Cyclic octapeptides containing thiazole. Effect of stereochemistry and degree of flexibility on calcium binding properties †

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Solution conformation and calcium binding properties have been investigated for the two cyclic octapeptides cyclo-(-D-Thr-D-Val(Thz)-Ile-)$_2$ (**4**) and cyclo(-Thr-Gly(Thz)-Ile-Ser-Gly(Thz)-Ile-) (**5**) and the results are compared to those for the cyclic octapeptides previously studied; ascidiacyclamide (**1**), patellamide D (**2**), cyclo(-Thr-D-Val(Thz)-Ile-)$_2$ (**3**), and cyclo(-Thr-D-Val-αAbu-Ile-)$_2$ (**6**). Both **4** and **5** contain two heterocyclic thiazole ring constraints but the latter has a larger degree of flexibility as a consequence of the glycine residues within the cyclic framework. The solution conformation of **4** and **5** was determined from ^{1}H NMR spectra and found to be a "twisted figure of eight" similar to that for **2**. Complexation studies using ^{1}H NMR and CD spectroscopy yielded 1 : 1 calcium–peptide binding constants (log$K$) for the two peptides (2.3 (**4**) and 5.7 (**5**)). For **5** the magnitude of the binding constant was verified by a competition titration using CD. The different calcium-binding affinities of **3** (log$K$ = 4.0) and **4** is attributed to the stereochemistry of the threonine residue. The magnitude of the binding constant for **5** compared to **3** and **4** (all peptides containing two thiazole ring constrains) demonstrates that the increase in flexibility of the cyclic peptide has a dramatic effect on the Ca^{2+} binding ability. The affinity for Ca^{2+} thus decreases in the order (**6** ~ **5** > **3** > **2** ~ **1** > **4**). The number of carbonyl donors available on each peptide has only a limited effect on calcium binding. The most important factor is the flexibility, which allows for a conformation of the peptide capable of binding calcium efficiently.

Introduction

Cyclic peptides have been the subject of extensive investigation due to their involvement in metal binding and transport in biological systems. Our interest in this field stems from the isolation of cyclic peptides from the aplousobranch ascidian (Sea Squirt) *Lissoclinum patella*.[1–4] Many of these peptides, known as patellamides, contain unusual constraining amino acid residues in the form of amino(thiazoles) and amino(oxazolines) which have the effect of limiting the conformations of the macrocyclic backbone to only three distinct shapes (Scheme 1). Type I is an intermediate in the conversion from Type II (described as a "saddle") to Type III (described as a "twisted figure of eight").[5] Ascidiacyclamide (**1**, Chart 1) is an example of a Type II conformation and has been crystallised with a number of different solvates.[5–8] Crystallographic and NMR spectroscopic studies revealed the structure of ascidiacyclamide to be a "saddle" with a rectangular cavity of 6.1 Å by 7.5 Å with the amide hydrogen atoms and nitrogen lone pairs of the heterocyclic rings orientated towards the centre of the cavity.[5,8,9] Patellamide D (**2**, Chart 1) has a Type III conformation. The structure appears to be stabilised by four transannular hydrogen bonds, as well as a π-stacking interaction between the two thiazole rings.[10,11] Energy minimisation studies reveal that the conformation of **2** is approximately 10 kcal mol^{-1} lower in energy than that of **1**.[10]

Metal complexes of **1** and **2** have been investigated.[12–14] Quantitative studies have been undertaken to determine the binding constants of the cyclic peptides with metal ions. The interactions of zinc(II) with **1**, as well as a synthetic analogue cyclo(Ile(Oxn)-D-Val(Thz))$_2$, have been monitored by ^{1}H NMR and CD spectroscopy and binding constants calculated to be $K_{(Zn(L-H))} = 10^{7\pm2}$ M^{-1} and $K_{ZnClL} = 10^{7.2\pm0.1}$ M^{-2}.[15] In this study, the importance of investigating the binding order and the rate of the complexation reaction for zinc, and accordingly any other metal with slow water exchange, was established. Freeman *et al.*[16] reported the formation of copper(II) and zinc(II) complexes of other patellamides (B, C and E). Binding constants were obtained using only one technique, CD spectroscopy. An unusual tetra-silver(I) complex with a cyclic hexapeptide, westiellamide was reported by Wipf *et al.*[17] In that study, the complex was characterised by X-ray crystallography, revealing it to be composed of a tetra-silver(I) cluster sandwiched by two neutral peptide molecules. A binding constant ($K = 2.8 \times 10^{13}$ M^{-5}) was determined by NMR spectroscopy.[17]

Calcium is a biologically relevant metal ion, being involved in many signaling pathways in animals and is present in quite high concentrations *in vivo* (~1 mM).[18,19] It was also found that the concentration of calcium in the body of the ascidians could be up to 500 times higher than in the surrounding seawater, suggesting that these animals must have a mechanism for sequestering the metal ion.[12] Additionally, calcium provides a simple system for a metal binding study, as complexation typically takes place at the carbonyl oxygens of the cyclic peptide. As a result, modeling of the complexation behaviour does not require consideration of coordination to deprotonated amide nitrogens, as would be the case for copper and zinc.[13–15,20]

