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Engineering New Peptidic Inhibitors from a Natural Chymotrypsin Inhibitor

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Abstract: Three model peptides of different sizes (17–24 amino acid residues), mimicking the chymotrypsin inhibitor SCGI (a peptide of 35 amino acid residues) isolated from *Schistocerca gregaria* were designed and prepared by convergent peptide synthesis. Selective formation of disulphide bridges in the closing step was achieved without selective protection of cysteine residues. The natural pattern of the two disulphide bridges was determined by 2D homonuclear ^{1}H NMR techniques. All three model peptides were characterized by amino acid analysis, MS and CD spectra. Preliminary results revealed that the two smaller model peptides exhibit no inhibitory activity, whereas the larger one shows limited inhibition of chymotrypsin.

Keywords: chymotrypsin inhibitor; peptide design; molecular modelling; convergent peptide synthesis; selective disulphide bridge formation

INTRODUCTION

Nowadays, one of the most interesting and dynamically developing fields of chemistry is the investigation of the protein–small molecule interaction. Although numerous researchers have dedicated their work to the understanding of the structure and function of canonical serine protease inhibitors (reviewed e.g. in [1]), there are still many unanswered questions. All known canonical inhibitors have one common structural motif, a protease-binding loop [2], and a scissile bond, between P1–P1′ sequential positions [3], which is responsible for binding to protease, usually in a reversible manner. The choice of the target enzyme of this inhibitor family, e.g. trypsin or chymotrypsin, is determined primarily by the P1 position which is very close to the active site of the protease.

In this paper we focus on the modelling of the small serine protease inhibitor peptide SGCI (*Schistocerca gregaria* chymotrypsin inhibitor). SGCI was first isolated in 1998, and its structure (see Figure 1) and biological activity were described together with the synthesis based on solid-phase technique and standard Fmoc methodology [4]. The overall fold of SGCI, determined later by NMR spectroscopy [see Figure 3 in ref. 5], corresponds to the typical structure of the grasshopper inhibitor family [5,6]. The polypeptide inhibitor SGCI is composed of 35 amino acid residues, exhibiting a compact structure. The molecule contains three slightly twisted antiparallel β-sheets (9–10, 16–19, and 26–28), three disulphide bonds (Cys^4–Cys^{19}, Cys^{17}–Cys^{28} and Cys^{14}–Cys^{33}), and a type II β-turn with amino acids

Abbreviations: protecting groups: Acm, acetamidomethyl; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; cHex, cyclohexyl; Fmoc, fluorenyloxycarbonyl; O^{t}Bu, *tert*-butoxy; Tos, tosyl; Trt, triphenylmethyl; Z, benzyloxycarbonyl symbols were used. For other abbreviations see Materials Section.

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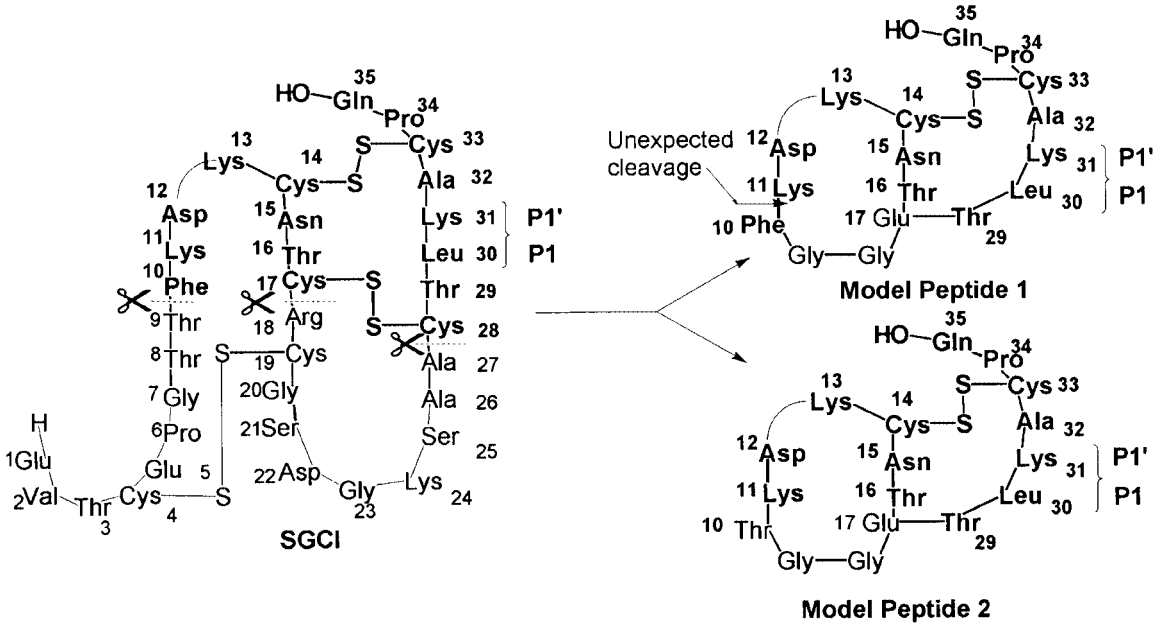


Figure 1 Model peptides **1** and **2** derived from SGCI; the amino acid preserved from SGCI structure are marked by bold letters.

from 5 to 8. In SGCI the P1–P1′ position corresponds to the Leu^{30}–Lys^{31} bond, which is cleaved by chymotrypsin very slowly. The Leu^{30}–Lys^{31} part is held by two adjacent disulphide linkages (Cys^{17}–Cys^{28} and Cys^{14}–Cys^{33}) in a well-defined antiparallel β-sheet. SGCI has a hydrophobic core organized around the aromatic side chain of Phe^{10}, which plays an important role in stabilizing the 3D-structure. This phenyl ring is almost completely embedded thus protected from chymotrypsin cleavage. The three antiparallel β-sheets are the most rigid parts of the structure. As expected, the two terminal regions of the molecule (from 1 to 4 and from 33 to 35) are flexible, and the binding loop (from 28 to 33) is also less determined.

Based on P1–P1′ (Leu^{30}–Lys^{31}) positions in the sequence of SGCI, this peptide is expected to be a good substrate of chymotrypsin. In contrast, SGCI was shown to be an excellent inhibitor. The question arises, why an apparently good substrate behaves, at the same time, as a good inhibitor. It may be assumed that the three-dimensional structure of SGCI is responsible for the inhibitory activity. Our purpose was to find the minimal building block in the structure of SGCI, which is necessary for this inhibitory effect to occur. In our opinion, if we retain the active P1–P1′ site of SGCI and its immediate neighbourhood within the chain as well as in the adjacent part of the molecule, the inhibitory activity should be retained. To test this view, three smaller peptides, model peptides **1**, **2** and **3**, were modelled and synthesized (Figures 1 and 2).

In Figures 1 and 2 one can see that all three model peptides are composed of a large cyclopeptide (10–14 residues) and of a linear 'tail-peptide' part (7–10 residues), which is linked to the cyclopeptide ring by an amide bond and disulphide bridge(s). The synthesis of these peptides was performed by a convergent peptide synthetic method including the formation of a large cyclopeptide part; cf. refs. [7–10] and [11–13], respectively. A similar modelling of a natural peptide is not common in the literature. However, the Bowman–Birk inhibitor [14,15] should be mentioned, modelled by a simple cyclopeptide composed of nine amino acid residues; for other studies see refs. [16,17].

