisation of peptides to promote the formation of specific three-dimensional structures represents an important strategy in achieving conformational restriction [1]. The conversion of linear peptides to their cyclic analogues not only generates conformational constraints within the peptide backbone and substantially reduces the degrees of freedom of the side chain orientations, but also often creates structures with enhanced metabolic stability. A range of methods have been applied to generate cyclic peptides from linear precursors, including procedures based on thioester ring contraction [2,3], disulphide bridge construction [4,5] and pseudopeptide cyclisation [6]. Methods for the synthesis of $i-(i+n)$ side chain to side chain macrocyclic lactams have also been developed and applied in the preparation of cyclic peptides with enhanced potency and/or metabolic stability with a range of target peptides, including gonadotropin releasing hormone (GnRH) antagonists, α-melanotropins and growth hormone releasing factor (GRF) analogues [7–10]. Cyclic peptides have also been frequently used as model systems for the induction of reverse turn structures with particular L-α- and D-α-amino acids, and as peptidomimetics of bioactive peptides and proteins [2,11,12].

As part of our structure-function investigations into the molecular basis of the hypoglycaemic properties of some hGH[6–13]-related peptides [13], we required a general protocol for the synthesis of cyclic analogues which would allow the preparation of libraries of cyclic peptides containing, β-turn motifs. On-resin head to tail and side-chain to side chain cyclisation of linear peptides to form macrocyclic lactams is a challenging, yet convenient, strategy which is beginning to find wider application [6,14–16]. The selection of appropriate conditions for the cyclisation is, however, critical to successful synthesis, with guidelines to achieve this outcome still incomplete. Arising from our previous investigations into the preparation of cyclic peptides from their linear progenitors, we have reported [17] the synthesis of a number of cyclic analogues of bioactive linear peptides using the BOP reagent [18]. However, in some cases it was found that signifi-

* Correspondence to: Centre for Bioprocess Technology, Monash University, Clayton 3168, Australia.

© 1998 European Peptide Society and John Wiley & Sons, Ltd.
CCC 1075–2617/98/050335-09$17.50

Table 1 Peptide sequences attached to the MBHA-resin

Peptide	Sequence position							
	1	2	3	4	5	6	7	8
1		Fmoc	Lys	Phe	D-Ala	Pro	Glu	Gly
2		Fmoc	Glu	Phe	D-Ala	Pro	Lys	Gly
3		Fmoc	Lys	Phe	D-Ala	Pro	Glu	Leu
4	Fmoc	Gly	Lys	Phe	D-Ala	Pro	Glu	Gly
5	Fmoc	Gly	Glu	Phe	D-Ala	Pro	Lys	Gly

cant levels of competing side reactions precluded the successful generation of the cyclic peptide library [17]. In subsequent studies, we have examined several variations to this approach in order to optimise the cyclisation reaction. Here, we report on the synthesis of model peptides incorporating $i-(i+4)$ macrocyclic head-to-tail Lys→Glu and Glu→Lys lactams and the D-Ala^{11}-Pro^{12} reverse turn motif [19] (Table 1). Three features of the optimisation approach have been examined: firstly, the effectiveness of various reagents for achieving on-resin cyclisation, secondly, the importance of the position of the reacting side chains of the Glu and Lys residues; and thirdly, the influence of the protecting groups and the nature of the side chains of the amino acid residues distal to the Glu/Lys residues which form the cyclic portion of the molecule and which impact on a successful outcome for the cyclisation.

MATERIAL AND METHODS

Chemicals and Equipment

Peptide syntheses were performed in plastic columns with plastic sinters at their base for solvent removal under suction. Unless otherwise stated, all solvents were analytical grade. Thioanisole, ethanedithiol, 1,3- diisopropylcarbodiimide (DIC), and trifluoromethanesulfonic acid (TFMSA) were obtained from Aldrich Chemical (Milwaukee, USA), whilst *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), Castro's reagent (BOP), diisopropylethylamine (DIEA), piperidine, *N*,*N*-dimethylformamide (DMF), 4-methylbenzhydrylamine resin (MBHA) hydrochloride (0.83 mmol equiv/g), trifluoroacetic acid and all protected amino acids were obtained from Auspep (Melbourne, Australia).