The present work complements our previous study in which the interaction of calcium with cyclic octapeptides, designed to explore the effects of increasing both the conformational flexibility and the number of carbonyl oxygen donors, was

† Electronic supplementary information (ESI) available: NOE and CD titration data for **5**. See http://www.rsc.org/suppdata/p2/b1/b109168a/

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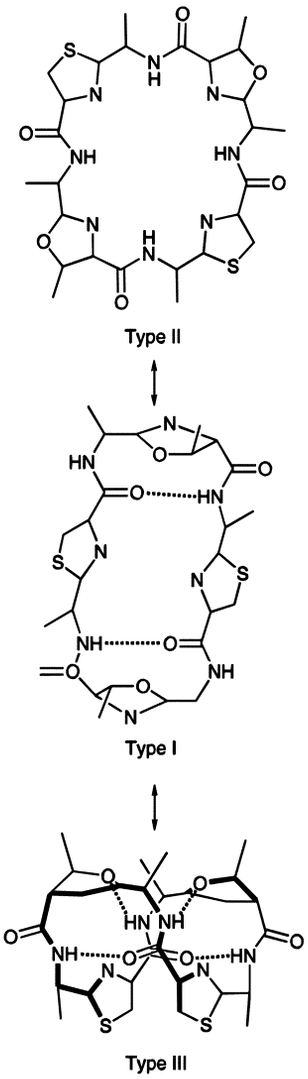


Scheme 1 Conformations of the patellamides (taken from ref. 5).

reported.[21] It was found that the affinity for Ca^{2+} decreases with increasing numbers of 5-membered ring constraints on the macrocycle. However, it was not clear from the range of cyclic peptides investigated whether the increase in stability of the Ca^{2+} complex arises from the increase in flexibility of the peptide or the increase in the number of carbonyl oxygen donors, or a combination of both. In order to explore the effects of subtle changes to the structure of the cyclic octapeptides on complexation with calcium ions, a cyclic octapeptide analogue of **3** was prepared, namely cyclo(-D-Thr-D-Val(Thz)-Ile-)$_2$ (**4**) in which the D-isomer of threonine has been substituted for the L-isomer. Additionally, the interaction of calcium has been explored with the previously reported cyclic peptide cyclo(-Thr-Gly(Thz)-Ile-Ser-Gly(Thz)-Ile-) (**5**), in which glycine amino acids theoretically introduce enhanced flexibility to the peptide backbone.[20]

Experimental

Materials were of analytical grade unless otherwise stated, and were used without further purification. cyclo(-Thr-Gly-(Thz)-Ile-Ser-Gly(Thz)-Ile-) (**5**) was synthesized as described previously.[20] Preparative scale reversed phase (RP) HPLC separations were performed on a Waters Delta-Pak PrepPak C_{18} 40 mm × 100 mm cartridges (100 Å). Linear peptides were purified using gradient mixtures of water (MilliQ, 18.2 MΩ)–0.1% TFA and water 10%–acetonitrile 90%–TFA 0.1%. Cyclic peptides were purified using gradient mixtures of water (MilliQ, 18.2 MΩ) and water 10%–acetonitrile 90%.

Mass spectra were obtained with a triple quadrupole mass spectrometer (PE SCIEX API III) equipped with an Ion-Spray (pneumatically assisted electrospray) atmospheric pressure ionisation source (ISMS). Solutions of compounds were injected by syringe infusion pump at mM–pM concentrations and flow rates of 2–5 mL min^{-1} into the spectrometer. Molecular ions, $\{[M + nH]^{n+}\}/n$, were generated by ion evaporation and focused into the analyser of the spectrometer through a 100 mm sampling orifice. Full scan data were acquired by scanning quadrupole-1 from *m*/*z ca.* 300–1200 Da with a scan step of 0.1 Da and a dwell time of 2 ms.

1D and 2D Nuclear Magnetic Resonance (NMR) spectra were recorded with either a Varian 300 at 300 K, a Bruker AMX400 spectrometer at 301 K or a Bruker Avance DRX500 at 298 K. 2D ^{1}H NMR NOESY experiments were recorded using a mixing time of 500 ms unless otherwise stated. Coupling constants (J) are reported in Hz. ^{1}H and ^{13}C NMR spectra were recorded with either a Bruker AMX400 spectrometer at 301 K or a Bruker DRX500 spectrometer at 298 K. 2D ^{1}H–^{1}H TOCSY[22,23] (TOtal Correlation SpectroscopY), and NOESY (Nuclear Overhauser Enhancement correlation SpectroscopY)[24] spectra were employed to assign signals in the 1D ^{1}H NMR spectrum of each peptide. In the latter experiment, various mixing times ranging from 100 ms to 500 ms were used to obtain proton–proton distance information. Variable temperature experiments were recorded with a Bruker DRX500 spectrometer.