Modelling

Model peptide 1. This model was designed to include the important features of the above introduced type of chymotrypsin inhibitor: the three β-sheet structures with the P1–P1′ environment. At the same time, some 'inactive parts' were assumed to contribute only indirectly to the inhibitory effect. Thus, fragments considered unnecessary were removed from the original structure by cleaving the 9–10, 17–18 and 27–28 bonds (Figure 1). Furthermore, two nontrivial modifications were made

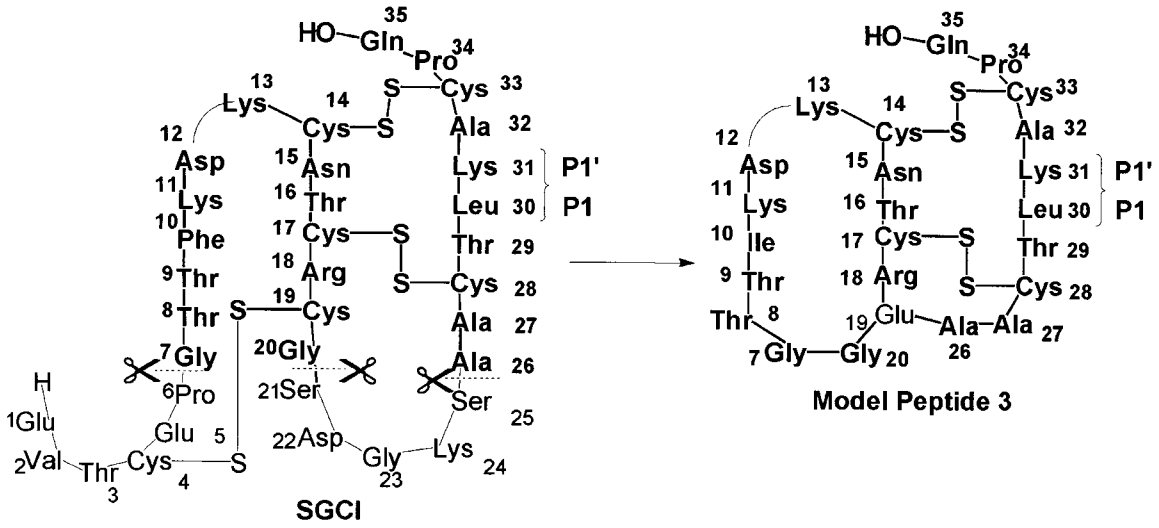


Figure 2 Model peptide **3** derived from SGCI; the amino acid residues preserved from SGCI structure are marked by bold letters.

during design. In order to retain the original β-sheet structure, one of the peptide fragments (10–17) with fewer amino acid residues was combined to a cyclopeptide by inserting a Gly-Gly bridge, to which the second peptide fragment (28–35) was joined as a tail-peptide. To avoid a problematic final step involving a selective formation of disulphide bridges, one of the original disulphide bridges (17–28) was replaced by an amide bond. Thus Cys^{28} was omitted and Cys^{17} was substituted by Glu and the γ-carboxyl group of the latter was condensed with the amino group of the Thr^{29} residue.

Model peptide 2. A preliminary biological test [18] showed that model peptide **1** had no inhibitory effect and was cleaved not only at the expected P1–P1′ position, i.e. at the Leu^{30}–Lys^{31} bond, but also at the Phe^{10}–Lys^{11} bond in the cyclopeptide part, as well. Whereas Phe^{10} in SCGI is the seed of a hydrophobic region, the same amino acid residue in model peptide **1** is easily approached by the enzyme. Because peptides containing Phe are good substrates of chymotrypsin, in model peptide **2** Phe was replaced by Thr (Figure 1), in order to prevent undesired cleavage.

Model peptide 3. Since model peptide **2** did not exhibit any inhibitory effect [18], a third larger model was designed incorporating 24 amino acid residues instead of 17, with two disulphide bridges instead of one, and Phe^{10} was substituted by Ile^{10}. A larger cyclopeptide ring was formed by cutting the chain in SGCI (Figure 2) between residues 6 and 7, and similarly between 20 and 21, and the terminal Gly^{7} and Gly^{20} residues of the first peptide fragment was joined to form a ring. The tail-peptide was also larger by cutting the original chain between positions 25 and 26 to yield the second peptide fragment. In the cyclopeptide part Cys^{19} was substituted by Glu, and its side chain was linked via a γ-peptide bond to the *N*-terminal (Ala^{26}) of the second peptide fragment to form the tail-peptide part. This amide bond, however, was not called upon to substitute any disulphide bridge, but was expected to simplify the synthesis.

Synthesis

Model peptides **1**, **2** and **3** were synthesized by using a convergent peptide synthetic method [10,11], because linear syntheses could not be carried out efficiently. Syntheses were carried out as follows. (i) Preparation of starting peptide fragments (**4**–**6**) by solid phase technique [19], used as linear cyclopeptide-precursors. (ii) Cyclization of the linear peptides to give protected cyclopeptides **7**–**9**. (iii) Removal of the side-chain protecting groups from glutamic acid and from asparagine residues leading to **10**–**12**. (iv) Preparation of peptide fragments **13**–**14** by solid phase technique, used as Boc-protected tail-peptide precursors. (v) Preparation of glutamine benzyl ester **(15)** used in the subsequent step. (vi) Coupling of Boc-protected tail-peptide precursors with glutamine benzyl ester to yield **16**–**17**. (vii) Removal of the Boc-protecting group from the protected tail-peptides

yielding **18**–**19**. (viii) Fragment condensation of partially protected cyclopeptides and tail-peptides to give **20**–**22**. (ix) Removal of all protecting groups except Acm leading to **23**–**25**. (x) Removal of Acm protecting groups (isolated only **26**) and formation of disulphide bridge(s) [20] to yield model peptides **1**–**3**. A schematic representation of synthetic steps (i) → (x) is shown in Figure 3. Peptide intermediates (**4**–**26**) obtained in the syntheses of model peptides **1**–**3** are listed in Figure 4.

The side-chain protecting groups used in our work may be classified into three categories. The first category includes O^{t}Bu on Asp[12], Trt on Asn[15] and Boc on the *N*-terminal of tail-peptide precursors, which can be selectively removed by TFA even in the presence of other protective groups. The second category covers the hydrogen fluoride labile protecting groups, such as Bzl, cHex, Z and Tos. The Acm protecting group of Cys, stable under HF-cleaving conditions, falls in the third category.

MATERIALS AND METHODS

Butanol (*n*-BuOH), chloroform ($CHCl_3$), dichloromethane (DCM; distilled from phosphorus pentoxide), diethyl ether (Et_2O; distilled from $LiAlH_4$), *N*,*N*-dimethylformamide (DMF; distilled from ninhydrin), ethyl acetate (EtOAc), glacial acetic acid (AcOH), oxidized glutathione (GSSG), reduced glutathione (GSH), iodine (I_2), methanol (MeOH), mercaptoethanol (distilled *in vacuo*), 1-methyl-2-pyrrolidone (NMP; distilled from ninhydrin), 2-propanol (*i*-PrOH), triethylamine (TEA) and trifluoroacetic acid (TFA) were purchased from Reanal (Budapest, Hungary). Anisole (PhOMe), benzotriazole-1-ol hydrate (HOBt), benzotriazol-1-yl–oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP), *N*,*N*′-diisopropylcarbo-diimide (DIC), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), diphenylphosphorylazide (DPPA), hydrogen fluoride (HF) and trifluoroethanol (TFE) were from Fluka (Buchs, Switzerland). *N*,*N*-Diisopropylethylamine (DIEA; distilled from ninhydrin), mercury(II) acetate [$Hg(OAc)_2$] and *N*-methylmorpholine (NMM) were from Merck (Hohenbrunn, Germany). Benzyl bromide, piperidine were obtained from Aldrich (Steinheim, Switzerland). Acetonitrile (MeCN) of HPLC quality was purchased from Lab-Scan (Dublin, Ireland). O-Benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) was from Quantum Biotechnologies (Carlsbad, Canada). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was from Cambridge Isotope Laboratories, Andover, USA). *o*-Chlorotrityl resin was prepared in our laboratory [21].