Peptide Synthesis Procedures

The peptides described in this work were synthesised using a Fmoc-solid-phase method on 4-methylbenzhydrylamine resin as described previously [20]. Couplings were performed using HBTU/HOBt activation of Fmoc-amino acids in DMF. The Fmoc-protected peptidyl-resins were washed with dichloromethane (3×5 ml) and side chain deprotection to liberate the free amino and carboxyl side chains was carried out using 50% TFA in dichloromethane (2×5 ml). The peptide resin was then washed with DCM (2×5ml) and DMF (3×5ml) and transferred to a 250 ml round bottomed flask. On-resin cyclisation was achieved by treating the resin with 3 equivalents of the one of the following reagent mixtures, A–D, 4 mM in DMF over a time course of 7 h. Reagents were refreshed after 2.5 and 5 h. The reagent mixtures were: (A) BOP/DIEA (B) BOP/HOBt/DIEA, (C) HBTU/HOBt/DIEA or (D) DIC/HOBt. The resin was then washed with DMF (3×5 ml). For the synthesis of the peptides **1**, **2**, **3** and **5**, the N-terminal Fmoc protection was removed with 20% piperidine in DMF (l0 ml). The resin was then washed with DCM (2×10 ml) and diethyl ether (2×10 ml) and dried under vacuum.

Resin Cleavage Methods

The suction dried peptide-resin was treated with thioanisole (1 ml) and ethanedithiol (0.5 ml) followed by TFA (l0 ml) and the mixture stirred at RT for 5 min. The mixture was then cooled in ice and TFMSA (1 ml) added dropwise while stirring. The flask was stoppered and the mixture stirred at RT for 1.5 h. Ice-cold diethyl ether (50 ml) was then added and the mixture was stirred vigorously for 1 min and filtered. The peptide was extracted into TFA (3×3 ml) and the solution concentrated *in vacuo* and then treated with cold ether to precipitate the peptide. The solid was filtered and then dis-

© 1998 European Peptide Society and John Wiley & Sons, Ltd.

solved in 50% aqueous acetonitrile before lyophilisation.

Peptide Purification and Analysis Procedures

The crude products were analysed by RP-HPLC using a gradient of 5–40% Buffer B (Buffer A, 0.1% aq. TFA; Buffer B, 60% acetonitrile/0.1% aq. TFA) over 60 min at a flow rate of 1 ml/min on a TSK ODS-120T column (150×4.6 mm ID). The eluent was monitored at 214 nm. Chromatography was performed on a Waters Associates (Milford, MA) liquid chromatography system consisting of a Model 600 solvent delivery pump, a Wisp Model 712 Sample Processor and automated gradient controller.

FAB-mass spectra were acquired using a JEOL JMS-DX300 spectrophotometer at an acceleration voltage of 1.5 keV with a FAB primary ion energy of 4 keV and emission current of 20 mA. Xenon was employed as the bombardment gas. The scan range was 100–1600 m/z. Electrospray mass spectra were acquired using a Perkin Elmer-SciEx mass spectrometer model PE Sciex API, using a scan range between 100–2400 amu. Amino acid compositions were determined by hydrolysis of the sample *in vacuo* in constant boiling 6 M HCl for 22 h at 110°C. Samples were then analysed using the PICOTAG method [21].

Characterisation of Peptides

The following analytical data were obtained for the cyclic and linear peptide analogues:

(**IA**) Cyclo(Lys$^1 \rightarrow$Glu^5)-Lys-Phe-D-Ala-Pro-Glu-Gly-$CONH_2$: ES-MS (M + H): Calc. 628.8, Found 628.2; Amino acid composition: Glu 0.9(1), Gly 0.97(1), Ala 1.0(1), Pro 0.97(1), Phe 1.0(1), Lys 0.96(1).

(**1B**) Linear Lys-Phe-D-Ala-Pro-Glu-Gly-$CONH_2$: ES-MS (M + H): Calc. 646.8, Found 646.4.

(**2A**) Cyclo(Glu$^1 \rightarrow$Lys^5)-Glu-Phe-D-Ala-Pro-Lys-Gly-$CONH_2$: ES-MS (M + H): Calc. 628.8, Found 628.2; Amino acid composition: Glu 0.8(1), Gly 0.8(1), Ala 1.0(1), Pro 1.0(1), Phe 0.85(1), Lys 0.9(1).

(**2B**) Linear Glu-Phe-D-Ala-Pro-Lys-Gly-$CONH_2$: ES-MS (M + H): Calc. 646.8, Found 647.2.

(**3A**) Cyclo(Lys$^1 \rightarrow$Glu^5)-Lys-Phe-D-Ala-Pro-Glu-Leu-$CONH_2$: FAB-MS: Calc. 684.9, Found 685; Amino acid composition: Glu 1.0(1), Ala 1.2(1), Pro 1.2(1), Leu 1.2(1), Phe 1.2(1), Lys 0.9(1).

(**3B**) Linear Lys-Phe-D-Ala-Pro-Glu-Leu-$CONH_2$: FAB-MS: Calc. 702.9, Found 703.

