CHAPTER 5

Role of Disulfide Linkages in Arthropod Defensins and the Fungal Defensin Plectasin

5.1 Introduction

In chapter 4, investigations on antibacterial activity on C-terminal linear analogs of arthropod defensins were described. These studies indicated that the C-terminal region of arthropod defensins have sufficient features to exhibit antibacterial activities. In order to delineate the role of disulfide linkages and the central α-helix towards the antibacterial activity of arthopod defensins, peptides were synthesized spanning the α-helix and β-hairpin region of the arthropod defensins as deduced from the known three-dimensional structures of insect defensins (Hanzawa et al., 1990; Bonmatin et al., 1992; Cornet et al., 1995). Recently, an antibacterial peptide was isolated from a fungus, *Pseudoplectania nigrella*, which had structural similarities to insect defensins (Mygind et al., 2005). Hence, analogs corresponding to plectasin were also synthesized. A C-terminal analog of plectasin, which is similar to the linear peptide analogs in chapter 4, was also synthesized except that it possessed a single disulfide linkage. The sequences and net charge of the synthetic peptides are indicated in Table 5.1 alongwith the parent peptides.

5.2 Experimental Procedures

5.2.1 Peptide Synthesis

PNC, OMC2, SCC2 and PNC2 were synthesized on PAC-PEG-PS resin (0.18 mmol/g). PAC2 was synthesized on PAC-R resin (0.29 mmol/g) using the procedures mentioned in chapter 2, section 2.2 (page 40).

The removal of acetamidomethyl was effected by Hg(II) acetate and β-mercaptoethanol treatment (Veber et al., 1972) followed by gel filtration on a Biogel P-2 column. Two equivalents of mercury (II) acetate for each equivalent of cysteine was added to the peptide dissolved in 30% acetic acid and stirred for 1 h. Then 20 equivalents of β-mercaptoethanol was added and stirred for 1 h. Grey mercury sulphide precipitate was spun down. Supernatant was loaded on P-2 Biogel column (20 cm x 1 cm) to separate out salts and β-mercaptoethanol using buffer containing 50% acetonitrile with

Table 5.1. Primary structures of Arthropod defensins and synthetic peptides

Peptide	Sequence	Net charge[a]
Pa	ATC[1]DILSFQSQWVTPNHAGC[2]ALHC[3]VIKGYKGGQC[4]KITVC[5]HC[6]RR	+4
PAC2	GCALHCVIKGYKGGQKITVCHCRR	+5
Om-C	GYGC[1]PFNQYQC[2]HSHC[3]SGIRGYKGGYC[4]KGLFKQTC[5]NC[6]Y	+4
OMC2	QCHSHCSGIRGYKGGYKGLFKQTCNCY	+4
Sc-2	ATC[1]DLLSMWNVNHSAC[2]AAHC[3]LLLGKSGGRC[4]NDDAVC[5]VC[6]RK	+1
SCC2	ACAAHCLLLGKSGGRNDDAVCVCRK	+2
Plectasin	GFGC[1]NGPWDEDDMQC[2]HNHC[3]KSIKGYKGGYC[4]AKGGFVC[5]KC[6]Y	+1
PNC2	QCHNHCKSIKGYKGGYAKGGFVCKCY	+5
PNC	KSIKGYKGGYAKGGFVCKCY	+5

[a]Net charge at neutral pH. Cysteines are indicated in bold and their connectivities are C^1-C^4, C^2-C^5 and C^3-C^6. Pa, Om-C and Sc-2 are defensins from *Pyrrhocoris apterus*, *Ornithodoros moubata* and *Stomoxys calcitrans* respectively. PAC2, OMC2, SCC2 and PNC2 are synthetic analogs with two disulfide linkages while PNC consists of a single disulfide linkage.

0.01% TFA. Peptides were then oxidized in 20% DMSO-water (Tam et al., 1991) at a peptide concentration of 0.2 to 0.4 mM. This was followed by gel filtration (P-2 Biogel column) with 50% acetonitrile-water containing 0.01% TFA to remove DMSO.

Peptides were purified on a Zorbax C8 Reversed Phase column (250 mm x 4.6 mm). The gradient used to separate disulfide linked peptides was: 0-5 min, 15% B; 5-10 min, 20% B; 10-100 min 50% B; 100-110 min, 100% B. Solvent A consisted of 0.1% TFA in water and solvent B consisted of 0.1% TFA in 1:1 acetonitrile/isopropanol. Stock solutions of peptides were made by dissolving peptides in water and the concentration was determined by monitoring absorbance of tyrosine at 280 nm or using the formula: Concentration (μg/mL) = 144 (A_{215}-A_{225}), where A_{215} is the absorbance at 215 and A_{225} is the absorbance at 225.

5.2.2 Other Procedures

Bactericidal activity was determined as described in section 2.3 of chapter 2 (page 45). The values mentioned are mean of three independent experiments. The kinetics of bacterial killing were determined as described in chapter 2, section 2.4 (page 46). The values mentioned in the results are the averages of two independent experiments. Salt sensitivity was assayed as mentioned in section 2.5 (page 46). The values determined are the averages of three independent experiments. The variation was within ±5%. Membrane damage caused by peptides was visualized by fluorescence microscopy using a lipophilic dye FM 4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4(diethylamino) phenylhexatrienyl) pyridinium dibromide) which preferentially stains the inner membrane as mentioned in section 2.12 (page 48). Human erythrocytes were used to evaluate the hemolytic activity of the peptides analogs as mentioned in 2.9 (page 47). The values determined were the averages of three independent experiments. The variation was within ±5%. Circular dichroism spectra were recorded as described in section 2.11 (page 48).

5.3 Results

5.3.1 Choice and Synthesis of Peptides

Plectasin, the first defensin identified in a fungus, was recently isolated from the saprophytic ascomycete, *Pseudoplectania nigrella* (Mygind et al., 2005). The primary, secondary and tertiary structures of plectasin are very similar to that of arthropod defensins (Mygind et al., 2005). The three dimensional structure of plectasin was solved using proton NMR spectroscopy and the disulfide bonds were determined by NMR, mass spectroscopic analysis and molecular dynamic simulations (Mygind et al., 2005). Peptides corresponding to the α-helical and β-hairpin region of Pa, Om-C, Sc-2 and plectasin having four cysteines were synthesized. The net charge varies between +2 and +5. The sequences are shown in Table 5.1.

Peptides were purified using RPHPLC and the chromatogram of two disulfide and single disulfide peptide analogs were obtained. Two peaks were obtained for PAC2 that gave identical m/z of 2668.46, which corresponded to the two disulfide linked peptide with +42 m/z (Figure 5.1). The additional 42 m/z may be due to acetylation at the N-terminus during the procedure to remove Acm group from cysteines as acetic acid was used. Peak 1, peak 2 and peak 3 of OMC2 displayed identical m/z of 3034.47 corresponding to completely oxidized cysteines in OMC2 (Figure 5.1). The chromatogram of SCC2 showed seven peaks with identical m/z of 2556.22, all of which corresponds to two disulfide linked peptide (Figure 5.2). PNC2 displayed five peaks of which peak 1, peak 2 and peak 3 gave correct m/z of 2875.49, which corresponds to two disulfide linked peptide (Figure 5.2). Peak 4 and peak 5 of PNC2 also displayed a m/z of 3075.49 that is an additional +200 m/z, which can be assigned to mercury adduct. Two peaks were obtained for PNC (Figure 5.3). Peak 1 of PNC yielded an additional 200 m/z (2355.18), which is mercury adduct of peptide. Peak 2 displayed m/z of 2155.18 that corresponds to the single disulfide linked peptide.