Synthesis of 4 (cyclo(-D-Thr-D-Val(Thz)-Ile-)$_2$)

Abbreviations: Boc = butyloxycarbonyl; BOP = [benzotriazol-1-yl-oxytris(dimethylamino)phosphonium] hexafluorophosphate; DIPEA = diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; PAM = phenylacetamidomethyl resin; TFA = trifluoroacetic acid; Thz = thiazole; Oxn = oxazoline; NOE = Nuclear Overhauser Effect.

Boc-D-Val(Thz)-OH. Boc-D-Val(Thz)-OEt was synthesised according to a modified Hantzsh method.[25] A solution of Boc-D-Val(Thz)-OEt (0.996 g, 3.04 mmol) in ethanol (25 mL) was stirred for 5 min at 0 °C. Lithium hydroxide (0.255 g, 6.07 mmol) was added and the solution was stirred overnight. A solution of citric acid (10% w/v) was added until the pH was 3. The solvent was removed *in vacuo* and the residue was extracted with ethyl acetate (3 × 50 mL) and water (50 mL). The organic extracts were combined, dried over sodium sulfate and filtered. The solvent was removed *in vacuo* to give Boc-D-Val(Thz)-OH as a yellow solid (0.868 g, 98%). ^{1}H NMR ($CDCl_3$, 500 MHz, 298 K): δ_H 8.16 (1H, s, NH), 4.87 (1H, br s, αH), 2.38 (1H, br s, βH), 1.43 (9H, s, BocH), 0.92 (6H, s, CH_3).

(D-Thr-D-Val(Thz)-Ile)$_2$. Boc-Ile-PAM resin (1.02 g, substitutional value (S.V.) = 0.4 mmol g^{-1}) was deprotected with TFA (5 mL, 2 × 1 min), washed with DMF and then a solution of Boc-D-Val(Thz)-OH (0.296 g), HBTU (0.5 M in DMF, 2 mL) and DIPEA (256 μL) was added. The reaction mixture was shaken for 10 min and monitored by the negative Ninhydrin test (>99.6% coupling). The resin was washed with DMF. The synthesis was then completed by the sequential coupling of Boc-D-Thr-OH (4 equiv.), Boc-Ile-OH (4 equiv.), Boc-D-Val-(Thz)-OH (2 equiv.), Boc-D-Thr-OH (4 equiv.) using the same procedure. The peptide was cleaved from the resin with HF, washed with ether and extracted with water–acetonitrile (50/50). The extract was then lyophilised to give a crude powder (303 mg, 65.6% from Boc-Ile-PAM resin). The crude powder was purified by HPLC (isocratic; 70% water–0.1% TFA and 30% (acetonitrile 90%–water 10%–TFA 0.1%) over 90 min) to

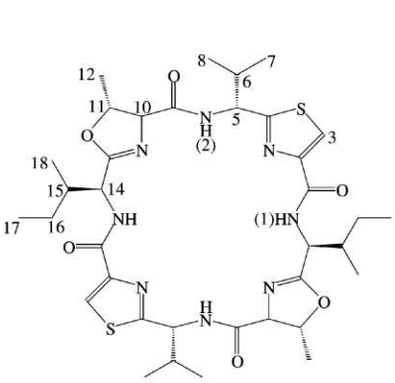
give (D-Thr-D-Val(Thz)-Ile)$_2$ as a white powder (187 mg, 40.6% from Boc-Ile-PAM resin). ISMS Calc.: *m*/*z* 811, Found: 811 Da $[M + H]^+$. ^{1}H NMR (d_3-methanol, 500 MHz, 298 K): δ_H 9.10 (1H, d, *J* 8.3, V7-NH), 8.70 (1H, d, *J* 8.4, V3-NH), 8.28 (1H, d, *J* 8.5, I1-NH), 8.24 (1H, d, *J* 7.8, T4-NH), 8.22 (1H, d, *J* 8.5, I5-NH), 8.02 (1H, s, ThzH), 8.01 (1H, s, ThzH), 4.93 (1H, m, V7-αH), 4.85 (1H, m, V3-αH), 4.31 (2H, m, I1-αH, T4-αH), 4.27 (1H, m, I5-αH), 4.13 (1H, m, T8-βH), 4.10 (1H, m, T4-βH), 3.84 (1H, d, *J* 5.6, T8-αH), 2.22 (2H, m, V3-βH, V7-βH), 1.92 (1H, m, I1-βH), 1.78 (1H, m, I5-βH), 1.41 (1H, m, I1-γH), 1.31 (1H, m, I5-βH), 1.25 (3H, d, *J* 6.5, T8-γCH$_3$), 1.17 (1H, m, I5-γH), 1.13 (3H, d, *J* 6.4, T4-γCH$_3$), 1.05 (1H, m, I5-γH), 0.88 (9H, m, I1-γCH$_3$, V3-γCH$_3$, V7-γCH$_3$), 0.80 (9H, m, I1-δCH$_3$, V3-γCH$_3$, V7-γCH$_3$), 0.68 (6H, m, I5-γCH$_3$, I5-δCH$_3$).