(**4A**) Cyclo(Lys$^2 \rightarrow$Glu^6)-Gly-Lys-Phe-D-Ala-Pr-Glu-Gly-$CONH_2$): FAB-MS: Calc. 908.1, Found 909 (Fmoc group still attached)

(**4B**) Linear -Gly-Lys-Phe-D-Ala-Pro-Glu-Gly-$CONH_2$: FAB-MS: Calc. 926.1, Found 927 (Fmoc group still attached); Amino acid composition: Glu 0.9(1), Gly 2.1(2), Ala 1.0(1), Pro 1.0(1), Phe 0.9(1), Lys 0.8(1).

(**5A**) Cyclo (Glu$^2 \rightarrow$Lys^6)-Gly-Glu-Phe-D-Ala-Pro-Lys-Gly-$CONH_2$: No cyclic peptide **5A** was obtained in the attempted cyclisation reactions.

(**5B**) Linear -Gly-Glu-Phe-D-Ala-Pro-Lys-Gly-$CONH_2$: FAB-MS: Calc. 703.9, Found 704; Amino acid composition: Glu 0.9(1), Gly 2.3(2), Ala 1.2(1), Pro 1.2(1), Phe 1.2(1), Lys 1.0(1).

RESULTS

As part of the development of constrained cyclic hGH[6–13] peptide analogues, a synthetic strategy was chosen for the construction of the corresponding resin-bound linear peptide since this represented the simplest approach to the preparation of Lys and Glu side chain deprotected resin-bound peptide precursors, whilst still being characterised by the absence of reactive side chains apart from those targeted for cyclisation. Thus, the resin-bound linear protected peptides synthesised using Fmoc- chemistry on MBHA resin could be treated with TFA to remove the acid labile side chains of Lys and Glu, while retaining Fmoc-protection on the amino terminus. After the cyclisation step, Fmoc removal could be achieved by piperidine deprotection with cleavage of the peptide from the resin was performed using standard TFMSA cleavage methods [20]. In the absence of the cyclisation step, high yields of the corresponding linear peptides with minimal amounts of side products were obtained. The strategy for the preparation of the cyclic peptides is summarised in Figure 1.

Comparison of the Effect of the Cyclisation Reagents

In this investigation, conditions for the cyclisation reaction were examined using two isomeric model linear pentapeptides **1A** and **2A** of the same composition but involving Lys/Glu residue reversal. In both cases, the synthesis of the desired linear peptides proceeded smoothly in very high yield, thus enabling the effects of cyclisation conditions on product formation to be directly examined. The results obtained for the cyclisation of the linear peptides Fmoc-Lys-Phe-D-Ala-Pro-Glu-Gly-MBHA-resin **1A** and Fmoc-Glu-Phe-D-Ala-Pro-Glu-Gly-MBHA-resin **2A** using triple couplings of reagent mixtures:

© 1998 European Peptide Society and John Wiley & Sons, Ltd.

BOP, BOP/HOBt, HBTU/HOBt or DIC/HOBt over a time course of 7 h in DMF are shown in Table 2, whilst the RP-HPLC profiles for the separation of the crude reaction products are shown in Figures 2 and 3, respectively. In all cases, the respective linear and cyclic peptide products were characterised by high sensitivity amino acid analysis as well as electrospray mass spectroscopy (ES-MS) and/or fast atom bombardment mass spectroscopy (FAB-MS).

As is evident from these results, the success of cyclisation was found to be dependent upon the choice of the cyclisation reagent, with the products

A: SPPS Procedure:

2eq. HOBt, HBTU & Fmoc-A.A.

Fmoc-Xxx-Phe-D-Ala-Pro-Yyy-Gly-MBHA-Resin

Xxx = Lys(Boc) Xxx = Glu(OtBu)
Yyy = Glu(OtBu) Yyy = Lys(Boc)

B: Side Chain Deprotection

50% TFA , DCM

Fmoc-Xxx-Phe-D-Ala-Pro-Yyy-Gly-MBHA-Resin

C: Cyclisation Conditions:

A) BOP (3eq.)
B) BOP, HOBt (3eq.)
C) HOBt, HBTU (3eq.)
D) DIC, HOBt (3eq.)