Peaks were further characterized to know their disulfide connectivities. Three different disulfide linkages are theoretically possible: C^1-C^2, C^3-C^4 or C^1-C^3, C^2-C^4 or C^1-

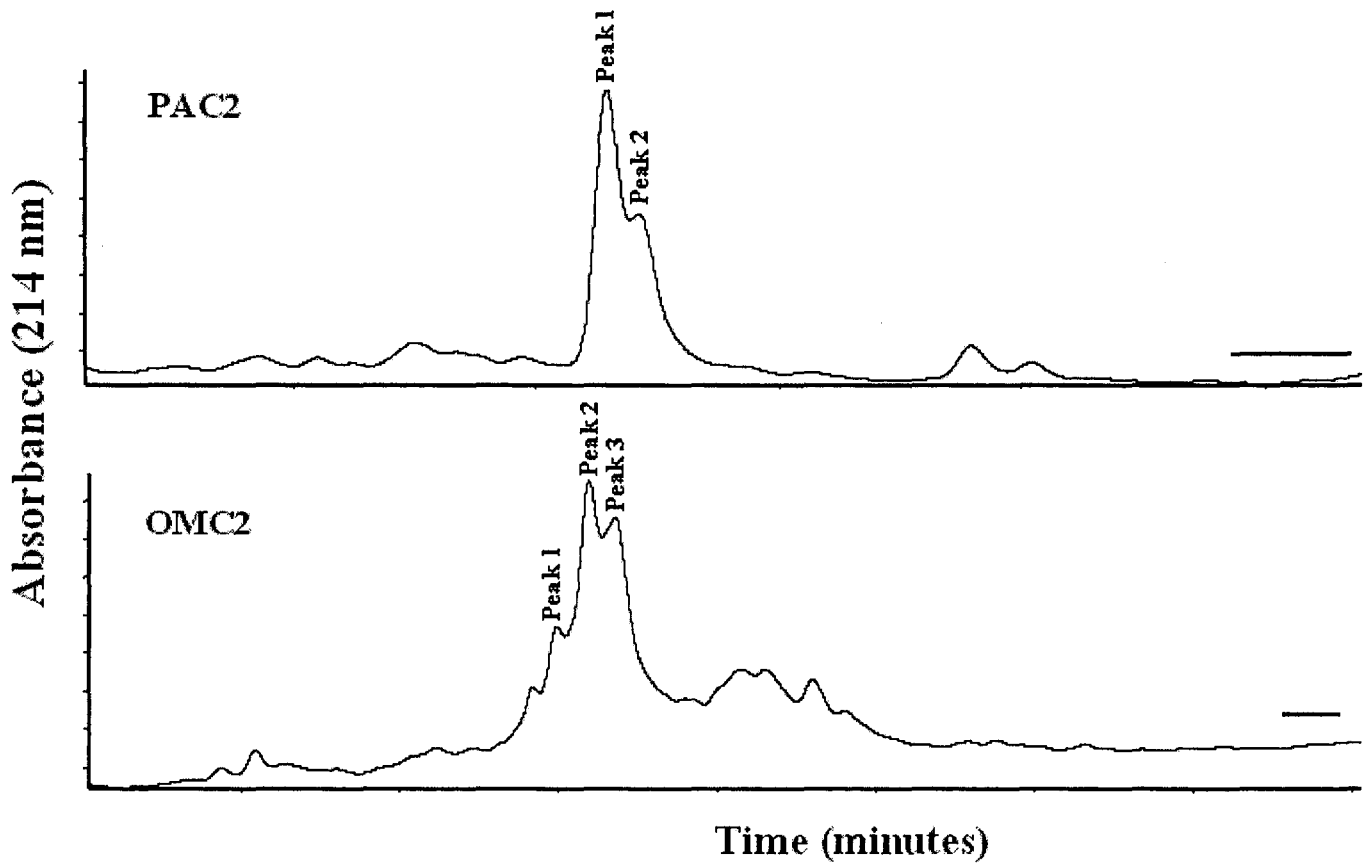


Figure 5.1. Chromatogram of two disulfide linked PAC2 and OMC2.
Both peak 1 and peak 2 in PAC2 showed +42 m/z (2626.34 + 42). Peak 1, peak 2 and peak 3 of OMC2 displayed correct m/z of 3034.47 corresponding to two disulfide bridges. The theoretical m/z (MH^+) corresponding to two disulfide linkages for PAC2 and OMC2 are 2626.3410 and 3034.3600, respectively. Bars denote a scale of 1 minute.