cyclo(-D-Thr-D-Val(Thz)-Ile-)$_2$ (4). A solution of (D-Thr-D-Val(Thz)-Ile)$_2$ (150 mg, 185 μmol), HBTU (263 mg, 690 μmol) and DIPEA (201 μL, 1.2 mmol) in DMF (200 mL) was stirred at room temperature for one hour. The reaction was quenched with HCl, the solvent was removed *in vacuo* and the residue was purified using HPLC (gradient: 100% water–0.1% TFA to 100% (acetonitrile 90%–water 10%–TFA 0.1%) over 75 min). The appropriate fractions were combined and lyophilised to give cyclo(-D-Thr-D-Val(Thz)-Ile-)$_2$ as a white powder (25.2 mg, 17.1%). ISMS Calc.: *m*/*z* 793.4, Found: 792.9 Da $[M + H]^+$. (Calculated for $C_{36}H_{60}N_8S_2O_{10}$: C 52.20, H 7.30, N 13.53. Found: C 52.04, H 7.13, N 13.61%). ^{1}H NMR for **4** (d_3-acetonitrile, 500 MHz, 300 K): δ_H 9.17 (2H, d, *J* 6.4, NH(1)), 7.56 (2H, d, *J* 9.6, NH(2)), 7.41 (2H, s, H3), 7.37 (2H, d, *J* 8.4, NH(3)), 5.22 (2H, dd, *J* 9.5, 2.9, H5), 4.40 (2H, m, H11), 4.15 (2H, m, H10), 4.10 (2H, dd, *J* 11.2, 6.6, H15), 3.30 (2H, d, *J* 4.8, H13), 2.49 (2H, m, H16), 2.35 (2H, m, H6), 1.68 (2H, m, H17), 1.29 (6H, d, *J* 6.4, H12), 1.27 (2H, m, H17), 1.08 (6H, d, *J* 4.8, H7/8), 1.06 (6H, d, *J* 4.6, H7/8), 0.96 (6H, d, *J* 6.8, H19), 0.88 (6H, t, *J* 7.4, H18). ^{1}H NMR for 4 (d_3- methanol, 500 MHz, 298 K): δ_H 9.28 (2H, d, *J* 6.0, NH(1)), 8.47 (2H, d, *J* 7.5, NH(3)), 7.52 (2H, d, *J* 9.5, NH(2)), 7.48 (2H, s, H3), 5.29 (2H, d, *J* 5.5, H13), 4.43 (2H, m, H11), 4.31 (2H, dd, *J* 7.5, 2.0, H10), 4.12 (2H, dd, *J* 7.0, 11.2, H15), 2.53 (2H, m, H16), 2.38 (2H, m, H6), 1.75 (2H, m, H17), 1.33 (6H, d, *J* 6.5, H12), 1.28 (2H, m, H17), 1.14 (6H, d, *J* 6.5, H7/8), 1.13 (6H, d, *J* 6.0, H7/8), 0.98 (6H, d, *J* 7.0, H19), 0.88 (6H, t, *J* 7.5, H18).

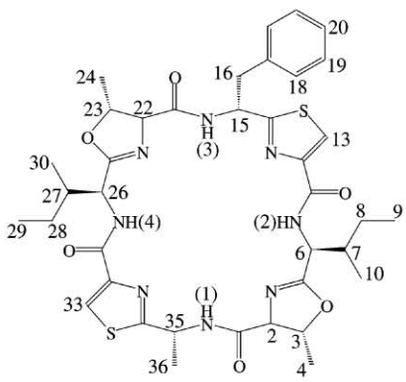
cyclo(-Thr-Gly(Thz)-Ile-Ser-Gly(Thz)-Ile-) (5). ^{1}H NMR (d_3-acetonitrile, 500 MHz, 300 K): δ_H 9.31 (1H, d, *J* 5.0, NH(1)), 8.90 (1H, d, *J* 6.0, NH(4)), 7.92 (1H, d, *J* 9.0, NH(5)), 7.92 (1H, d, *J* 7.0, NH(2)), 7.92 (1H, d, *J* 7.4, NH(6)), 7.80 (1H, d, *J* 6.5, NH(3)), 7.49 (2H, s, H3, H18), 5.11 (2H, br m, H5, H20), 4.52 (1H, m, H23), 4.24 (2H, br m, H5, H20), 4.01 (1H, dd, *J* 6.5, 10.6, H27), 3.92 (3H, br m, H7, H11, H22), 3.68 (1H, m, H8), 3.26 (1H, d, *J* 5.0, H9), 2.28 (2H, m, H12, H28), 1.72 (1H, m, H29), 1.63 (1H, m, H13), 1.17 (1H, m, H29), 1.17 (3H, d, *J* 6.5, H24), 1.12 (1H, m, H13), 1.02 (3H, d, *J* 6.7, H31), 0.95 (3H, d, *J* 6.7, H15), 0.89 (3H, t, *J* 7.4, H30), 0.85 (3H, d, *J* 7.4, H14). ^{1}H NMR (d_3-methanol, 400 MHz, 301 K): δ_H 9.60 (1H, d, *J* 6.8, NH(6)), 9.50 (1H, d, *J* 6.5, NH(1)), 9.15 (1H, d, *J* 6.8, NH(4)), 8.92 (1H, d, *J* 7.0, NH(3)), 8.25 (1H, dd, *J* 9.5, 1.9, NH(5)), 8.15 (1H, dd, *J* 8.3, 1.9, NH(2)), 5.20 (1H, dd, *J* 9.5, 1.9, H20), 5.14 (1H, dd, *J* 9.5, 1.9, H20), 4.64 (1H, m, H23), 4.47 (1H, dt, *J* 5.3, 5.4, H7), 4.31 (1H, dd, *J* 8.3, 1.9, H5), 4.30 (1H, dd, *J* 8.3, 1.9, H5), 4.15 (1H, dd, *J* 3.0, 3.1, H11), 3.98 (1H, m, H8), 3.97 (1H, m, H22), 3.89 (1H, m, H8), 2.50 (1H, m, H28), 2.40 (1H, m, H12), 1.80 (1H, m, H29), 1.70 (1H, m, H13), 1.26 (3H, d, *J* 6.6, H24), 1.20 (1H, m, H29), 1.17 (1H, m, H13), 1.07 (3H, d, *J* 6.8, H31), 0.99 (3H, d, *J* 6.8, H15), 0.91 (3H, t, *J* 7.4, H30), 0.90 (3H, t, *J* 7.4, H14).