N-Fmoc- and *N*-Boc-amino acid derivates Fmoc-Gly-OH, Fmoc-Lys(Z)-OH, Fmoc-Arg(Tos)-OH, Fmoc-Asp(OcHex)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Cys(Acm)-OH, Fmoc-Thr(Bzl)-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Glu(O^{t}Bu)-OH, Boc-Ala-OH and Boc-Gln-OH were purchased from Reanal (Budapest, Hungary). Fmoc-Asn(Trt)-OH was obtained from Bachem (Bubendorf, Switzerland).

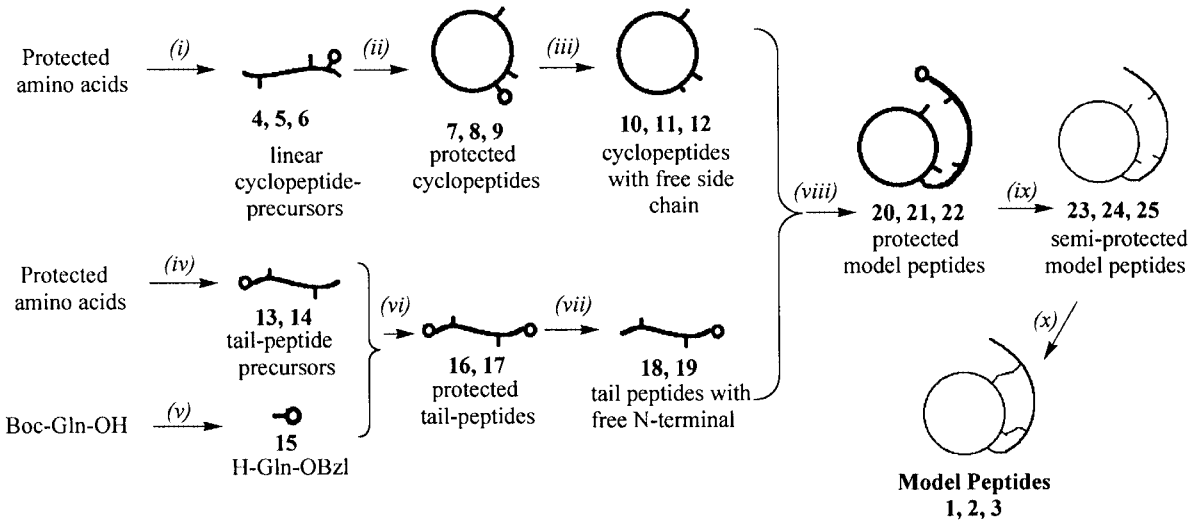
Chromatography

The first quality control for homogeneity (R_f) of peptides was thin-layer chromatography on Kieselgel 60 F254 (from Merck). The following eluents were used (indicated as subscripts in the text): (1) EtOAc–pyridine–AcOH–water (240 : 20 : 6 : 11 v/v); (2) EtOAc–pyridine–AcOH–water (120 : 20 : 6 : 11 v/v); (3) EtOAc–pyridine–AcOH–water (60 : 20 : 6 : 11 v/v); (4) EtOAc–pyridine–AcOH–water (30 : 20 : 6 : 11 v/v); (5) chloroform–MeOH–AcOH (9 : 0.8 : 0.2 v/v); (6): chloroform–MeOH–AcOH (9 : 0.7 : 0.3 v/v); (7) *n*-BuOH–AcOH–pyridine–water (1 : 1 : 1 : 1 v/v); (8) chloroform–MeOH–AcOH (3 : 1 : 0.1 v/v).

The system for analytical and semi-preparative liquid chromatography consisted of two Shimadzu LC-6A pumps and a Shimadzu SPO-6 AU variable wavelength spectrophotometer (Shimadzu, Kyoto, Japan), with a detector operating at 220 nm. A 250 × 4.6 mm 218TP54 Vydac 5μ C18 (Hesperia, CA, USA) analytical column was used. Analytical chromatographic runs were made at a constant flow rate of 1 ml/min at ambient temperature. The eluents were water (A; double-distilled) and acetonitrile (B) containing 0.045% and 0.036% TFA, respectively. The following linear gradients were used: (1) 5%–95% eluent B over 30 min. (2) 5%–40% eluent B over 30 min. (3) 30%–100% eluent B over 30 min. (4) 0–35% eluent B over 30 min.

The semi-preparative column was a 250 × 10 mm 218TP1010 Vydac 8 μ C18 (Hesperia, CA, USA) operating at a constant flow rate of 4 ml/min at ambient temperature. The eluents were the same as described above.

The preparative liquid chromatography and gel filtration were performed on a system consisting of two Pharmacia LKB P500 Pumps (Pharmacia,



4: H-Gly-Phe-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn(Trt)-Thr(Bzl)-Glu(OtBu)-Gly-OH
5: H-Gly-Thr(Bzl)-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn(Trt)-Thr(Bzl)-Glu(OtBu)-Gly-OH
6: H-Gly-Thr(Bzl)-Thr(Bzl)-Ile-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn(Trt)-Thr(Bzl)-Cys(Acm)-Arg(Tos)-Glu(OtBu)-Gly-OH
7: cyclo[Gly-Phe-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn(Trt)-Thr(Bzl)-Glu(OtBu)-Gly]
8: cyclo[Gly-Thr(Bzl)-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn(Trt)-Thr(Bzl)-Glu(OtBu)-Gly]
9: cyclo[Gly-Thr(Bzl)-Thr(Bzl)-Ile-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn(Trt)-Thr(Bzl)-Cys(Acm)-Arg(Tos)-Glu(OtBu)-Gly]
10: cyclo[Gly-Phe-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn-Thr(Bzl)-Glu-Gly]
11: cyclo[Gly-Thr(Bzl)-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn-Thr(Bzl)-Glu-Gly]
12: cyclo[Gly-Thr(Bzl)-Thr(Bzl)-Ile-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn-Thr(Bzl)-Cys(Acm)-Arg(Tos)-Glu-Gly]
13: Boc-Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-OH
14: Boc-Ala-Ala-Cys(Acm)-Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-OH
15: H-Gln-OBzl
16: Boc-Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-Gln-OBzl
17: Boc-Ala-Ala-Cys(Acm)-Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-Gln-OBzl
18: H-Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-Gln-OBzl.HCl
19: H-Ala-Ala-Cys(Acm)-Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-Gln-OBzlHCl
20: cyclo[Thr(Bzl)-Glu-Gly-Gly-Phe-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn]
Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-Gln-OBzl
21: cyclo[Thr(Bzl)-Glu-Gly-Gly-Thr(Bzl)-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn-]
Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-Gln-OBzl
22: cyclo[Arg(Tos)-Glu-Gly-Gly-Thr(Bzl)-Thr(Bzl)-Ile-Lys(Z)-Asp(cHex)-Lys(Z)-Cys(Acm)-Asn-Thr(Bzl)-Cys(Acm)]
Ala-Ala-Cys(Acm)-Thr(Bzl)-Leu-Lys(Z)-Ala-Cys(Acm)-Pro-Gln-OBzl
23: cyclo[Thr-Glu-Gly-Gly-Phe-Lys-Asp-Lys-Cys(Acm)-Asn]
Thr-Leu-Lys-Ala-Cys(Acm)-Pro-Gln-OH
24: cyclo[Thr-Glu-Gly-Gly-Thr-Lys-Asp-Lys-Cys(Acm)-Asn]
Thr-Leu-Lys-Ala-Cys(Acm)-Pro-Gln-OH
25: cyclo[Arg-Glu-Gly-Gly-Thr-Thr-Ile-Lys-Asp-Lys-Cys(Acm)-Asn-Thr-Cys(Acm)]
Ala-Ala-Cys(Acm)-Thr-Leu-Lys-Ala-Cys(Acm)-Pro-Gln-OH
26: cyclo[Arg-Glu-Gly-Gly-Thr-Thr-Ile-Lys-Asp-Lys-Cys-Asn-Thr-Cys]
Ala-Ala-Cys-Thr-Leu-Lys-Ala-Cys-Pro-Gln-OH