Fmoc-Xxx-Phe-D-Ala-Pro-Yyy-Gly-MBHA-Resin

D: Fmoc Deprotection - Cleavage - & RP-HPLC

H_2N-Xxx-Phe-D-Ala-Pro-Yyy-Gly- $CONH_2$

Figure 1 Synthesis strategy for the generation of the cyclic peptides from the resin-bound partially deprotected linear peptide precursors. Cyclisation scheme for the synthesis of cyclic peptides **IA** and **2A** The peptide **3A** has a Leu at the C-terminus of the sequence instead of Gly, whilst peptides **4A** and **5A** have an additional Gly introduced into the sequences of **1** and **2,** respectively at the N-terminus prior to the cyclisation reaction.

obtained upon deprotection and cleavage containing different abundances of the linear, cyclic and oligomeric peptides. For both peptide **1A** and **2A**, the rank order of reactivity of the reagents was found to be the same (BOP $\approx$ HBTU/HOBt $\approx$ BOP/HOBt $\gg$ DIC) with this increased reactivity concomitantly associated with increased oligomer formation. For example, with Fmoc-Lys-Phe-D-Ala-Pro-Glu-Gly-MBHA-resin **1A**, no linear product was recovered using the BOP reagent, but 56% of the reacted product was higher oligomers. On the other hand, with DIC/HOBt, 33% of the peptide-resin remained unreacted after 7 h, but with over 90% of the reacted product present as the desired cyclic product **1B**. Thus, in the case of the cyclisation of **1A** to **1B**, DIC gave the highest yield of cyclic product. It should be noted that in preparative isolation circumstances, the cyclic peptides **1B** and **2B** were more easily purified from oligomeric side products than from the corresponding linear peptides **1A** and **2A**, and thus the BOP and HBTU methods resulted in higher overall yields of the cyclic peptides.

One feature to note regarding the cyclisation reaction was that the Kaiser and TNBSA tests with the crude product showed the presence of free amino groups even when the crude reaction mixture was found be devoid of unreacted, linear peptide precursors. This observation can be attributed to the presence of unreacted amino groups on the oligomeric adducts, and so these tests were not applicable as a direct assay procedure to monitor the cyclisation reaction *per se*. Further, lengthened exposure to the reagents under circumstances where all the linear peptide progenitor was exhausted served no beneficial purpose in the synthesis and appeared to propagate other unwanted side reactions. In the optimised syntheses, small scale cleavage and HPLC analysis was thus used to determine the exhaustion of the corresponding linear peptide precursors.

Comparison of the Effect of Side Chain Groups on the Cyclisation Efficiency

Because the ring size and overall conformational flexibility of the cyclic peptide products derived from the resin-bound peptides **1A** and **2A** was the same in each case, on these grounds substantial differences in the cyclisation propensity of the corresponding resin-bound linear peptides was not expected. However, in terms of stereo-electronic effects associated with the formation of the transition

© 1998 European Peptide Society and John Wiley & Sons, Ltd.

Table 2 Comparison of the efficacy of the reagents for the cyclisation of the resin-bound partially deprotected linear peptides **1** and **2** to their corresponding cyclic peptides **1A** and **2A** as well as the linear peptide species **1B** and **2B** and oligomeric products respectively

Method	Product ratios (%)					
	Peptide **1**			Peptide **2**		
	Linear	Cyclic	Oligomeric	Linear	Cyclic	Oligomeric
BOP/DIEA	0	42	56	4	24	67
BOP/HOBt/DIEA	2	51	46	16	30	54
HBTU/HOBt/DIEA	0	35	56	13	32	54
DIC	33	61	6	87	7	5

state intermediates for the reaction with DIC, BOP and HBTU which leads ultimately to the two isomeric lactams, differences in the cyclisation kinetics for peptide **1A** *vis-a-vis* peptide **2A** could be expected. This behaviour was in fact observed with significant differences evident for the cyclisation efftciency with these two peptides under identical reaction conditions. Under all examined conditions, the linear peptide **1A** was much more readily cyclised than the linear peptide **2A**, the reaction of the latter peptide being characterised by the presence of a higher residual amount of unreacted linear peptide precursor as well as larger amounts of side products. Under the DIC/HOBt cyclisation conditions ca. 30% of the resin-peptide **1A** remained unreacted, while nearly 90% of the resin-peptide **2A** remained unreacted after 7 h, as shown by the recovery of a high yield of linear peptide **2A** following deprotection, cleavage from the resin and purification. The yields of oligomeric products were relatively independent of this reversal of the sequence, suggesting that the rate of the oligomerisation reaction was not sequence dependent.

Effects of Distal Residues on the Cyclisation Efficiency

The marked effect of the Lys/Glu residue reversal on the cyclisation efficiency suggested that the neighbouring bulky Fmoc- group as well as the MBHA-resin might have exerted an effect upon the cyclisation kinetics. By substituting Leu for Gly adjacent to the Glu residue at the C-terminus, the steric bulk at the C-terminus will be effectively increased. Alternatively, by adding a Gly residue at the amino terminus, the location of the Fmoc-protecting group was made more remote from the two cyclising residues.