Binding constant determinations

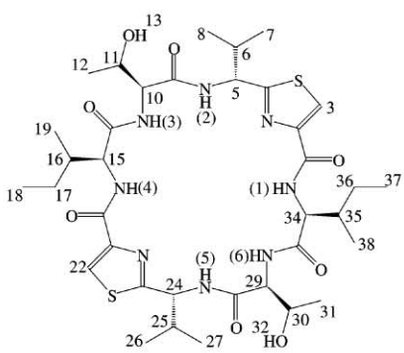
For titrations monitored by ^{1}H NMR spectroscopy, a solution of metal perchlorate in d_3-acetonitrile (*ca.* 0.1 M) was added to



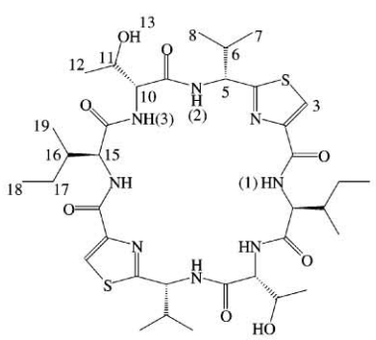
1 ascidiacyclamide



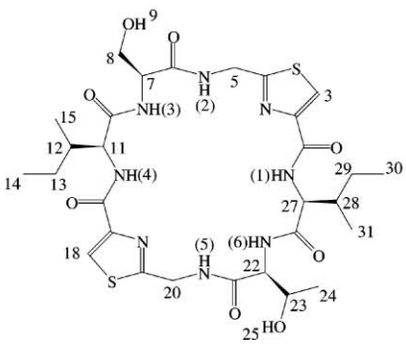
2 patellamide D



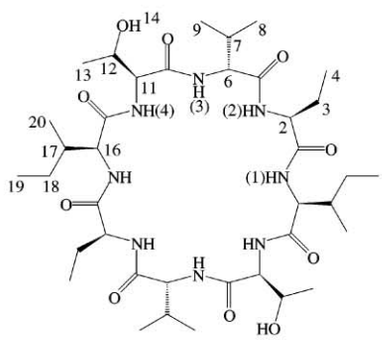
3 c(Thr-D-Val(Thz)-Ile)$_2$



4 c(D-Thr-D-Val(Thz)-Ile)$_2$



5 c(Thr-Gly(Thz)-Ile-Ser-Gly(Thz)-Ile)$_2$



6 c(Thr-D-Val-αAbu-Ile)$_2$

Chart 1 Structures of cyclic peptides.

a solution of peptide in d_3-acetonitrile. The volume of peptide solution ranged from 500–1000 μL, whilst the concentration was 0.4 mM for **4** and 1.0 mM for **5**. The titration of **4** was performed by the addition of 37 equivalents of Ca^{2+} and the titration of **5** was performed by the addition of 1.2 equivalents. The peptide solution was diluted by 15% for **4** upon addition of the calcium solution. Data were analysed by following the change in the chemical shifts of proton resonances as a function of metal concentration. The effect of dilution and of non coordinating anions has been investigated previously.[21]

Circular Dichroism (CD) spectra were recorded with a JASCO J-710 spectrophotometer. The measurements were carried out in the range 200–300 nm using a 0.1 cm path length cell. The cell was maintained at 298 ± 1 K by a Neslab RTE-111 temperature controller. In the CD titration, solutions of cyclic peptides in acetonitrile (0.2–0.3 mM) were placed in the cell (initially containing 200–250 μL of peptide solution) and titrated with a solution of calcium perchlorate in 95% acetonitrile–water. Titrations were continued until 24 equivalents of calcium had been added in the titration of **4** and until 2 equivalents of calcium had been added in the titration of **5**. In the CD competition titration an acetonitrile solution of **2** (0.124 mM) and calcium perchlorate (0.124 mM) was titrated with an acetonitrile solution of **5** (0.276 mM) until 1.1 equivalents had been added. At least 10 spectra were used to calculate stability constants. Data were analysed using Specfit, a global least squares fitting program.[26]