Figure 3 Schematic presentation of the syntheses of model peptides **1**, **2** and **3**. Protected and unprotected peptides are indicated by thick and thin lines, respectively. Strategic protective groups are marked by **0-** signs. Protected cysteine residues are indicated by spikes on the peptide chain. Disulphide bridges are shown by thin lines joining the cyclopeptide and tail-peptide parts. Peptide intermediates correspond to model peptides **1**–**3**, are listed.

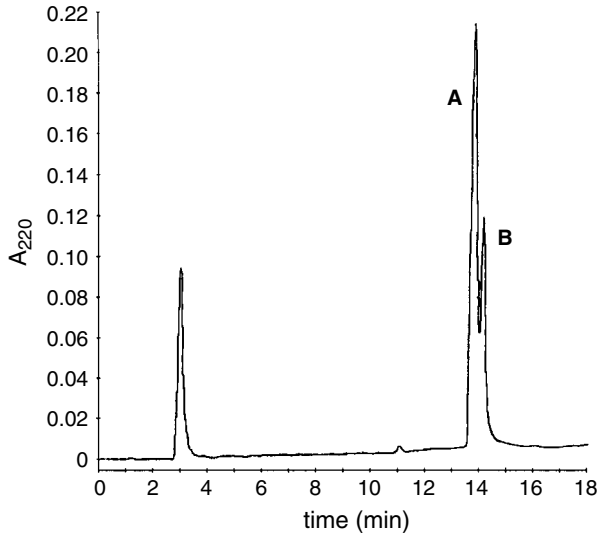


Figure 4 Chromatogram of model peptide **3** (peak A; Rt = 13.9 min) + Model Peptide **3*** (peak B; Rt = 14.1 min) mixture. 200 × 4.6 mm 218TP54 Vydac 5 μ C18 (Hesperia, CA, USA) analytical column 5%–26% eluent B over 18 min, 220 nm, 1 ml/min.

Uppsala, Sweden) and a Büchi UV/VIS Filter Photometer (Knauer, Berlin, Germany) operating at 220 nm. For preparative liquid chromatography a 450 cm × 5.4 cm Vydac 20μ C18 (Hesperia, CA, USA) column was used, running at a constant flow rate of 14 ml/min, at ambient temperature.

A 500 cm × 3.6 cm column filled with Sephadex G25 gel (Pharmacia, Uppsala, Sweden) was used for gel filtration, running at a constant flow rate of 1 ml/min, at ambient temperature.

Amino Acid Analysis

The amino acid composition of peptides was determined by amino acid analysis using a Beckman (Fullerton, CA, USA) Model 6300 amino acid analyser. Prior to analysis peptides were hydrolysed in 6 M HCl in evacuated and sealed tubes at 110 °C for 24 h.

ES-MS Spectra

Mass spectra were recorded on a Perkin Elmer Sciex API2000 triple quadrupole instrument (Toronto, Canada) equipped with Turbo Ionspray source. By using a flow rate of 100 μl/min samples in aqueous methanol solution were directly injected.

NMR Spectra

1D and 2D homonuclear ^{1}H NMR spectra of model peptide **3** dissolved in H_2O–D_2O (1 : 10 v/v) were recorded at 500 MHz, at 297 K on a Bruker DRX 500 spectrophotometer in order to determine the patterns of the disulphide bridges. Data processing in t_2 dimension was carried out by zero filling up to 2 K and by applying a Kaiser window function. A spin lock of 60 ms and mixing time of 150 ms were used for TOCSY and NOESY measurements, respectively. Spectra were referenced to internal DSS. Resonance assignment was carried out with the TRIAD module of the software package SYBYL [22], running on SGI Octane R12000 work stations.

CD Spectra

CD measurements were performed at room temperature over the range 300–180 nm on a Jasco-810 spectropolarimeter. The optical path length was 0.02 cm. Peptide concentration was in the range 0.60–0.83 mM in water–TFE mixtures of various concentrations. In the spectra reported $[\theta]_R$ represents the ellipticity value per mole of peptide residue (deg.cm^2/dmol).

Peptide Syntheses

(i) Synthesis of linear cyclopeptide-precursors. The syntheses of cyclopeptide-precursors (**4**–**6**) were carried out by Fmoc/Bzl technique using 1 g *o*-chlorotrityl resin of 0.6 mmol/g capacity. In the chain-elongation step 2 equivalents of Fmoc-protected amino acid were used with 1.95 equivalents of HBTU dissolved in DMF containing 3 equivalents of DIEA. The Fmoc protecting group was cleaved by piperidine–DMF (3 : 7 v/v) mixture for 3 + 17 min. After the synthesis the resin was washed with DMF, DCM, MeOH, *i*-PrOH and diethyl ether, the dried in high *vacuo*. Products were displaced from the resin by DCM–AcOH–MeOH (8 : 1 : 1 v/v) mixture (3 × 10 ml) for 3 × 1 h. The combined cleavage mixtures were evaporated *in vacuo*, the residue was treated with diethyl ether to obtain the peptide products which were reprecipitated from DMF (8 ml containing 200 μl of a 5.3 M HCl–dioxane solution) and diethyl ether (80 ml).

4. Yield 1.16 g (90%); the homogeneity of the crude product was 81%. Rt$_1$[min]: 23.2; Rf$_1$: 0.19, Rf$_5$: 0.44. Amino acid analysis for the HF-cleaved and purified product gave the following values: Asx: 1.90 (2); Cys: 0.98 (1); Gly: 2.03 (2); Glu: 1.02 (1); Lys: 1.96 (2); Phe: 1.01 (1); Thr: 0.95 (1). — **5**. Yield 700 mg (60%); the homogeneity of the crude product was 75%. Rt$_1$ [min]: 25.1; Rf$_1$: 0.18, Rf$_5$: 0.45. Molecular weight: $[M + H]^+$ (found): 1952.1, M(calculated):

1950.9. — **6**. Yield 618 mg (40%); the homogeneity of the crude product was 70%. R_{t_1} [min]: 19.9; R_{f_2}: 0.21, R_{f_8}: 0.36. Molecular weight: $[M + 2H]^{2+}$ (found): 1371.8, M(calculated): 2741.8.

(ii) Cyclization of linear cyclopeptide-precursors. The crude linear cyclopeptide-precursors **4** (1.11 g, 0.56 mmol)**, 5** (700 mg, 0.36 mmol) and **6** (610 mg, 0.26 mmol) were cyclized in a diluted (10^{-3} M) DMF solution containing 4 equivalents of BOP, 4 equivalents of HOBt and 6 equivalents of DIEA.

After 1 day, the reaction mixture was evaporated *in vacuo*, and the product was precipitated by diethyl ether to give the protected cyclopeptides (**7**–**9**).