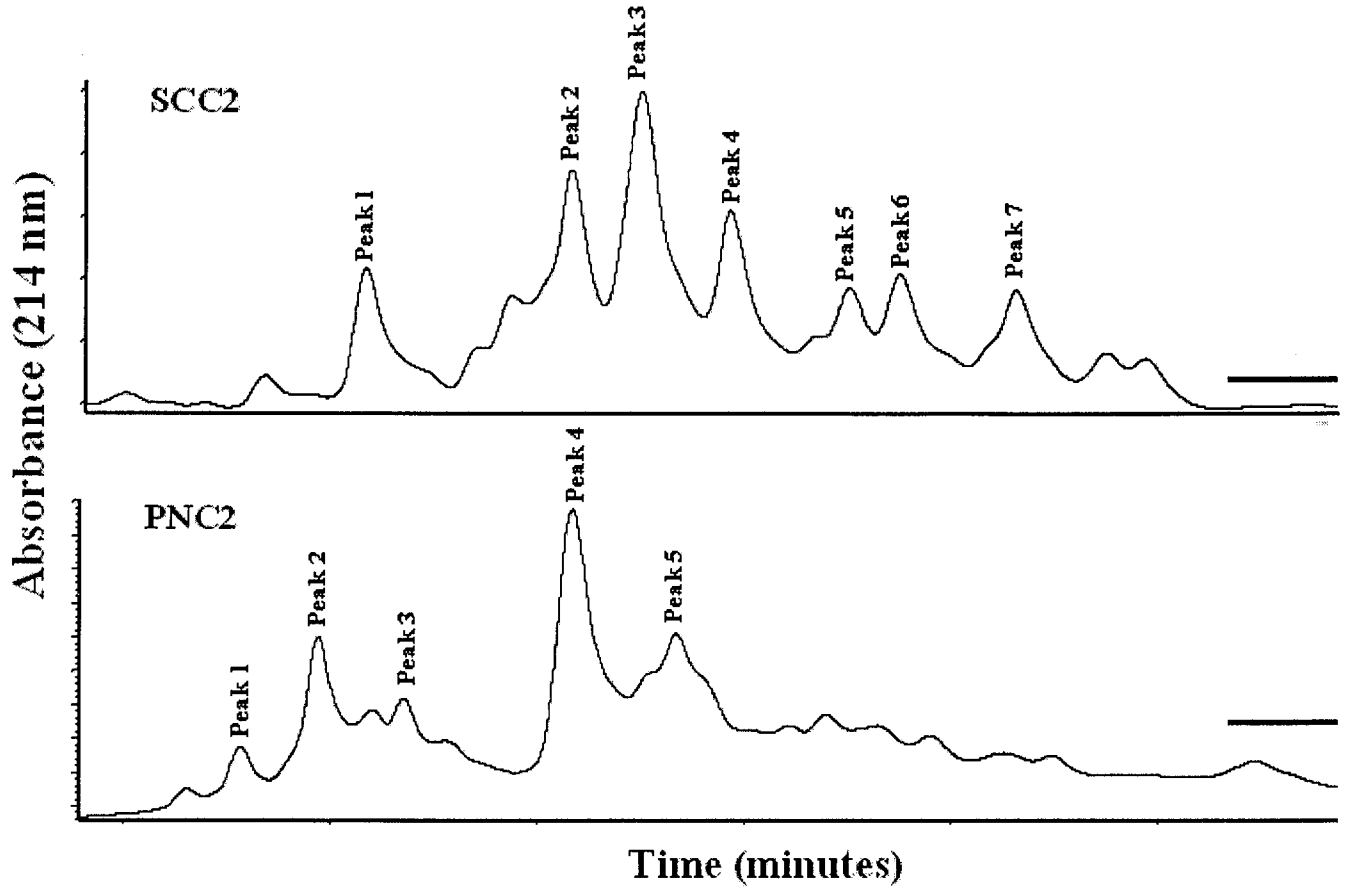


Figure 5.2. Chromatogram of two disulfide linked SCC2 and PNC2.
All peaks of SCC2 displayed the correct m/z corresponding to two disulfide linked peptide of 2556.22. Peak 1, peak 2 and peak 3 showed correct m/z (2875.49) corresponding to two disulfide linked peptide. Peak 4 and peak 5 of PNC2 displayed +200 m/z (3075.49) alongwith the correct m/z of two disulfide linked PNC2 (2875.49). The theoretical m/z (MH^+) corresponding to two disulfide linkages for SCC2 and PNC2 are 2556.2363 and 2875.3360, respectively. Bars denote a scale of 1 minute.

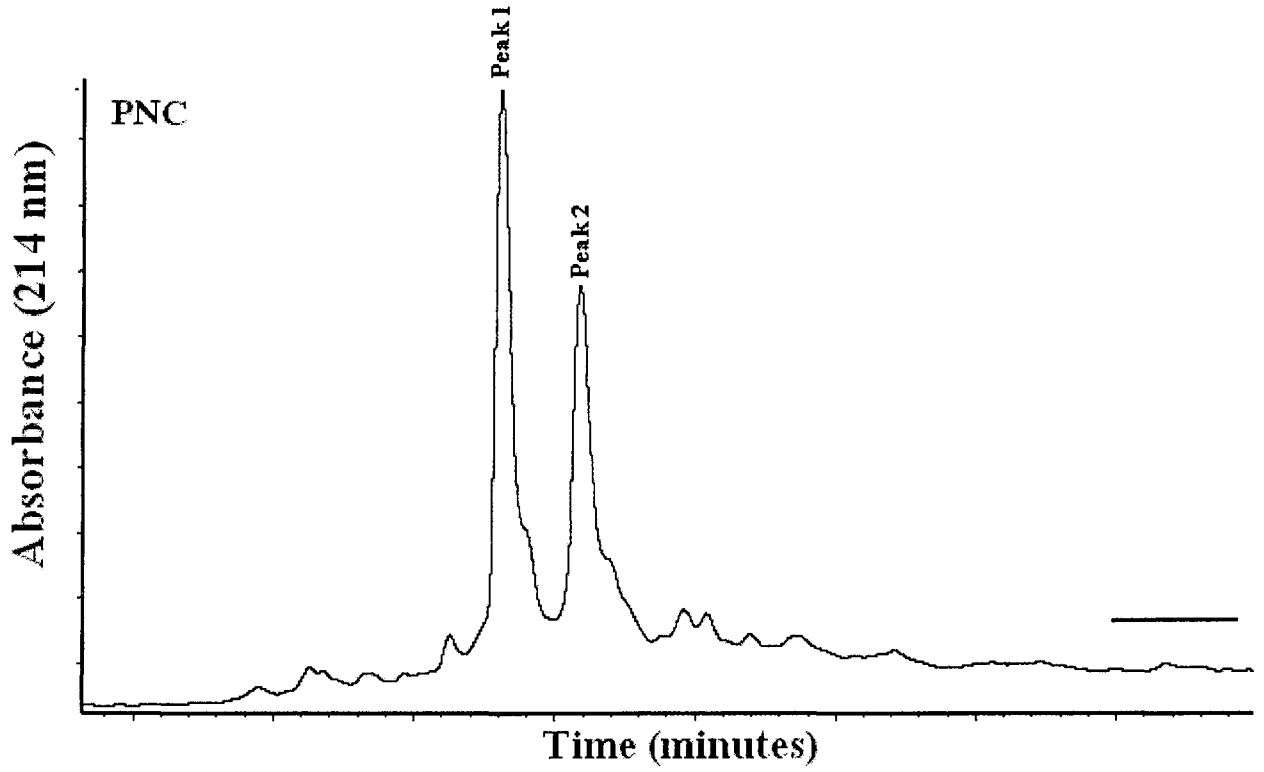


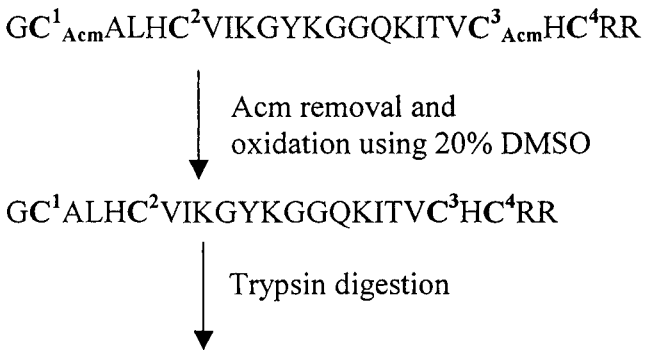
Figure 5.3. Chromatogram of one disulfide linked PNC.
Peak 1 displayed the correct peptide m/z corresponding to single disulfide linkage (2155.18) as well as +200 m/z (2355) denoting mercury adduct. Peak 2 displayed only correct m/z of 2155.18. Bars denote a scale of 2 minutes. The theoretical m/z (MH^+) corresponding to single disulfide linkage for PNC is 2155.0983.

C^4, C^2-C^3. Out of these possible combinations, it is only possible to distinguish whether the connectivities are C^1-C^2, C^3-C^4 or C^1-C^3, C^2-C^4/C^1-C^4, C^2-C^3 when trypsin or chymotrypsin is used. The analysis of PAC2 shows that the peaks, peak 1 and peak 2, consist of isomers with scrambled disulfide linkages as analyzed by trypsin digestion (Figure 5.4). The additional 42 m/z observed on N-terminal fragment having m/z 982.59 indicates that acetylation is on the N-terminus of PAC2. The analysis of OMC2 revealed that scrambled disulfide linked peptides are present in each of the three peaks (Figure 5.5). Analysis of disulfide linkages in SCC2 showed that all seven peaks have a mixture of scrambled disulfide linked peptides (Figure 5.6). Trypsin fragments obtained for PNC2 peaks demonstrated similar results as observed by the presence of scrambled disulfide peptides in all the five RPHPLC peaks (Figure 5.7).

Disulfide linkage analysis indicated that all the two disulfide peptide analogs possessed scrambled disulfide bridges. The HPLC profiles indicate the presence of all possible combinations of disulfide pairings. The peptide SCC2 should yield three peaks if all possible combinations of disulfide bridges are formed. The presence of additional peaks with correct m/z values could arise as a result of aggregation.