Results and discussion

The solution conformation and the calcium ion complexation ability were studied for the two analogues of the patellamides, peptides **4** and **5**. Peptide **4** contains threonine residues in place of the oxazoline rings, as these are believed to be the precursor amino acids used in nature to form the oxazoline groups.[27] Peptide **4** is an analogue of **3** in which the D-isomer of threonine has been substituted for the L-isomer. Peptide **5** which is prepared with glycine amino acids to prevent isomerisation of the peptide during synthesis, has been reported previously.[20] Incorporation of glycine residues in cyclic peptides is known to increase backbone flexibility.[20] The yields of the cyclisation reactions were found to vary, with those for **5** being very good (>35%), whilst that for **4** was lower (~17%).

Solution conformation studies

There is evidence that some cyclic octapeptides can adopt multiple conformations in solution, depending on the solvent and the constraints present in the molecules.[28,29] As part of the investigation of the calcium binding properties, the solution conformations of the cyclic peptides were investigated. Studies were conducted with d_3-acetonitrile solutions having a peptide concentration less than 0.4 mM.

The 1D ^{1}H NMR experiment of **4** in d_3-acetonitrile suggests a C_2-symmetric molecule with the isoleucine amide proton resonance appearing at 9.12 ppm, at lower field than observed for **3** (8.12 and 8.09 ppm) and **2** (7.54 and 7.51 ppm). Peptide **3**, which contains an L-threonine, exhibits an NMR spectrum indicative of a C_1-symmetric molecule. The large downfield shift of the isoleucine signal for **4** suggests that this proton may be involved in a hydrogen bond,[30] and suggests that the solution conformations of **3** and **4** are different.

A variable temperature study undertaken to probe the presence of hydrogen bonds in **4** showed that two of the three distinguishable amide protons, assigned to isoleucine and valine, had temperature coefficients of 1.6 and 1.1 ppb K^{-1}, respectively, suggesting that they were either hydrogen bonded or solvent shielded.[31] The threonine amide protons exhibited a temperature coefficient only slightly higher (2.4 ppb K^{-1}) which, given the dilute solution, was not enough evidence to state unequivocally whether it was exposed to solvent. A titration involving the addition of d_6-DMSO to a solution of **4** in d_3-acetonitrile resulted in a ~0.7 ppm change in the chemical shifts of the threonine amide protons when up to 9% v/v of d_6-DMSO was added to the solution. This suggested that the threonine amide proton is exposed to the solvent. The other amide proton signals showed very little (< 0.05 ppm) shift over this range, consistent with their involvement in hydrogen bonds.

A variable temperature experiment involving **4** in d_3-methanol was conducted to elucidate solvent exposed amide protons. The results demonstrate that the threonine amide protons of **4** are exposed to the solvent, evident from the large ($\Delta\delta/T$ = 7.7 ppb K^{-1}) temperature coefficient. The remaining amide protons from isoleucine and valine show quite low temperature coefficients (~2–3 ppb K^{-1}), indicative of solvent shielding and/or involvement in intramolecular hydrogen bonds. This behaviour is similar to that in d_3-acetonitrile, as well as to its analogue, **3** and **5**. In all of these cases, it is the threonine (and serine for **5**) residues that are exposed to solvent.

A 2D NOESY experiment of peptide **4** in methanol demonstrated that the thiazole protons are able to strongly couple to the valine methyl protons. Similar coupling was not observed in the NOESY spectrum of **3** in methanol or acetonitrile. The crystal structures of **1**[5–8] reported distances from the thiazole protons to the neighbouring valine methyl protons of at least 4.4 Å, comparable to the maximum distance between two protons (5 Å) that would give rise to a cross peak in the NOESY spectrum.[24] This distance is, however, towards the limits of detection and, given the non-ideality of the NOESY experiment conditions, *i.e.* recording at room temperature, it was anticipated that such coupling would either be very weak or not observed. Hence, the observed coupling is most likely due to close approach of the thiazole protons and valine methyl protons from opposite sides of the macrocyclic ring, suggesting that **4** adopts a conformation similar to the Type III conformation (Scheme 1) in both d_3-acetonitrile and d_3-methanol.

The ^{1}H NMR spectrum of **5** in methanol solution has been reported and from the results of variable temperature studies it was inferred that the amide protons of the serine and threonine residues (temperature coefficients of 8.5 and 12.0 ppb K^{-1}) were exposed to the solvent.[20] The two isoleucine amide protons have intermediate (~3–6 ppb K^{-1}) coefficients, indicative of weak hydrogen bonding and/or solvent exposure. The low ($\Delta\delta/T \leq 2$ ppb K^{-1}) temperature coefficients for the two glycine amide protons indicate that these are involved in hydrogen bonds within the molecule. Potentially, these hydrogen bonds can form with the carbonyl oxygens of either the serine, threonine, isoleucine and thiazole residues. Formation of a H-bond with either the serine or threonine residues would not be expected, as these atoms are adjacent to the glycine amide protons.