7. Yield 980 mg (96%); the homogeneity of the crude product was 72%. R_{t_3} [min]: 21.1; R_{f_5}: 0.55, R_{f_1}: 0.69. — **8**. Yield 630 mg (91%); the homogeneity of the crude product was 74%. R_{t_3} [min]: 20.1; R_{f_5}: 0.55, R_{f_1}: 0.66. Molecular weight of HF-cleaved product: $[M + H]^+$ (found): 1105.8, M(calculated): 1104.6.–**9**. Yield 580 mg (91%); the homogeneity of the crude product was 72%. R_{t_3} [min]: 24.8; R_{f_6}: 0.54, R_{f_2}: 0.68. Molecular weight: $[M + 2Na]^{2+}$ (found): 1407.2, M(calculated): 2723.8.

(iii) Selective cleavage of the side chain protecting group of glutamic acid and asparagine residues. The crude protected cyclopeptides **7** (630 mg, 0.32 mmol), **8** (610 mg, 0.36 mmol) and **9** (580 mg, 0.22 mmol) were dissolved in TFA–DCM (1 : 1 v/v) solvent mixture (10 ml) containing water (0.1 ml). The reaction proceeded at room temperature for 1 h. The solution was then evaporated *in vacuo*, and products (**10**–**12**) were precipitated by treating with diethyl ether.

10. Yield 366 mg (67%); the homogeneity of the crude product was 72%. R_{t_3} [min]: 16.5; R_{f_2}: 0.76, R_{f_5}: 0.29. Molecular weight of HF-cleaved product: $[M + H]^+$ (found): 1151.8, M(calculated): 1150.5. — **11.** Yield 540 mg (91%); the homogeneity of the crude product was 70%. R_{t_1} [min]: 20.2, R_{f_1}: 0.48, R_{f_6}: 0.21. — **12**. Yield 380 mg (72%); the homogeneity of the crude product was 71%. R_{t_3} [min]: 18.2; R_{f_3}: 0.25, R_{f_8}: 0.62.

(iv) Synthesis of Boc-protected tail-peptide precursors. The syntheses of the Boc-protected tail-peptide precursors **13** and **14** were carried out by Fmoc/Bzl strategy on *o*-chlorotrityl resin (1 g) of 0.6 mmol/g capacity. Two equivalents of Fmoc-protected amino acid were used, except the last residue, which was a Boc-protected amino acid derivative. The coupling reagent was composed of 1.95 equivalents of HBTU dissolved in DMF containing 3 equivalents of DIEA. The Fmoc protecting group was cleaved by piperidine–DMF (3 : 7 v/v) mixture in 3 + 17 min. After the synthesis the resin was washed with DMF, DCM, MeOH, *i*-PrOH and diethyl ether, then dried in high *vacuo*. The product was displaced from the resin by DCM–AcOH–MeOH (8 : 1 : 1 v/v) mixture (3 × 10 ml) in 3 × 1 h. Collected solutions were evaporated *in vacuo*, the residue was treated with diethyl ether to obtain **13** and **14** which was reprecipitated from DMF and diethyl ether as described earlier.

13. Yield 572 mg (89%); the homogeneity of the crude product was 92%. R_{t_1} [min]: 21.0; R_{f_5}: 0.22 R_{f_3}: 0.68. — **14**. Yield 660 mg (91%); the homogeneity of the crude product was 89%. R_{t_3} [min]: 11.7; R_{f_5}: 0.18 R_{f_3}: 0.62.

(v) Synthesis of glutamine benzyl ester. To the cooled (0 °C) solution of Boc-Gln-OH (5 g, 20.3 mmol) in DMF (50 ml) was slowly added benzyl bromide (3.82 g, 2.7 ml, 22.3 mmol) and triethylamine (3.1 ml, 22.3 mmol). The mixture was stirred for 2 h at room temperature, and finally evaporated *in vacuo*. The yellow oil obtained was dissolved in EtOAc (20 ml) and the organic layer was washed three times with aq. 10% $KHSO_4$ (10 ml), and three times with saturated $NaHCO_3$ aq. (10–10 ml), then with brine and finally dried over anhydrous $MgSO_4$. The solvent was evaporated to afford Boc-Gln-OBzl as a pale oil. The oily product obtained (6.2 g, 18.5 mmol) was dissolved in 50 ml of a 5.3 M HCl–dioxane solution. After 2 h, the clear solution was evaporated *in vacuo* to yield 3.9 g (14.5 mmol, 71%) of **15** (H-Gln-OBzl.HCl) as pale yellow oil; R_{f_2}: 0.78. R_{f_5}: 0.15.

(vi) Coupling of N-terminal Boc-protected tail-peptide precursors with glutamine benzyl ester. One equivalent of crude *N*-terminal Boc-protected tail-peptide precursors **13** (570 mg, 0.54 mmol) and **14** (498 mg, 0.37 mmol) and 1.1 equivalents of glutamine benzyl ester hydrochloride **15** were dissolved in a minimal amount of DMF (4 ml and 4.3 ml, respectively) containing 2.1 equivalents of DIEA. As a coupling reagent the combination of 2 equivalents of BOP, 2 equivalents of HOBt and 2 equivalents of DIEA was used. After 4 h the solution was evaporated *in vacuo*, and the product was precipitated by water to give the Boc-protected tail-peptides **16** and **17**.

16. Yield 620 mg (90%); the homogeneity of the crude product was 83%. R_{t_1} [min]: 22.1; R_{f_2}: 0.86,

Rf_5: 0.56. — **17**. Yield 515 mg (89%); the homogeneity of the crude product was 75%. Rt_1 [min]: 19.4; Rf_2: 0.67, Rf_5: 0.50.

(vii) Selective cleavage of N-terminal Boc-protecting group from protected tail-peptides. The crude fully protected tail-peptides **16** (596 mg, 0.47 mmol), **17** (390 mg, 0.25 mmol) were dissolved in TFA–DCM (1 : 1 v/v) cleavage mixture (10 ml). The reaction was complete within 1 hour at room temperature. The solution was then evaporated *in vacuo*, and the products were precipitated by treating with diethyl ether. The product was purified on FPLC, and the collected fractions were lyophilized in the presence of 5 equivalents of hydrochloric acid to give pure **18** and **19**.

18. Yield 432 mg (78%); the homogeneity of the product was 98%. Rt_1 [min]: 17.0; Rf_3: 0.56, Rf_5: 0.26. Molecular weight: $[M + H]^+$ (found): 1179.5, M(calculated): 1178.4. — **19**. Yield 260 mg (71%); the homogeneity of the product was 98%. Rt_1 [min]: 16.8, Rf_3: 0.42, Rf_5: 0.23. Molecular weight: $[M + H]^+$ (found): 1461.2, M(calculated): 1460.4.

(viii) Fragment condensation of partially protected cyclopeptides and tail-peptides. One equivalent of crude cyclopeptides with free side chain **10** (97 mg, 0.058 mmol), **11** (144 mg, 0.088 mmol) and **12** (430 mg, 0.177 mmol), and 1.1 equivalents of purified tail-peptides with free *N*-terminal **18** (73 mg, 0.062 mmol for **10** and 115 mg, 0.097 mmol for **11**, respectively) and **19** (260 mg, 0.18 mmol for **12**) were dissolved in a minimal amount of NMP (1.2 ml, 3 ml and 4 ml, respectively) containing 2.1 equivalents of DIEA. As coupling reagents 2 equivalents of BOP, 2 equivalents of HOBt and 2 equivalents of DIEA were used. After 1 day, the product (**20**–**22**) was precipitated by treating with water.