The Leu substitution had a significant inhibitory effect upon the rate of cyclisation of the resin-bound linear peptide **3A**, with significant levels of the linear peptide **3A** recovered after treatment with the BOP reagent. Insertion of a Gly spacer between the Fmoc-group and the reacting N-terminal amino acid also inhibited the cyclisation reaction using DIC, with the extent of cyclisation only 29% for the resin bound linear peptide **4A** whilst the resin-bound linear peptide **5A** failing to undergo cyclisation. This result indicates that the adjacent Fmoc protecting group actually exerted a favourable effect upon the cyclisation reaction.

DISCUSSION

The fundamental objective of this investigation was to find conditions suited to generating cyclic $i-(i+4)$ linked peptide libraries related to hGH[6–13] in a relatively sequence independent manner. Our initial attempts at synthesising such peptides had resulted in mixed success and we were eager to adjust the conditions to reduce some of the more significant side reactions. The syntheses of model hexapeptides were carried out in order to permit an examination of the effects mediated via on resin cyclisations using a variety of reagents over a 7 h period.

In solid phase cyclic peptide synthesis, the efficacious BOP and HBTU have been promoted as reagents for effecting intramolecular head-to-tail cyclisation, usually in preference to the more sluggish carbodiimide reagents [14]. In the present study, while this ability was confirmed, a significant extent of competing inter-chain reaction was observed resulting in large amounts of higher oligomeric pep-

© 1998 European Peptide Society and John Wiley & Sons, Ltd.

tides being obtained. This was not anticipated, since peptide cyclisation on solid support was originally introduced to effect a pseudo-dilution phenomenon with the idea of eliminating polymerisation and cyclooligomerisation indicative of solution phase cyclisation effects [14]. Thus, whilst DIC proved to be sluggish with the linear hGH[6–13] resin-bound peptide, very low proportions of oligomeric peptides were retrieved, offering the prospect that increased reaction times would furnish cyclic peptides in high yield and purity. In subsequent investigations we have found this to be the case (Cavallaro *et al.*, unpublished results).

The reversal of the position of Lys and Glu in the hGH[6–13] related sequence had a major impact on the success of cyclisation. This outcome was illustrated as a significant influence of apparently conservative amino acid changes upon overall conformation. Rao *et al.* [12] have gone some way to addressing this influence in their study of $i-(i+3)$ model peptides. Whilst the sequence reversal was not examined, differences in the yield of peptides and in the reaction time conditions were noted for peptides of the same ring size. For example, peptide cyclo^{1,4}-Ac-Dab-Pro-Gly-Glu-NH_2 was recovered in 24% yield, whilst the peptide cyclo^{1,4}-Ac-Orn-Pro-Gly-Asp-NH_2 could only be achieved in 7% yield, despite both cyclisation reactions involving the formation of isomeric 16-membered ring structures.

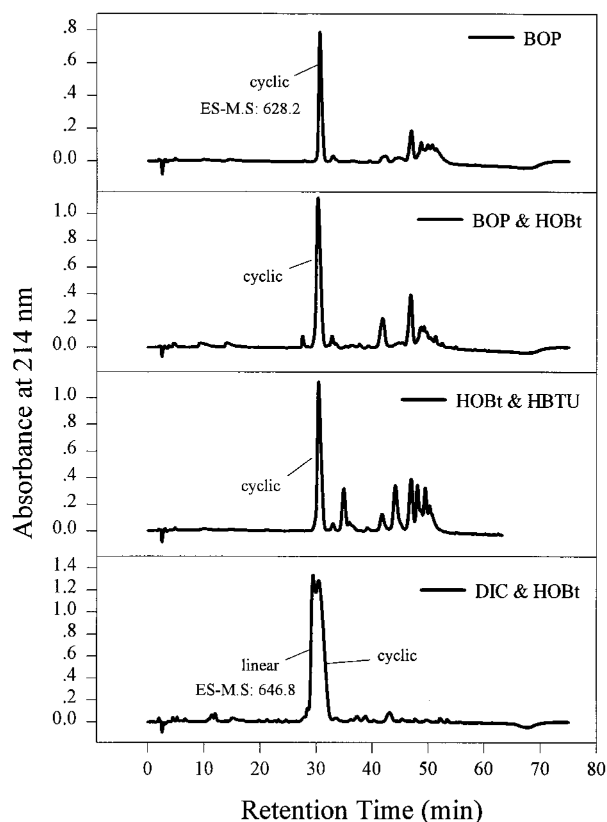

Figure 2 Analytical RP-HPLC chromatograms of crude peptide products obtained after cyclisation of the resin immobilised partially deprotected peptide **1** with either BOP, BOP/HOBt/DIEA, HBTU/HOBt/DIEA or DIC/HOBt and cleavage of the *in situ* generated cyclic peptide **1A**, linear peptide **1B** and oligomeric products from the resin with TFMSA. Gradient elusion was achieved with 5–40% Buffer B (60% acetonitrile/0.1% aq. TFA) over 60 min. at a flow rate of 1 ml/min. Other experimental details are given in the Materials and Methods section.