5.3.2 Antibacterial Activities of Peptides

Antibacterial activities of disulfide-linked peptides with scrambled disulfides were examined against Gram-positive and Gram-negative bacteria. The mixture of scrambled disulfide linked peptides was used for antibacterial activity. Lethal concentrations of peptides are mentioned in Table 5.2. PAC2 and PNC2 exhibited comparable potencies against both Gram-positive and Gram-negative bacteria. OMC2 was comparatively less potent than PAC2 and PNC2 against both Gram-negative and Gram-positive bacteria. SCC2 was inactive against both Gram-negative and Gram-positive bacteria. No activity was observed even upto 200 μg/mL. The single disulfide analog corresponding to the C-terminus of plectasin was the most potent among all the analogs. All the analogs displayed more activity against Gram-positive as compared to Gram-negative bacteria as observed by their low LCs against Gram-positive bacteria.



Peptide fragments obtained	Theoretical m/z MH^+	Obtained m/z MH^+	Disulfide linkage
GCALHCVIK	941.4852	982.59*	C1-C2 and C3-C4
GYKGGQKITVCHCR	1549.7726	1549.96	C1-C3 and C2-C4 or C1-C4 and C2-C3

All HPLC peaks gave identical fragments after trypsin digestion. *denotes acetylation.

Figure 5.4. Analysis of disulfide linkages in two disulfide peptide PAC2.
Peptide fragments corresponding to missed cleavages during trypsin digestion are also shown.

$QC^{1}{}_{Acm}HSHC^{2}SGIRGYKGGYKGLFKQTC^{3}{}_{Acm}NC^{4}Y$

↓ Acm removal and oxidation using 20% DMSO

$QC^{1}HSHC^{2}SGIRGYKGGYKGLFKQTC^{3}NC^{4}Y$

↓ Trypsin digestion

Peptide fragments obtained	Theoretical m/z MH^+	Obtained m/z MH^+	Disulfide linkage
QCHSHCSGIR	1125.4833	1125.46	C1-C2 and C3-C4
GLFKQTCNCY	1174.5176	1174.50	C1-C2 and C3-C4
QCHSHCSGIRGYK	1473.6631	1473.64	C1-C2 and C3-C4
GGYKGLFKQTCNCY	1579.7188	1579.71	C1-C2 and C3-C4
QCHSHCSGIRGYKGGYK	1878.8643		C1-C2 and C3-C4
GYKGGYKGLFKQTCNCY	1927.8986	1927.89	C1-C2 and C3-C4
QCHSHCSGIR QTCNCY	1836.7142	1836.67	C1-C3 and C2-C4 or C1-C4 and C2-C3

All HPLC peaks gave identical fragments after trypsin digestion.

Figure 5.5. Analysis of disulfide linkages in two disulfide peptide OMC2.
Peptide fragments corresponding to missed cleavages during trypsin digestion are also shown.

$AC^{1}_{Acm}AAHC^{2}LLLGKSGGRNDDAVC^{3}_{Acm}VC^{4}RK$

↓ Acm removal and oxidation using 20% DMSO

$AC^{1}AAHC^{2}LLLGKSGGRNDDAVC^{3}VC^{4}RK$

↓ Trypsin digestion

Peptide fragments obtained	Theoretical m/z MH^+	Obtained m/z MH^+	Disulfide linkage
ACAAHCLLLGK	1097.5751	1097.55	C1-C2 and C3-C4
SGGRNDDAVCVCR	1349.5841	1349.56	C1-C2 and C3-C4
ACAAHCLLLGKSGGR	1454.7511	1454.72	C1-C2 and C3-C4
SGGRNDDAVCVCRK	1477.6791	1477.65	C1-C2 and C3-C4
ACAAHCLLLGK NDDAVCVCR	2071.9653	2071.94	C1-C3 and C2-C4 or C1-C4 and C2-C3

All HPLC peaks gave identical fragments after trypsin digestion.

Figure 5.6. Analysis of disulfide linkages in two disulfide peptide SCC2.
Peptide fragments corresponding to missed cleavages during trypsin digestion are also shown.

$QC^{1}_{Acm}HNHC^{2}KSIKGYKGGYAKGGFVC^{3}_{Acm}KC^{4}Y$

↓ Acm removal and oxidation using 20% DMSO

$QC^{1}HNHC^{2}KSIKGYKGGYAKGGFVC^{3}KC^{4}Y$

↓ Trypsin digestion

Peptide fragments obtained	Theoretical m/z MH^+	Obtained m/z MH^+	Disulfide linkage
QCHNHCKSIK	1195.5616	1178.52*	C1-C2 and C3-C4
QCHNHCKSIKGYK	1543.7413	1526.70*	C1-C2 and C3-C4
QCHNHCKSIKGYKGGYAK	2019.9796	2002.94*	C1-C2 and C3-C4
QCHNHCK GGFVCKCY	1723.7069	1723.66	C1-C3 and C2-C4 or C1-C4 and C2-C3

All HPLC peaks gave identical fragments after trypsin digestion.
*denotes loss of $-NH_3$

Figure 5.7. Analysis of disulfide linkages in two disulfide peptide PNC2.
Peptide fragments corresponding to missed cleavages during trypsin digestion are also shown.

Table 5.2. Antibacterial activity of arthropod defensin analogs

Bacteria	Lethal concentration (μM)[a]				
	PAC2	OMC2	PNC2	SCC2	PNC
E. coli MG 1655	30	40	30	Inactive	15
P. aeruginosa NCTC 6750	33	45	35	Inactive	20
S. aureus NCTC 8530 2.5	20	32	17	Inactive	10

[a]The values reported for the analogs are the average of at least three independent experiments. The variation was within ±5% around mean.

The kinetics of bacterial killing by disulfide-linked peptides was examined (Figure 5.8). PNC exhibited the most rapid bactericidal rate against both *E. coli* and *P. aeruginosa*. All two disulfide analogs exhibited similar bactericidal rates against Gram-negative bacteria. All peptide analogs except OMC2 exhibited similar rates of killing against *S. aureus*. OMC2 displayed a comparatively slower rate of killing against *S. aureus*.

5.3.3. Effect of Salts on Antibacterial Activity

The effect of salts on the antibacterial activity of peptides is summarized in Figure 5.9. The antibacterial activity of all the analogs except PAC2 was completely abolished against *E. coli* in presence of 100 mM NaCl (Figure 5.9). Only PNC displayed substantial activity against *P. aeruginosa* in presence of 100 mM NaCl. PAC2, PNC2 and PNC showed antibacterial activity against *S. aureus* in the presence of NaCl. However, PNC displayed the least sensitivity towards NaCl against *P. aeruginosa* and *S. aureus*. OMC2 did not display any antibacterial activity against both Gram-negative and Gram-positive bacteria in the presence of 100 mM NaCl. All analogs were inactive in the presence of 1 or 2 mM $MgCl_2$ (Table 5.3). PAC2 and PNC2 exhibited about 50% killing against *E. coli* in the presence of 2 mM $CaCl_2$. Complete loss of antibacterial activity was observed for OMC2 and PNC in the presence of $CaCl_2$.