20. Yield 108 mg (71%); the homogeneity of the crude product was 70%. Rt_3 [min]: 12.1 Rf_3: 0.85, Rf_5: 0.40. — **21**. Yield 230 mg (93%); the homogeneity of the crude product was 69%. Rt_1 [min]: 24.0, Rf_2: 0.29, Rf_5: 0.37. — **22**. Yield 493 mg (71%); the homogeneity of the crude product was 69%. Rt_3 [min]: 18.5; Rf_3: 0.58, Rf_8: 0.50.

(ix) Preparation of semi-protected model peptides. To cleave all protecting groups (except Acm), fully protected model peptides **20** (72 mg, 0.037 mmol), **21** (230 mg) and **22** (490 mg) were treated between −5° and 0 °C with HF (20 ml) containing anisole (2 ml). After 1.5 h, HF was evaporated *in vacuo*, and the products were precipitated by treating the residues with diethyl ether. Crude **23** and **24** were extracted by water (3 × 10 ml) and lyophilized in the presence of 5 equivalents of hydrochloric acid. Both products obtained were purified on semi-preparative FPLC. Collected fractions were lyophilized in the presence of 5 equivalents of hydrochloric acid to give pure **23** and **24**. The crude product **25** was isolated by filtration then dried *in vacuo*.

23. Yield 10 mg (20%); the homogeneity of the product was 99%. Rt_4 [min]: 17.9; Rf_6: 0.32. Molecular weight: $[M + 2H]^{2+}$ (found): 982.3, M(calculated): 1962.6. — **24**. Yield 38 mg (25%); the homogeneity of the product was 99%. Rt_2 [min]: 10.9; Rf_6: 0.23. — **25**. Yield 620 mg (crude product; the homogeneity was 25%). Rt_2 [min]: 14.6. Rf_7: 0.78, Rf_4: 0.22. Molecular weight: $[M + 2H]^{2+}$ (found): 1390.0, M(calculated): 2778.1.

(x) Preparation of unprotected peptides with intramolecular disulphide bridges. Both purified semi-protected peptides **23** (10 mg, 0.023 mmol) and **24** (38 mg, 0.020 mmol) were dissolved in a mixture of acetic acid and water (4 : 1 v/v; 10 and 38 ml for **23** and **24**, respectively), then 10 equivalents of iodine and 3 equivalents of hydrochloric acid were added to the solution. After 1 hour, the excess of iodine was extracted from the solution using chloroform, at least three times (4–4 and 10–10 ml, respectively). The colourless solution was diluted with water and lyophilized. The crude peptide was purified on semi-preparative HPLC and the collected fractions were lyophilized again in the presence of 5 equivalent of hydrochloric acid to give model peptides **1** and **2** (see structures in Figure 1).

Model peptide **1**. Yield 4 mg (67%); the homogeneity of the purified product was 99%. Rt_2 [min]: 10.6; Rf_7: 0.81, molecular weight: $[M + 2H]^{2+}$ (found): 911.0, M(calculated): 1820.1. Amino acid analysis gave the expected values: Ala: 1.01 (1); Asx: 1.89 (2); Cystine: 0,98 (1); Gly: 2.04 (2); Glx: 2.06 (2); Leu: 1.02 (1); Lys: 2.82 (3); Phe: 1.01 (1); Pro: 0.96 (1); Thr: 1.90 (2).

Model peptide **2**. Yield 12 mg (40%); the homogeneity of the purified product was 99%. Rt_2 [min]: 10.2; Rf_7: 0.79. Molecular weight: $[M + 2H]^{2+}$ (found: 887.8, M(calculated): 1772.9. Amino acid analysis gave the expected values: Ala: 1.03 (1); Asx: 2.11 (2); Cystine: 0,96 (1); Gly: 2.01 (2); Glx: 2.03 (2); Leu: 1.04 (1); Lys: 2.85 (3); Pro: 0.98 (1); Thr: 2.87 (3).

Crude semi-protected peptide **25** (600 mg) was dissolved in 60 ml AcOH–water (1 : 1 v/v) mixture, and the protecting groups were removed by $Hg(OAc)_2$ (303 mg) from each of the four Cys(Acm) parts.

After 2 h, mercaptoethanol (2 ml) was added to the mixture, and then stirred for 1 day in darkness. The solution was filtered and purified with gel filtration on Sephadex G 25 in AcOH–water (3 : 97 v/v) solution. The collected fractions were lyophilized to give peptide **26** with free SH groups (yield: 270 mg, 45%). Then 66 mg of the peptide obtained was dissolved in 0.1 M Tris buffer (1320 ml, pH 8,1), and 5 M guanidine hydrochloride aq. solution (264 ml), GSH (1.621 g) and GSSG (321 mg) were added to the buffer solution. After vigorous stirring for 1 day the solution of the peptide was purified by semi-preparative HPLC. Collected fractions were lyophilized in the presence of 5 equivalents of hydrochloric acid to give model peptide **3** (see structure in Figure 2).

Model peptide **3**. Yield 12.5 mg (19%); the homogeneity of the product was 99%. Rt_2 [min]: 13.9; Rf_7: 0.77, Rf_4: 0.21. Molecular weight: $[M + 2H]^{2+}$ (found): 1245.6, M(calculated): 2489.1. Amino acid analysis gave the expected values: Ala: 3.06 (3); Asx: 2.02 (2); Arg: 0.89 (1); Cystine: 1,86 (2); Gly: 2.06 (2); Glx: 2.09 (2); Ile: 1.00 (1); Leu: 1.05 (1); Lys: 2.89 (3); Pro: 0.97 (1); Thr: 3.82 (4). Selected ^{1}H NMR data (ppm): δ 2.70 (Cys^{33} Hβ1), 2.81 (Cys^{28} Hβ1), 2.82 (Cys^{17} Hβ), 2.86 (Cys^{28} Hβ2), 3.02 (Cys^{14} Hβ), 3.04 (Cys^{33} Hβ2), 4.51 (Cys^{28} Hα), 4.57 (Cys^{17} Hα), 4.61 (Cys^{14} Hα), 4.84 (Cys^{33} Hα).

RESULTS AND DISCUSSION

Synthesis

The complete synthesis of all three model peptides (composed of 17–24 amino acid residues), on solid phase might be presumably achieved, but seemed experimentally more complicated than the longer but more effective route of a convergent peptide synthesis. The joint application of *o*-chlorotrityl resin and Fmoc/Bzl strategy provided a means of synthesizing selectively protected peptide fragments used in the convergent peptide synthesis. Utilizing the advantage of such a combined method, we succeeded in preparing intermediates containing protecting groups with graduated acid sensitivity.

Some steps in the synthesis of model peptides **1**–**3** deserve a more detailed discussion. While the solid phase synthesis of the two linear, 10-membered cyclopeptide precursors **4** and **5** (step i) could be solved without any difficulty, the coupling of the amino acid derivatives Fmoc-Ile-OH(11) and Fmoc-Thr(Bzl)-OH(12) in the synthesis of the 14-membered **6** was not complete even after the fourth re-coupling step (the Kaiser test was positive). The remaining free *N*-terminal amino groups therefore were capped with benzoic anhydride, which accounts for the lower purity of the product. In all three cases the peptides obtained were displaced from the resin by DCM–MeOH–AcOH mixture. The next cyclization step (ii) required the complete removal of AcOH traces. By treating the crude product with 5 equivalents of a 5.3 M HCl–dioxane solution, an acetate–hydrochloride exchange occurred at the *N*-terminal, without loss of the protecting groups.