Hydrogen bonding to the isoleucine carbonyls would lead to a γ-turn, whereas a bond to the thiazole carbonyls would create a β-turn. From the hydrogen bonding patterns observed in other cyclic peptides,[10,32–36] it appears that the 10-membered ring would be most likely to form, leading to a β-turn. In order to create a picture of the structure of **5** in solution, and to decide between the two possibilities outlined above, a 2D NOESY experiment was conducted. From the NOESY experiment, a number of inter-residue couplings were observed. A few of the inter-residue couplings are particularly diagnostic in terms of deducing the conformation of the peptide in methanol solution. The first of these diagnostic cross peaks occurs between the thiazole protons and the α-protons of the transannular glycine residues.

Examination of crystal structures of **1**,[5–8] patellamide A[37] and **2**,[10] as well as the solution structure of **3**,[27] indicated that the thiazole proton and the neighbouring glycine α-protons would approach no closer than ~5.5 Å. The distance of 5.5 Å was too large to allow for coupling between these protons to be observed in the NOESY experiment. The large distance

Table 1 Stability constants for calcium-cyclic octapeptide complexes in acetonitrile

Peptide	Method	Log$K_{1:1}$	Log$K_{1:2}$	Log$K_{2:1}$	Reference
1	NMR [a]	2.9 ± 0.4			21
1	NMR [a,b]	2.6 ± 0.3			21
2	NMR [a], CD [c]	2.9 ± 0.4			21
3	CD [c]	4.0 ± 0.4	4.5 ± 0.4		21
4	NMR [a], CD [c]	2.3 ± 0.3		3.5 ± 0.5 [e]	This work
5	CD [c]	5.7 ± 0.5	5.2 ± 0.6 [e]	3.6 ± 1.1 [e]	This work
6	CD [c]	5.5 ± 0.3			21
6	CD [c,d]	5.5 ± 0.4			21

[a] d_3-Acetonitrile at 300 K. [b] Titration performed in the presence of 0.1 M sodium perchlorate. [c] 298 K. [d] Titration performed in the presence of 0.1 M tetraethylammonium perchlorate. [e] CD measurement only.

between the adjacent thiazole protons and glycine α-protons suggested that the coupling observed was more likely due to close approach of transannular glycine and thiazole groups. Of further interest was the observation of moderate (~2.7–3.5 Å) coupling between the two isoleucine amide protons. If the structure of **1** is considered, then the transannular amide protons are found to approach no closer than 3.9 Å. Since **1** is much more constrained than **5**, due to the presence of oxazoline as well as thiazole residues, it would be expected that replacement of these oxazoline rings with threonine and serine amino acid residues would lead to a flattening of the saddle shape and a resultant increase in the distance between these two protons. Hence, if **5** assumed a Type II conformation (Scheme 1) in solution, it would adopt an even "flatter" saddle and no coupling would be expected between these residues. If, however, the peptide adopted the conformation of **2** (Type III), then close approach of these protons would be expected. From the crystal structure of **2**, the distance between the isoleucine amide protons was found to be 3.1 Å, which would be expected to give rise to moderate coupling in the NOESY experiment, as was observed for **5**. As a result, **5** is suggested to adopt the Type III conformation.

The conformation of **5** in acetonitrile was investigated. A variable temperature NMR study suggested that the threonine amide proton was exposed to solvent ($\Delta\delta/T$ = 6.4 ppb K^{-1}). The temperature coefficients were inconclusive as to whether one of the isoleucine amide protons and the serine amide proton were H-bonding or simply exposed to solvent. The involvement of the two glycine amide protons in hydrogen bonds appears to be confirmed by their low temperature coefficients ($\Delta\delta/T$ = 1.9 and 2.4 ppb K^{-1}, respectively). The shifts in the positions of the amide protons were monitored employing a titration involving addition of d_6-DMSO to a solution of **5** in d_3-acetonitrile. The results suggest that the amide protons from the threonine and serine residues are exposed to solvent, evident from the large change (~0.8 ppm) in their chemical shifts. No significant change is evident for the remaining amide protons, suggesting that they are either hydrogen bonded or shielded from the solvent. The exposure to solvent of the threonine and serine residues is similar to that found for **5** in methanol. The observation that the conformations of other peptides, such as **1**, **2**, and **3**, are invariant on changing from methanol to acetonitrile, suggests that **5** might also maintain the Type III conformation (Scheme 1) in acetonitrile.

Binding constants

Binding constants were determined either by CD or NMR spectroscopy. However, in all cases NMR was used to obtain stoichiometry and extent of reaction upon Ca^{2+} addition. Acetonitrile was used as the solvent to allow comparisons with previous determinations of calcium binding constants.[18,38–40] Data are collected in Table 1.