The effect of peptides on the morphology of *E. coli* was studied using the lipophilic dye FM 4-64, which accumulates in the cytoplasmic membrane of Gram-negative bacteria (Fishov and Woldringh, 1999). The data are shown in Figure 5.10. All analogs displayed extensive membrane damage to the *E. coli* cells as seen by clustering of the dye at some places and spreading out of the dye over bacterial cell. SCC2 that was inactive against all the bacterial strains did not exhibit any damage to the *E. coli* cells as observed by the comparable staining of FM 4-64 for SCC2 treated and control cells.

5.3.4 Hemolytic Activity

The ability of peptides to lyse human RBCs was tested. Very low degree of hemolysis was observed with all the analogs. At 50 μM peptide concentration ~15-20% RBC lysis was observed. SCC2 did not show any lysis at 50 μM peptide concentration.

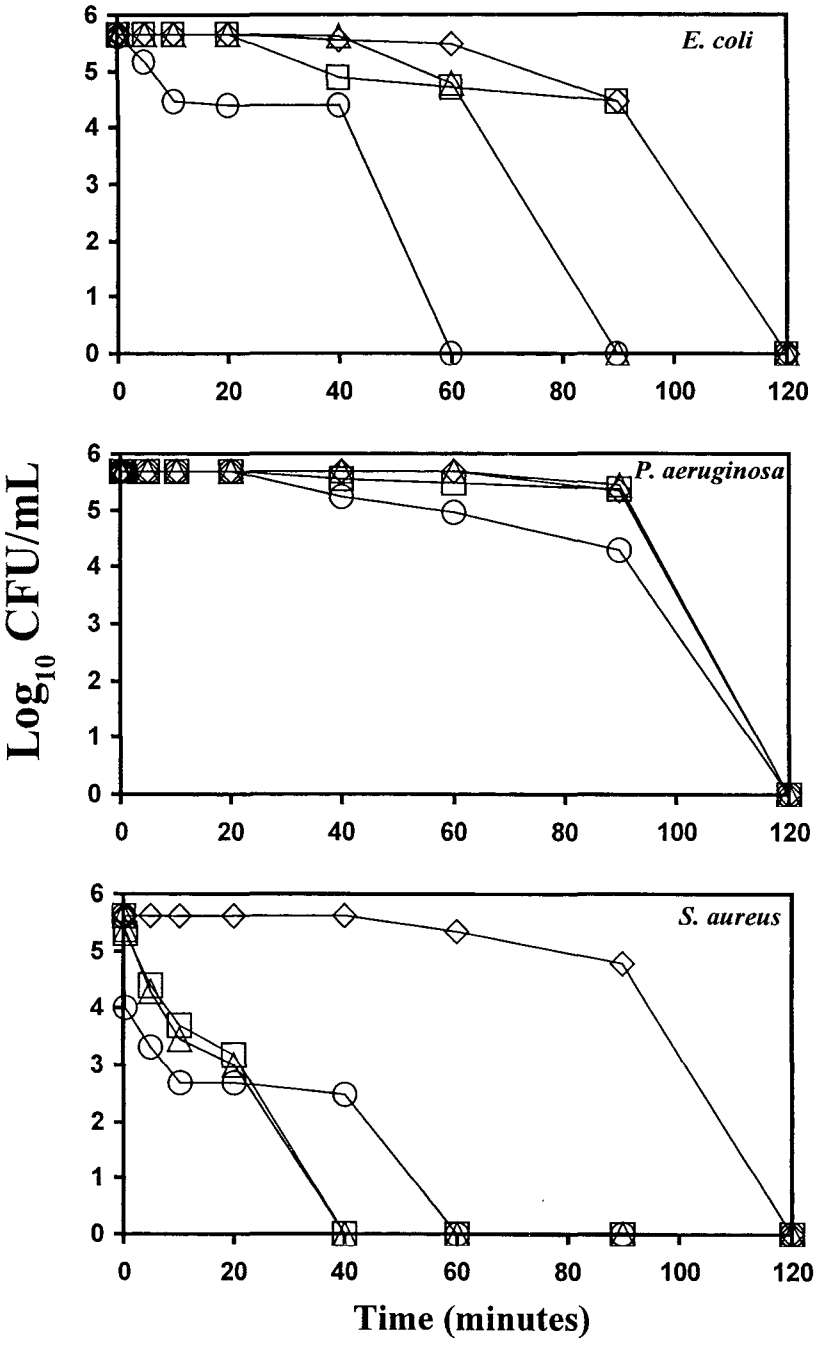


Figure 5.8. Kinetics of bacterial killing by peptides.
PAC2 (□); OMC2 (◊); PNC2 (Δ); PNC (○). Bacteria (10^6 CFU/ml) at mid-log phase were incubated with LCs of peptides.

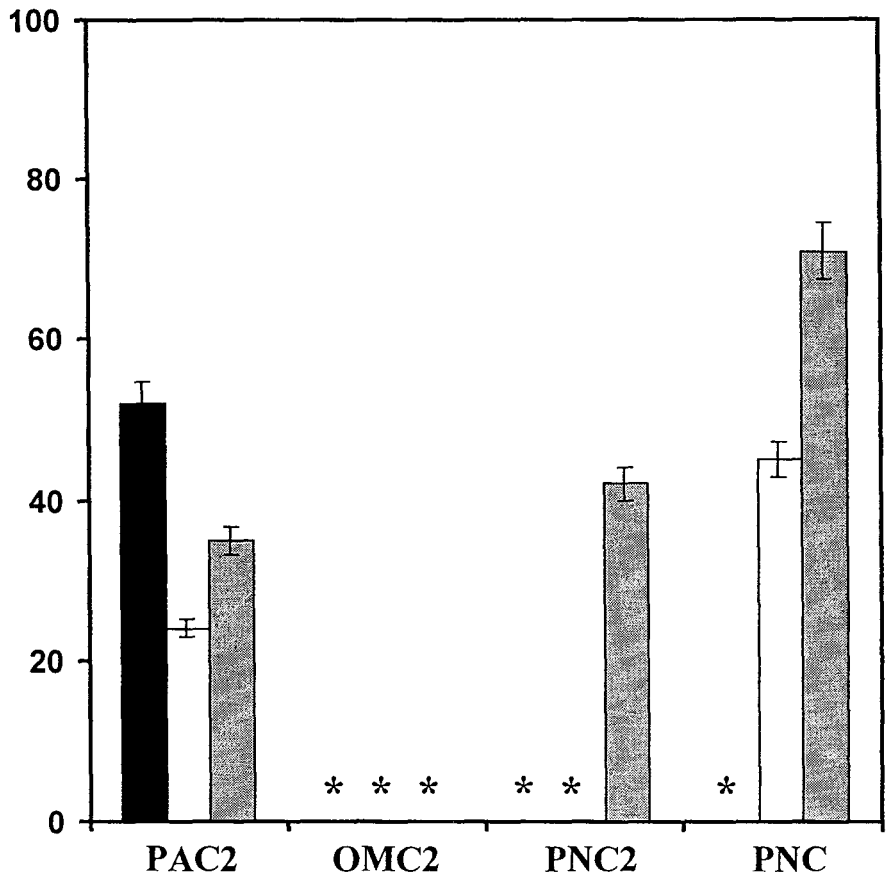


Figure 5.9. Effect of NaCl on antibacterial activity of peptides.
Mid-log phase bacteria (10^6 CFU/mL) were incubated with LCs of peptides in the presence of 100 mM NaCl. The values reported for the analogs are the average of at least three independent experiments. The variation was within ±5%. *E. coli* MG 1655 (black); *P. aeruginosa* NCTC 6750 (white); *S. aureus* (grey). '*' indicates zero percent killing.