The cyclization of linear precursors **4** and **5** (step ii) took place smoothly, but the conversion of **6** gave a poor yield. Different coupling methods were tested. (a) DPPA/$NaHCO_3$ coupling agent did not give any product. (b) DIC/HOBt coupling agent gave the expected product, but the yield (65%) and homogeneity (71%) were not acceptable. The reason for the poor yield and the formation of by–products may be ascribed to the *N*-terminal capping of the peptide by DIC, which is related to the slow reaction. (c) The cyclization effected by the BOP/HOBt/DIEA coupling reagent gave the best product (homogeneity 72%) with the highest yield (91%). None of the cyclopeptide derivatives prepared in steps (ii), (iii) and (viii) could be purified by HPLC method because they were soluble only in DMF and NMP. Although crude peptides were used as starting materials, the subsequent steps (viii) and (ix) could be conducted without trouble. Final purification was accomplished before the closing step x.

Owing to the very poor solubility of the protected cyclopeptides with free side chains (**10**, **11** and **12**) the fragment condensation (step viii) proceeded very slowly to yield the fully protected model peptides. Nevertheless, **20** and **21** could be prepared without difficulty, but the conversion of **12** and **17** into **22** proved to be rather difficult. A number of coupling agents and solvents (DMF, NMP, $CHCl_3$/TFE) were tested. (a) The fragment condensation carried out by the HBTU/HOBt/DIEA coupling agent in DMF or in NMP gave a product of low homogeneity (about 40%) in poor yield (about 30%). (b) DIC/HOBt coupling agent was also used in NMP. This method gave relatively good yield (62%), but the peptide homogeneity was low (33%). (c) EDC/HOBt coupling material in NMP led to a similar result, as in the former case. The poor yields and the low

homogeneity of products obtained in applying methods (a)–(c) are due both to *N*-terminal capping and a slow reaction rate. (d) Finally BOP/HOBt/DIEA was tried both in DMF and NMP. The conditions described in the Section 'Peptide syntheses' proved to be the most favourable. Because the solubility of the peptide derivatives is better in NMP than in DMF, the yield is better in the former solvent (71% and 45%, respectively). After 1 h the reaction mixture became coloured and a jelly; to obtain a clear solution a second portion of NMP was added to the mixture. It should be noted, however, that the reaction did not take place completely when started in a more diluted solution. If the fragment condensations (step viii) were carried out in $CHCl_3$/TFE solution by applying methods (c) or (d), the trifluoroethyl esters of cyclopeptides **10**–**12** were formed instead of fully protected model peptides **20**–**22.**

In step (ix) the cleavage of cHex and Tos protecting groups by HF required a longer reaction time, causing partial cleavage of the Cys-protecting Acm groups. Consequently, the semi-protected peptides **23**, **24** and **25** were contaminated by some disulphide-polymerized and oxidized products. Whereas the isolation of **23** and **24** in pure form was not problematic, **25** could be prepared only as a crude product containing about 25% of the desired semi-protected peptide.

For the closing step (x) a number of methods [19,20,23] for disulphide bridge formation were tested, and the products obtained were analysed by both HPLC and MS methods. First, the standard method and modified versions using iodine reagent were applied, which proved to be useful to synthesize model peptides **1** and **2** from **23** and **24**, respectively. Crude **25** was also attempted to convert into model peptide **3**; product obtained was

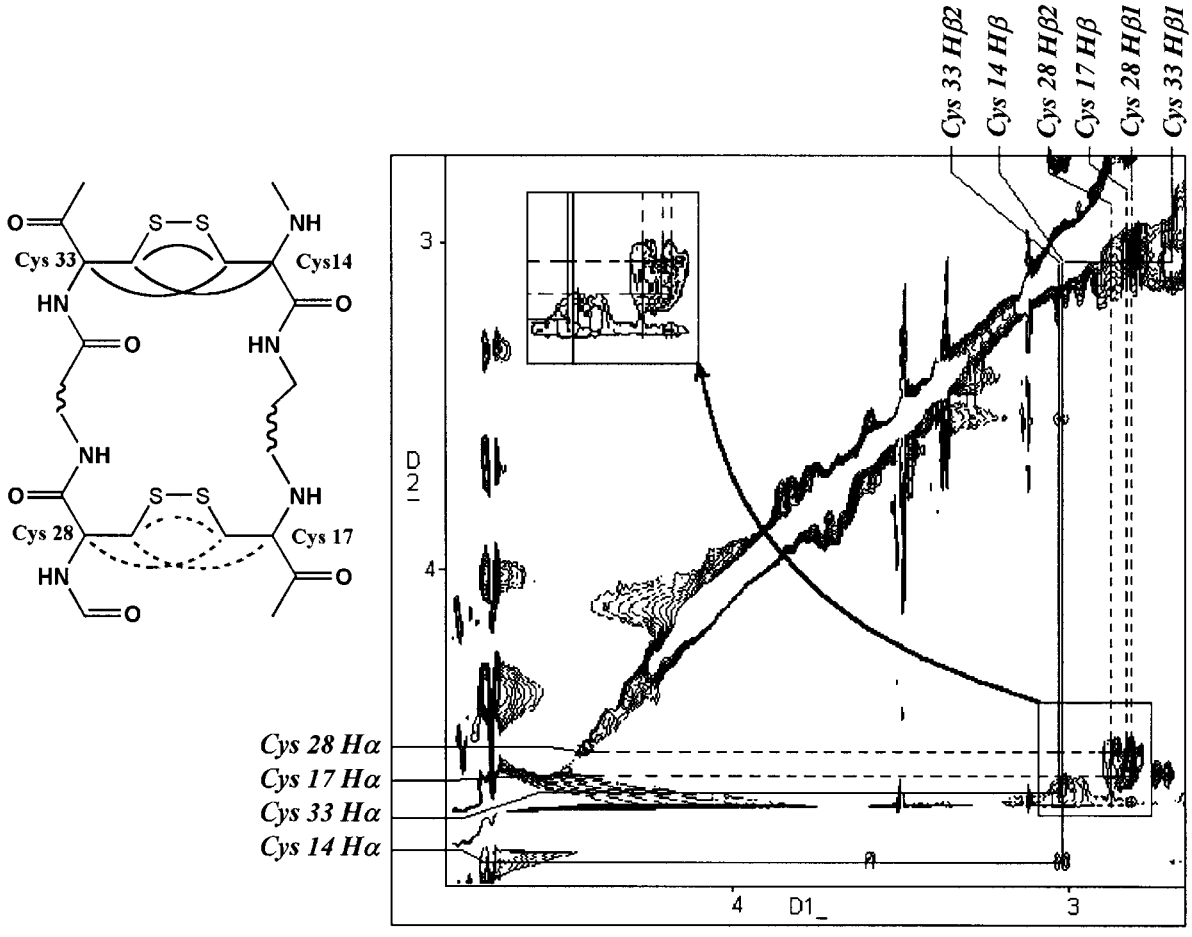


Figure 5 Assignment of disulphide bridges of model peptide **3** by using ^1H–^1H NOESY spectrum (mixing time 150 ms). The drawn and dotted lines represent the crosspeaks belonging to the disulphide bridges Cys14–Cys33 and Cys17–Cys28, respectively.

prepared by HPLC, and analysed by MS; $[M + 2H]^{2+}$ (found) = 1245.6, M(calculated) = 2489.1. Data confirmed the formation of disulphide bridges, but the yield was very poor. Subsequently, the 'reversible method' of disulphide bridge formation [23] was used for the conversion of crude **25**. The four Acm protecting groups from the cysteine residues were first removed by $Hg(OAc)_2$ to give peptide **26** which was treated with mercaptoethanol to regenerate partly the free SH groups in the by-products of disulphide type. The mixture was then submitted to gel filtration by which both the remainder of the polymer by-products and the smaller peptides from the incomplete fragment condensation (step viii) were separated. (It should be mentioned that polymer by-products of disulphide type cannot be reduced with $NaBH_4$.) After lyophilization **26** was obtained in an acceptable yield (45%) and homogeneity (73%). Owing to the unprotected SH groups, the peptide obtained was very sensitive to oxidation, requiring an instant conversion into model peptide **3**. The conditions to yield the model peptide **3** step were varied extensively: pH, redox potential by adding glutathione (oxidized) and glutathione (reduced) in different ratios, ionic strength by adding NaCl or guanidine hydrochloride. By applying the optimized conditions (see Section 'Preparation of peptides') model peptide **3** purified by HPLC was obtained in acceptable yield (19%). MS indicated the same molecular weight as in the case of the iodine oxidation, $[M + 2H]^{2+}$ (found): 1245.6, M(calculated): 2489.1, but HPLC gave different retention times: Rt_2 [min](I_2 oxidation): 14.1, Rt_2 [min](reversible way): 13.9 (Figure 5).