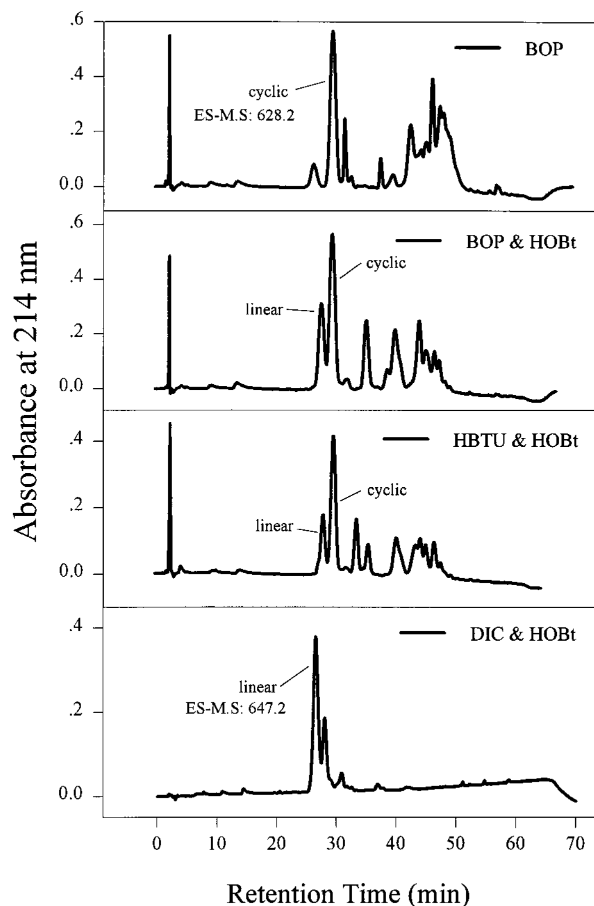

Figure 3 Analytical RP-HPLC chromatograms of crude peptide products obtained after cyclisation of the resin immobilised partially deprotected peptide **2** with either BOP, BOP/HOBt/DIEA, HBTU/HOBt/DIEA or DIC/HOBt and cleavage of the *in situ* generated cyclic peptide **2A**, linear peptide **2B** and oligomeric products from the resin with TFMSA. Gradient elusion was achieved with 5–40% Buffer B (60% acetonitrile/0.1% aq. TFA) over 60 min at a flow rate of 1 ml/min. Other experimental details are given in the Materials and Methods section.

© 1998 European Peptide Society and John Wiley & Sons, Ltd.

Table 3 Comparison of the product yields for the synthesis of the cyclic peptides **1A–5A**, respectively from the resin-bound partially deprotected linear peptide precursors

Method	Product ratios (%)					
	BOP			DIC		
	Linear	Cyclic	Higher	Linear	Cyclic	Other
Peptide (**1**)	0	42	56	33	61	6
Peptide (**2**)	4	24	67	87	7	5
Peptide 3-Leu^{6}-(**1**)	15	20	65		n.d.	
Peptide 4-Gly^{1}-(**1**)		n.d.		71	29	
Peptide 5-Gly^{1}-(**2**)		n.d.		100		

Notably, the resultant cyclic peptides also displayed markedly different conformational properties.

While a number of reports have documented side chain-to-side chain macrocyclic lactam formation, no specific examples have been described whereby an $i-(i+4)$ linkage has been used to stabilise a reverse turn sequence. Indeed, such a linkage is generally utilised to stabilise an α-helical structure [22]. In our case, the objective was to maintain a specific orientation of pharmacophoric groups of our active linear peptides [7]. Interestingly, Kapurniotu and Taylor recently reported a possible reverse turn structure in an $i-(i+4)$ lactam bridged analogue of human calcitonin (hCT), designed to stabilise an α-helix [23]. A dramatic difference was found in the CD spectra and biological activity of reversed sequences in analogues of hCT. In particular, it was observed that while cyclo^{17,21}-[Lys^{17}, Asp^{21}]hCT had very similar conformation features relative to the native hCT, cyclo^{17,21}-[Asp^{17}, Lys^{21}]hCT showed a more ordered non-helical structure and a 10–20-fold increase in activity. Unfortunately in this study by Kapurniotu and Taylor, no comparison could be made about the effects of the cyclisation reaction generating these cyclic peptides as they were synthesised using different strategies.