Table. 5.3 Effect of divalent cations on antibacterial activity of peptides.

Salt	% bacterial killing[a]			
	PAC2	OMC2	PNC2	PNC
$MgCl_2$ 1 mM	0	0	0	0
2 mM	0	0	0	0
$CaCl_2$ 1 mM	50	0	89	0
2 mM	50	0	48	0

[a]The values reported for the analogs are the average of three independent experiments. The variation was within ±5%. Mid-log phase *E. coli* MG 1655 (10^6 CFU/mL) were incubated with LCs of peptides in presence of 1 mM/2 mM $CaCl_2$ or $MgCl_2$.

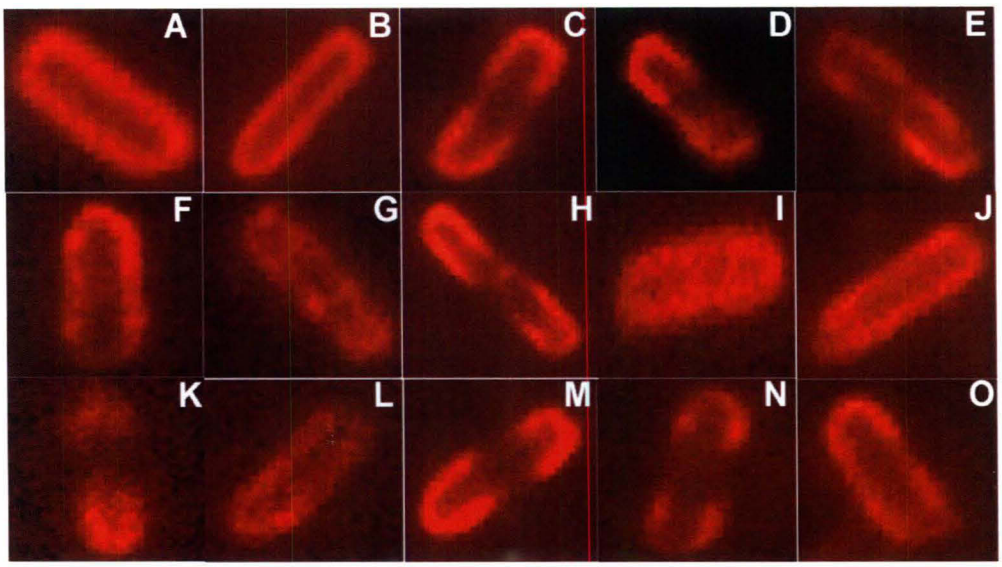


Figure 5.10. Visualization of the effect of disulfide linked analogs on *E. coli* by fluorescence microscopy.
Cells were stained with the lipophilic fluorescent dye FM 4-64. Control (A, B); PAC2 (C-E); OMC2 (F-H); SCC2 (I, J); PNC2 (K, L); and PNC (M-O). Mid-log phase bacteria (10^6 CFU/mL) were incubated with FM 4-64 for 10 min followed by incubation at LCs of peptides.

5.3.5 Conformation of Peptides

Circular dichroism (CD) spectroscopy was carried out for all the peptides in aqueous buffer and trifluoroethanol (TFE). The results are shown in Figures 5.11, 5.12 and 5.13. Spectra obtained were those of purified peaks. All analogs displayed unordered conformation in buffer (Figure 5.11). However, TFE induces the formation of secondary structures in the analogs (Figure 5.12). The spectra of PAC2 P1 and PAC2 P2 suggest the population of β-hairpin and helical conformation (Blanco et al., 1994; Blanco and Serrano, 1995; Viguera et al., 1996).

The spectra of SCC2 P5, SCC2 P7 and PNC2 P2, PNC2 P3, PNC2 P4 and PNC2 P5 also indicate a mixture of α-helical and β-hairpin conformation (Blanco et al., 1994; Blanco and Serrana, 1995; Viguera et al., 1996). The spectra of OMC2 P2 and OMC2 P3 suggest that the peptides adopt helical conformation. The spectra of PNC P1 and P2 suggest unordered conformation in buffer and helical conformation in TFE (Figure 5.13). The θ_{MRE} values indicate that a greater fraction of PAC2 P2, SCC2 P5 and PNC2 P2 populate unordered conformation. While the conformational propensities in the two different forms of these peptides are similar, the fraction populating ordered conformation depends on the pattern of disulfide linkage.

5.4 Discussion

Studies were carried out on antibacterial activities of disulfide-linked arthropod defensins analogs and plectasin analogs. All peptide analogs were constrained by disulfide linkages. The HPLC of all the two disulfide analogs displayed multiple peaks but with same molecular mass. Peak 1 and peak 2 of PAC2 displayed same m/z (2668.46) pertaining to the mass of two disulfide linkages (2626.34) with additional +42 Da. Extra mass was attributed to acetylation at the N-terminus of peptide. The disulfide analysis showed that different disulfide linkages are present in both the peaks. Peak 1, peak 2 and peak 3 in OMC2 showed same m/z (3034.47) and each peak showed the presence of peptides with different disulfide linkages. In SCC2, all peaks were well separated but

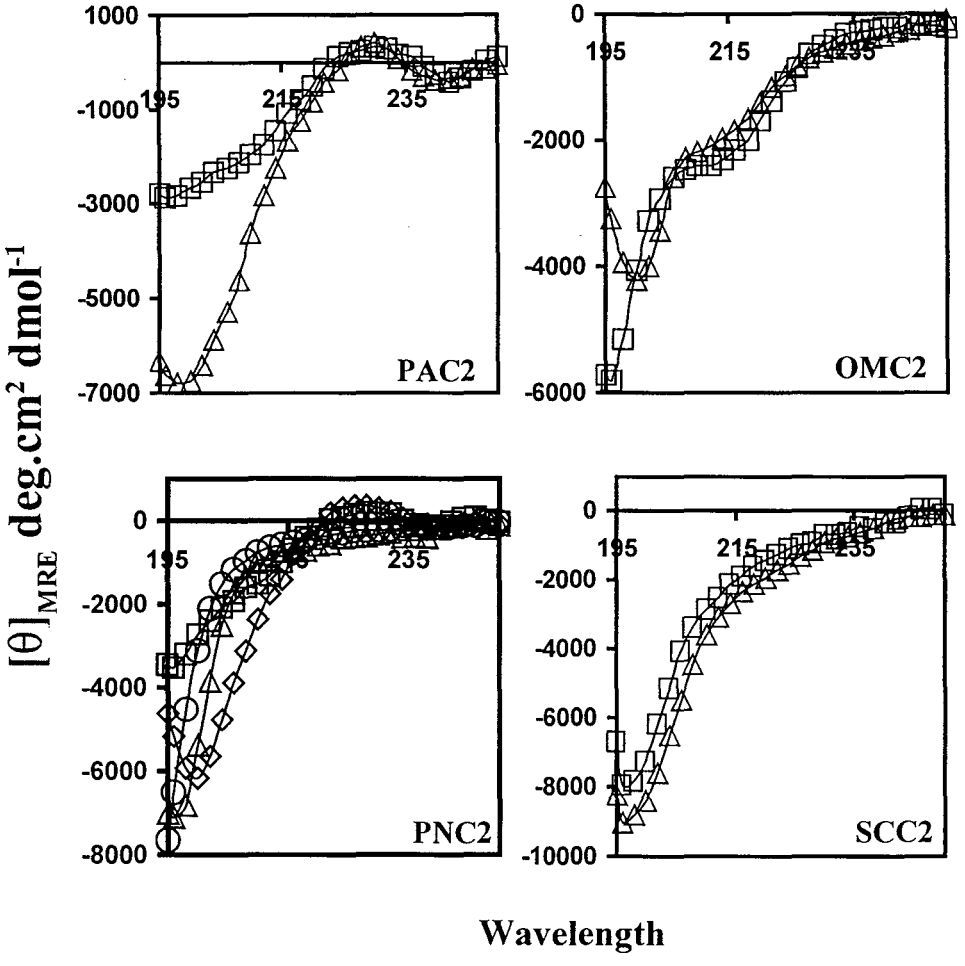


Figure 5.11. Circular dichroism (CD) spectra of two disulfide arthropod defensin and plectasin analogs.