Product obtained by iodine (model peptide **3***) contained either nonnatural cross-linked disulphide bridges (Cys^{14}–Cys^{28} and Cys^{17}–Cys^{33}) or disulphide bridges involving only the cyclopeptide and tail-peptide parts (Cys^{14}–Cys^{17} and Cys^{28}–Cys^{33}). The undesirable reaction pathways could be avoided if selective Cys protection methods were used, but in this case the synthesis leading to very poor yield might be more difficult.

The structure of disulphide patterns of model peptide **3** differing from that in model peptide **3*** was mapped by 2D homonuclear ^1H NMR techniques. The Hα–Hβ and Hβ–Hβ NOESY-crosspeaks of Cys carry the information about the disulphide bridges [24,25]. In model peptide **3** NOE data confirm the formation of the pairs Cys^{17}–Cys^{28} and Cys^{14}–Cys^{33}. Figure 6 demonstrates the NOE crosspeaks Cys^{14}(Hα)–Cys^{33}(Hβ), Cys^{14}(Hβ2)–Cys^{33}(Hα) and Cys^{14}(Hβ1)–Cys^{33}(Hβ) together with NOE crosspeaks Cys^{17}(Hα)–Cys^{28}(Hβ1, Hβ2) and Cys^{17}(Hβ)–Cys^{28}(Hα). In the latter case Cys^{17}(Hβ) and Cys^{28}(Hβ) peaks overlap, therefore Cys^{17}(Hβ)–Cys^{28}(Hβ) crosspeak can not be assigned.

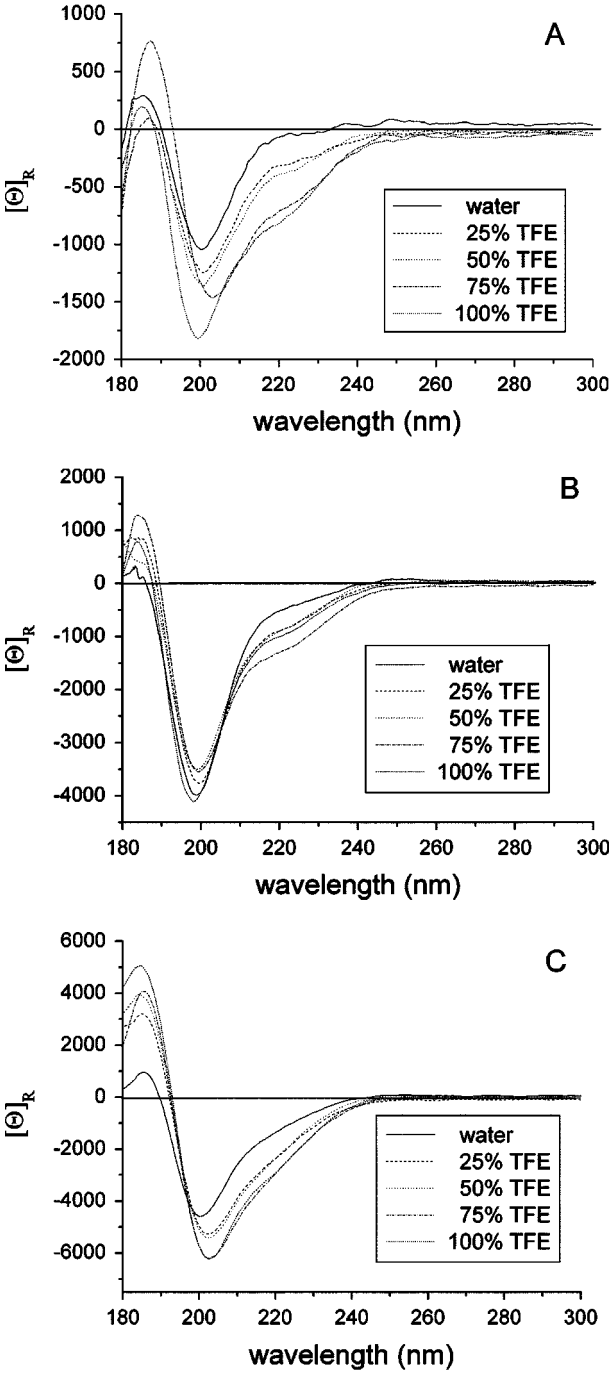


Figure 6 CD spectra of model peptides **1** (A), **2** (B) and **3** (C). For structures see Figures 1 and 2.

CD Spectroscopy

Model peptides **1**–**3** were studied in a water–TFE solvent system, containing 0, 25%, 50%, 75% and 100% of TFE. The CD spectra of model peptides **1**–**3** both in water and TFE indicate the absence of well-defined and characteristic secondary structures. The spectral properties do not depend considerably on the concentration of TFE, indicating that each model has a structure *relatively insensitive toward the permittivity* of the molecular environment. The spectra of each model peptide are of the typical U-type [cf. ref. 26], dominated by an intensive negative peak at about 200 nm. The CD spectra of model peptide **1** suggest that this molecule has the *lowest amount of ordered part*. The CD spectra of model peptide **2** are composed of a more intensive negative peak at 198 nm with a shoulder at 222 nm. The latter spectral feature indicates that model peptide **2** has a slightly more compact structure perhaps with more secondary structure content. According to CD spectroscopy model peptide **3** has the most compact and characteristic structure. Both the increase of the shoulder (n–π* transition) at 222 nm and the appearance of the positive part of the π–π* caplet at 185 nm show that this peptide is slightly more structured.

CONCLUSIONS

Three models with 17–24 amino acid residues of canonical serine protease inhibitor SGCI were synthesized by convergent peptide synthetic methods, in order to examine their inhibitory effects on chymotrypsin. The selective formation of the disulphide bridges proved to be the critical point in the multi-step syntheses. The disulphide patterns obtained were checked by 2D homonuclear ^{1}H NMR techniques. The CD spectra showed that Model Peptide **3** has a more ordered structure than model peptides **1** and **2**. The preliminary tests indicated that the two smaller model peptides **1** and **2** do not exert a remarkable inhibitory effect on chymotrypsin [18]. On the other hand, the larger model peptide **3** is more effective than model peptides **1** and **2**, but still less potent than SGCI. The different biological activities on mammalian chymotrypsin are obviously ascribed to the overall structures of these peptides, which are under going mapping by 2D homonuclear ^{1}H NMR techniques and theoretical internal dynamics. The results of the structure determinations together with the details of the measurements of biological activity will be published elsewhere (Mucsi Z, Gáspári Z, Orosz G, Perczel A: to be published). We plan to increase the inhibitory activity of our model by changing selected subunits, e.g. Gly-Gly bridge to Pro-Ala in the cyclopeptide moiety to form a more compact β-turn which could potentially enhance the effectiveness of the overall structure.

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