The present studies on the cyclisation reaction of several model peptides related to hGH[6–13] analogues provides additional insight into the relative propensity of resin-bound linear peptides to undergo intra-chain reaction and thus the results provide an interesting overview of the conformational preferences of the linear sequence, albeit under non-native conditions (immobilised to the resin and solvated in DMF). The conformational constraint imposed by peptide cyclisation represents an important strategy to study the conformational properties of specific peptide sequences. However, it appears that in many cases the lactam bond formed during the cyclisation reaction represents the dominant influence in determining the conformational preferences of the remaining sequence.

In considering further effects that could hinder intra-chain cyclisation, it was considered that the amino acid residue adjacent to those undergoing the reaction, but outside the cyclic structure to be formed, might also influence the success of cyclisation. This involvement was first suggested by the failure of cyclisation in the absence of a C-terminal glycine spacer of the resin-bound peptide Fmoc-Lys-Phe-D-Ala-Pro-Glu-MBHA resin, and was of particular interest in the development of suitable methodologies to synthesise cyclic peptide libraries related to hGH[6–13]. We also rationalised that if the Fmoc group could be placed more remote from the cyclising residue an increase in the rate of cyclisation might be observed. In fact, it was found that both the C-terminal Leu → Gly as well as the N-terminal Gly extension had a deleterious effect upon the rate of cyclisation (Table 3). As expected, the incorporation of a C-terminal Leu resulted in a significantly slower rate of cyclisation using the BOP reagent. Unexpectedly, substituting an Fmoc-Leu group with a Fmoc-Gly group also slowed down the cyclisation reaction using DIC, to such an extent that for peptide **5A** no cyclic product was observed whatsoever, whilst peptide **4A**, showed a much lowered yield. In this case therefore, the Fmoc-group represents a positive element in the induction of the cyclisation, and thus overall it can be considered that the side chain protecting groups external to the macrocyclic structure being formed can have a significant impact on the success or otherwise of the cyclisation reaction.

© 1998 European Peptide Society and John Wiley & Sons, Ltd.

In conclusion three findings of significance have emerged from this study. Firstly, whilst BOP, BOP/HOBt and HBTU all have enhanced reactivity relative to DIC, they all suffer from high levels of oligomer formation with resin-bound linear peptides. Based on these model studies, it appears that extended reactions using DIC may prove to be the most reliable source of cyclic hGH[6–13] analogues. Secondly, it is clear that the linear sequence Fmoc-Lys-Phe-D-Ala-Pro-Glu-Gly-MBHA resin, **1**, undergoes cyclisation much more readily than the 'reverse lactam' sequence, Fmoc-Glu-Phe-D-Ala-Pro-Lys-Gly-MBHA resin **2**. Thirdly, the results confirm that the nature of the protected amino acid residues adjacent to the Lys/Glu residues can significantly influence the cyclisation. From the viewpoint of a synthetic methodology, it is interesting to note that an adjacent Fmoc-group appears to enhance the cyclisation propensity with these partially protected resin-bound linear peptides, providing support for the strategy of mid-synthesis cyclisation. Based on the results of this investigation, the synthesis of the cyclic peptide libraries related to hGH[6–13], their conformational properties and their application as hypoglycaemic compounds are currently being examined and will be described in a subsequent manuscript.

Acknowledgements

These investigations were supported by the Australian Research Council and the National Health and Medical Research Council of Australia.