CD spectra were recorded in 5 mM Hepes 7.4 pH. PAC2 P1 (Peak 1) (triangles), PAC2 P2 (Peak 2) (squares) (page 119, Fig. 5.1); OMC2 P2 (Peak 2) (triangles), OMC2 P3 (Peak 3) (squares) (page 119, Fig. 5.1); PNC2 P2 (Peak 2) (rhombus), PNC2 P3 (Peak 3) (square), PNC2 P4 (Peak 4) (triangle), PNC2 P5 (Peak 5) (circle) (page 120, Fig. 5.2); SCC2 P5 (Peak 5) (triangle), SCC2 P7 (Peak 7) (square) (page 120, Fig. 5.2). Peaks are numbered as shown in the respective RPHPLC chromatograms.

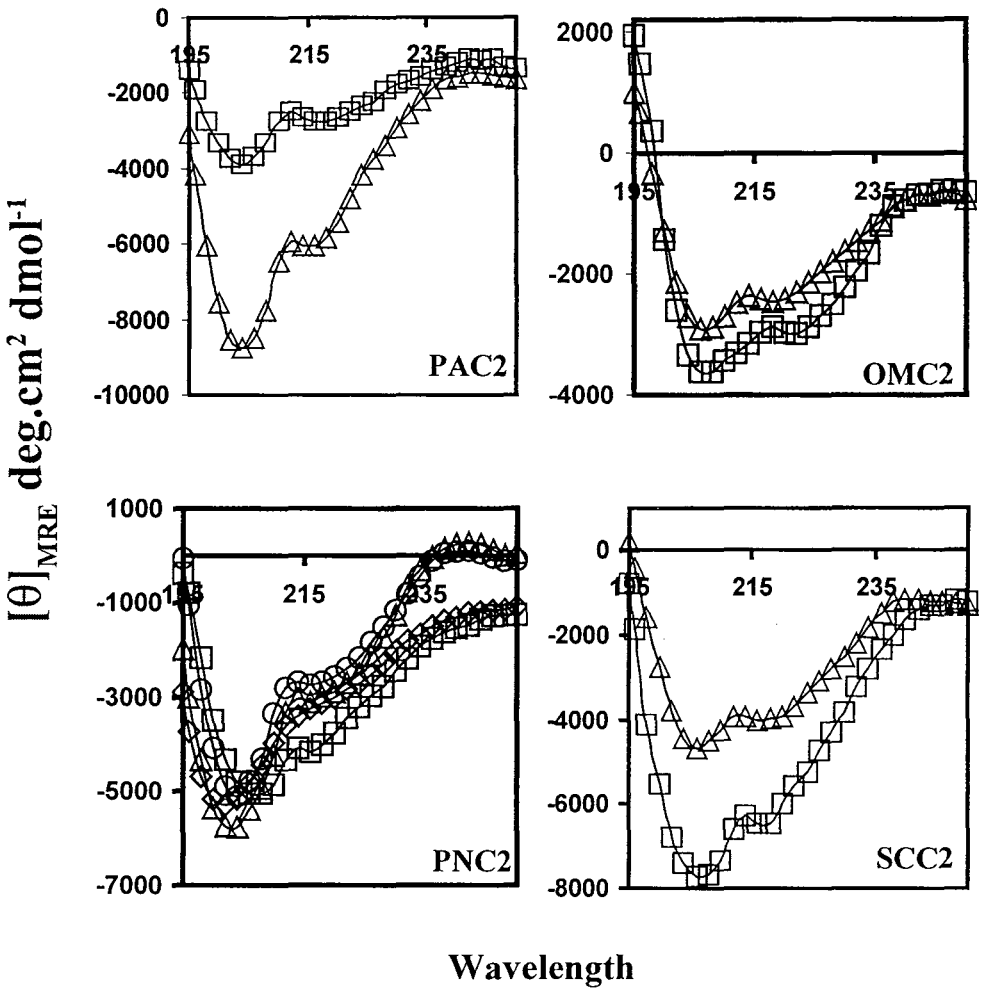


Figure 5.12. Circular dichroism (CD) spectra of two disulfide arthropod defensin and plectasin analogs.

CD spectra were recorded in TFE. PAC2 P1 (Peak 1) (triangles), PAC2 P2 (Peak 2) (squares) (page 119, Fig. 5.1); OMC2 P2 (Peak 2) (triangles), OMC2 P3 (Peak 3) (squares) (page 119, Fig. 5.1); PNC2 P2 (Peak 2) (rhombus), PNC2 P3 (Peak 3) (square), PNC2 P4 (Peak 4) (triangle), PNC2 P5 (Peak 5) (circle) (page 120, Fig. 5.2); SCC2 P5 (Peak 5) (triangle), SCC2 P7 (Peak 7) (square) (page 120, Fig. 5.2). Peaks are numbered as shown in the respective RPHPLC chromatograms.

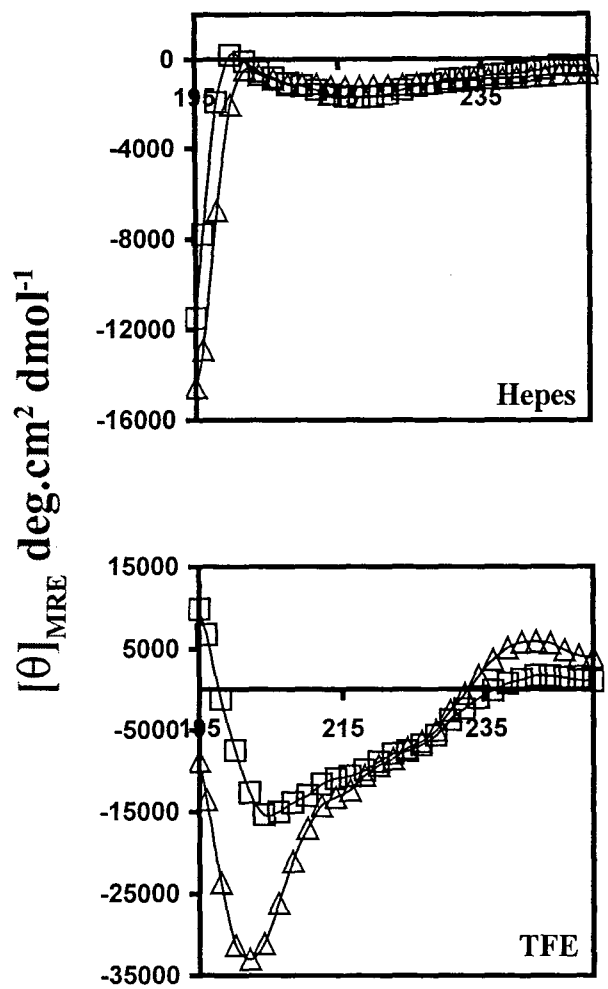


Figure 5.13. Circular dichroism (CD) spectra of one disulfide plectasin analog.

CD spectra were recorded in 5 mM Hepes 7.4 pH and TFE. PNC P1 (triangle); PNC P2 (square). P1 and P2 represent peak 1 and peak 2 shown in the RPHPLC chromatogram of PNC (page 121, Fig. 5.3).

yielded same m/z (2556.22) corresponding to the two disulfide linked peptide and mass spectral analysis indicated a mixture of scrambled disulfide linked peptides in each peak. Moreover, only three theoretical combinations of disulfide linkages are possible and the maximum peaks obtainable on this basis by RPHPLC analysis is three. Similar results were obtained with PNC2 in which peak 1, peak 2, peak 3, peak 4 and peak 5 showed expected m/z of 2875.49 corresponding to two disulfide linked PNC2. Individual peaks yielded scrambled disulfide linkages. In a recent report, which describes the synthesis and separation of β-defensin Defr 1, similar complexity involving multiple peaks in RPHPLC separation has been discussed (Taylor et al., 2007). Out of the 14 fractions separated by RPHPLC, each of the fractions clearly displayed different band intensity between ~5 to 13 kDa when further analyzed by native gel electrophoresis. The mass analysis, however, displayed only dimeric forms of the peptide. This complexity was attributed to different isoforms of Defr 1 dimer as theoretically more than 200 unique disulfide connectivities could be formed (Campopiano et al., 2004; Taylor et al., 2007). However, this is not possible with the two disulfide linked analogs of arthropods and plectasin defensins as only three unique disulfide linkages are feasible with four cysteines. Aggregation of peptides could be the reason for the presence of multiple peaks during HPLC separation. Mercury adduct with +200 Da was also obtained for peak 4 and peak 5 of PNC2. Peak 1 of PNC also displayed mercury adduct. An earlier report indicates that mercury adducts are formed in certain cases while using mercuric acetate to remove acetamidomethyl group from cysteine (Angeletti et al., 1996). The disulfide linkages were intact in the peptides with mercury adduct as observed by an exact +200 Da (3075) increase over 2875.49 m/z which relates to two disulfide linkages in PNC2 and an exact +200 Da (2355) increase over 2155.18 m/z which corresponds to a single disulfide linkage in PNC. Furthermore, Taylor et al. reported that each of the 14 fractions displayed antimicrobial activity similar to that of the parental unfractionated Defr 1 and hence confirming the earlier studies on defensins that showed dispensability of native disulfide linkages for antimicrobial activity (Mandal and Nagaraj, 2002; Mandal et al., 2002; Wu et al., 2003a; Hoover et al., 2003; Taylor et al., 2007). Folding of HBD-3 by DMSO oxidation yielded many isoforms with similar antimicrobial activity (Wu et al., 2003a). All the seven fractions obtained after RPHPLC separation exhibited similar bactericidal

activity (Wu et al., 2003a). Our study involving two disulfide linked peptides indicates that a similar structure-function relationship related to disulfide bonds exists in arthropod and fungal defensins.

The two disulfide analogs span the α-helical and β-hairpin region observed in the known three dimensional structures of insect defensins while one disulfide plectasin analog spans the C-terminal β-hairpin region (Hanzawa et al., 1990; Bonmatin et al., 1992; Cornet et al., 1995; Mygind et al., 2005). All two disulfide analogs displayed comparable LCs, although PAC2 and PNC2 were slightly more active than OMC2. In general, like the parent peptides two disulfide analogs were more active against Gram-positive bacteria unlike the peptides without disulfide bridges described in Chapter 4. The single disulfide C-terminal analog, PNC, exhibited broad-spectrum activity compared to the activity of plectasin that was directed only against Gram-positive bacteria. As observed in chapter 4, the C-terminal linear analogs exhibited broad-spectrum antibacterial activity in comparison to the parent peptides. The bactericidal activity of the two disulfide linked analogs seems to possess the specificity of their respective parent peptides. Hence, the central helical region and the disulfide linkages, although dispensable for antibacterial activity, might play a role in deciding the antibacterial specificity in arthropod defensins. SCC2 despite having a net +2 charge was inactive. The addition of α-helical region or disulfide linkages did not improve the activity of the linear analog SCΔC. The linear analogs of arthropod defensins are much more potent than the disulfide linked longer counterparts and the C-terminal plectasin analog, PNC, has got comparable LCs with the linear arthropod defensin analogs. The display of antibacterial activity by two disulfide linked peptides even in the presence of scrambled disulfide linkages suggests that native disulfide bonding is not essential for antibacterial activity.

CD spectra revealed that the two disulfide linked peptides were more ordered than their linear analogs or even the C-terminal single disulfide linked peptide. This could be due to the stabilization of the secondary structures by the disulfide linkages. The disulfide linkage in PNC might not play a major role since both the cysteines are separated by a single amino acid and hence the secondary structure stabilization would not be possible.

In the case of two disulfide linked peptides, the N-terminal is linked to the C-terminal via disulfide bonds and hence stabilizing the overall secondary structure of the peptide.

The rapid rate of killing observed for all the peptides and the fluorescence microscopy analyses suggests that the bacterial membrane could be the target of these peptides. This is similar to that observed for the linear analogs. Addition of disulfide linkages or the α-helical segment does not alter the mechanism of killing by these peptides. Insect defensins are known to be sensitive towards high salt concentrations (Cociancich et al., 1993a). The two disulfide linked peptides were more sensitive towards NaCl as compared to their linear counterparts. In fact, OMC2 was completely inactive against all the bacterial strains in the presence of 100 mM NaCl and PNC2 was active only against *S. aureus* in the presence of NaCl. Hence, linearization of defensins could be a useful strategy to develop salt insensitive peptides. Linear form of HBD-3 was shown to be more salt-resistant than the oxidized peptide with three disulfide linkages (Wu et al., 2003a). Differences in the sensitivity of peptides by divalent cations suggests towards different binding sites for the peptides on the bacterial membrane.

Full-length insect defensins are known to exhibit low level of hemolysis. All the analogs, irrespective of number of disulfide linkage, length or charge displayed very low hemolytic activity that is comparable to the parent peptides and the C-terminal linear analogs described in chapter 4.

It was observed that a peptide corresponding to the C-terminal β-sheet region of the insect defensin Tenecin, linked by a disulfide bridge, exhibited activity against both Gram-negative and Gram-positive bacteria unlike the parent defensin which was found to be active only against Gram-positive bacteria (Lee et al., 1998). Recently, it was shown for human α-defensins that disulfide bridges could have a role in protecting the peptide from enzymatic digestion *in vivo* (Tanabe et al., 2007). This could as well be the role of disulfide linkages in arthropod defensins. It was observed that the addition of disulfide linkages and the central helical segment observed in the parent sequences to the linear peptides restores to some extent the antibacterial specificity of the parent peptides.

However, there is a concomitant reduction in the potency and increase in the salt sensitivity of these disulfide linked peptides although the mechanism of antibacterial activity and hemolytic ability remains the same. Removal of disulfide linkages and designing of peptides from the vast number of sequences could be used to generate peptides with therapeutic applications.