REFERENCES

1. V.J. Hruby (1982). Conformational restrictions of biologically active peptides via amino acid side chain groups. *Life Sci. 31*, 189–199.
2. P. Botti, T. D. Pallin and J.P. Tam (1997). Cyclic peptides from linear unprotected precursors through thiazolidine formation. *J. Am. Chem. Soc. 118*, 10018–10024.
3. B.A. Katz, C.R. Johnson and R.T. Cass (1995). Structure-based design of high affinity streptavidin binding cyclic peptide ligands containing thioether crosslinks. *J. Am. Chem. Soc. 117*, 8541–8547.
4. L. Chen, I. Zoulikova, J. Slaninova and G. Barany (1997). Synthesis and pharmacology of novel analogues of oxytocin and deaminooxytocin: direct methods for construction of disulfide and trisulfide bridges in peptides. *J. Med. Chem. 40*, 864–876.
5. J.J. Leban, A. Spaltenstein, A. Landavazo, W. Chestnut, A.. Aulabaugh, L.C.E. Taylor and A.J. Daniels (1996). Synthesis, structure and stability of novel dimeric peptide-disulfides. *Int. J. Peptide Protein Res. 47*, 161–166.
6. J.J. Wen and A.F. Spatola (1997). A systematic approach to the solid-phase synthesis of linear and cyclic pseudopeptide libraries containing $\psi[CH_2NH]$ amide bond surrogates. *J. Pept. Res. 49*, 3–14.
7. J. Rizo and L.M. Gierasch (1992). Constrained peptides: Models of bioactive peptides and protein structures. *Ann. Rev. Biochem. 61*, 387–418.
8. R.J. Bienstock, R. Rizo, S.C. Koerber, J.E. Rivier, A.T. Hagler and L.M. Gierasch (1993). Conformational analysis of a highly potent dicyclic gonadotropin releasing hormone antagonist by nuclear magnetic resonance and molecular dynamics. *J. Med. Chem. 36*, 3265–3273.
9. F. Al-Obeidi, A.M. de L. Castrucci, M.E. Hadley and V.J. Hruby (1989). Potent and prolonged acting cyclic lactam analogues of α-melanotropin: design based on molecular dynamics. *J. Med. Chem. 32*, 2555–2561.
10. R.M. Campbell, J. Bongers and A.M. Felix (1995). Rational design, synthesis and biological evaluation of novel growth factor releasing factor analogues. *Biopolymers*, *37*, 67–88.
11. W.C. Ripka, G.V. De Lucca, A.C. Bach, R.S. Pottorf and J.M. Blaney (1993). Protein β-turn mimetics I. Design, synthesis, and evaluation in model cyclic peptides. *Tetrahedron 49*, 3593–3608.
12. M.H. Rao, W. Yang, H. Joshua, J.M. Becker and F. Naider (1995). Studies on conformational consequences of i to $i+3$ side-chain cyclization in model cyclic tetrapeptides. *Inf. J. Peptide Protein Res. 45*, 418–429.
13. P.E. Thompson, N. Lim, N.J. Ede, F.M. Ng, I.D. Rae and M.T.W. Hearn (1995). Structure and *in vivo* activity of hypoglycaemic analogues of human growth hormone hGH[6–13). *Drug Des. Discov. 13*, 55–72.
14. P.W. Schiller, T.M.-D. Nguyen and J. Miller (1985). Synthesis of side-chain to side-chain cyclized peptide analogs on solid supports. *Int. J. Peptide Protein Res. 25*, 171–177.
15. C. Taylor, R.J. Quinn and P. Alewood (1996). Synthesis of cyclic peptides modelled on the microcystin and nodularin rings. *Bioorg. Med. Chem. Lett. 6*, 2107–2112.
16. M.K. Hu, A. Badger and D.H. Rich (1995). Cyclosporin analogues modified in the 3,7,8-positions-substituent effects on peptidylprolyl isomerase inhibition and immunosuppressive activity are nonadditive. *J. Med. Chem. 38*, 4164–4170.
17. P.E. Thompson, V. Cavallaro and M.T.W. Hearn (1996). Potential applications of cyclic peptide libraries, in: *Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Libraries*, *Proceedings of the 4th International Symposium on Solid Phase Syn-*

© 1998 European Peptide Society and John Wiley & Sons, Ltd.

thesis and Combinatorial Libraries, R. Epton, Ed., p. 209–213, Mayflower, Birmingham.

18. A.M. Felix, C.-T. Wang, E.P. Heimer and A. Fournie (1988). Applications of BOP reagent in solid phase synthesis. *Int. J. Peptide Protein Res. 31*, 231–238.
19. P.E. Thompson, N. Lim, E. Wijaya, F.M. Ng and M.T.W. Hearn (1995). Hypoglycaemic activity of an analogue of human growth hormone hGH[6–13] incorporating a D-Ala-Pro dipeptide unit. *Biorg. Med. Chem. Lett. 3*, 1625–1628.
20. P.E. Thompson, H.H. Keah, P.T. Gomme, P.G. Stanton and M.T.W. Hearn (1995). Synthesis of peptide amides using Fmoc-based solid phase procedures on 4-methylbenzhydrylamine resins. *Int. J. Peptide Protein Res. 46*, 174–180.
21. S.A. Cohen and D.J. Strydom (1988). Amino acid analysis using phenylisocyanate derivatives. *Anal. Biochem. 174*, 1–16.
22. M.E. Houston, A.P. Campbell, B. Lix, C.M. Kay, B.D. Sykes and R.S. Hodges (1996). Lactam bridge stabilisation of α-helices: the role of hydrophobicity in controlling dimeric versus monomeric α-helices. *Biochemistry 35*, 10041–10050.
23. A. Kapurniotu and J.W. Taylor (1995). Structural and conformational requirements for human calcitonin activity: Design, synthesis, and study of lactam-bridged analogues. *J. Med Chem. 38*, 836–847.

© 1998 European Peptide Society and John Wiley & Sons, Ltd.