Due to the low solubility of **4** in acetonitrile, titrations were performed with peptide concentrations of less than 0.4 mM. Up to thirty seven equivalents of calcium were added to a solution of the peptide in d_3-acetonitrile. The binding constant for the 1 : 1 complex was calculated from the change in the chemical shift of a number of protons. Fitting a non-linear curve-fitting algorithm to the data,[41] allowed a binding constant of log$K_{1:1}$ = 2.3 ± 0.3 to be determined for the 1 : 1 complex when assuming a simple complexation model (eqn. (1)).

$$Ca^{2+} + L \rightleftharpoons [CaL]^{2+} \quad (1)$$

The NMR experiment presented no evidence for species other than the 1 : 1 complex.

The complexation reaction was also probed employing a CD titration. Up to forty-three equivalents of calcium were added to a solution of **4** in acetonitrile and the CD spectrum was recorded after each addition (Fig. 1), an isosbestic point

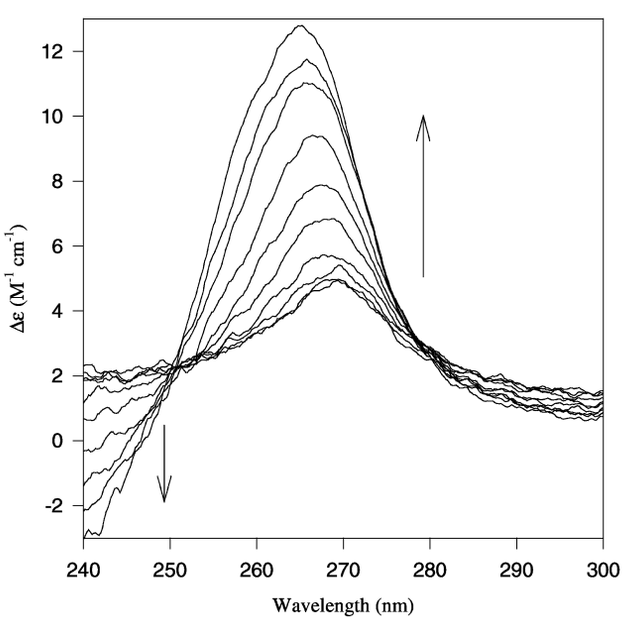


Fig. 1 CD titration of an acetonitrile solution containing **4** (0.276 mM) with added (from bottom to top) 0, 1.01, 3.04, 5.07, 8.83, 12.58, 16.34, 20.09, 22.12, 24.15 equivalents of calcium perchlorate (0.0166 M).

was observed at 251 nm. Continued addition of Ca^{2+} led to a further increase in the CD absorption, indicative of formation of higher complexes. The nature of the higher complexes, and even their reality, is unclear. Whilst a more complex model now including equations (1) and (2) was fitted to the data (log$K_{1:1}$ = 2.0 ± 0.5, log$K_{2:1}$ = 3.5 ± 0.5) (Fig. 2) the presence of species such as $[CaL_2]^{2+}$, suggested at low Ca^{2+} concentration, could not be accommodated in any model. The NMR and CD experiments both suggest the formation of a 1 : 1 complex (log$K$ ~ 2); the presence of other complexes ($[CaL_2]^{2+}$ and $[Ca_2L]^{4+}$ has not been definitively established.

$$Ca^{2+} + [CaL]^{2+} \rightleftharpoons [Ca_2L]^{4+} \quad (2)$$

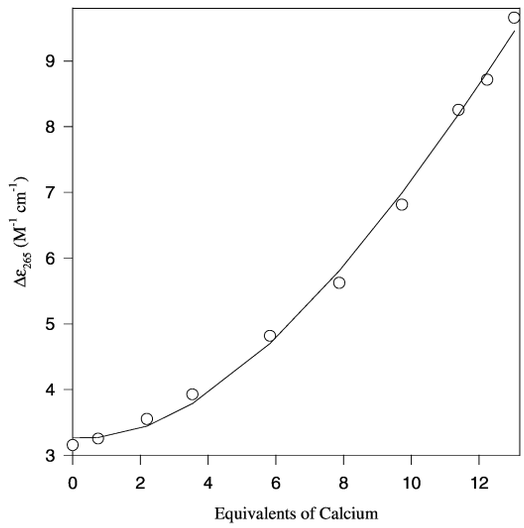


Fig. 2 Calculated (−) and experimental (○) at 265 nm for the titration of **4** and $Ca(ClO_4)_2$ in acetonitrile.

The behaviour of **4** towards calcium is in marked contrast to that of **3** which differs only in the stereochemistry of the threonine residue.[27] The Ca^{2+} binding modes to this ligand follows a complexation model including equations (1) and (3) with $\log K_{1:1} = 4.0 \pm 0.4$ and $\log K_{1:2} = 4.5 \pm 0.4$.[21] The selectivity of two orders of magnitude in the 1 : 1 complex induced by the simple difference in stereochemistry is intriguing. As the same functional groups and donor atoms are present in both peptides, the only reason for their different calcium binding behaviour stems from the arrangement of their donor atoms, *i.e.* their conformation. **3** adopts a chair conformation in solution,[27] whilst the Type III conformation is more likely for **4**. It appears that the reason for the difference in calcium binding is most likely due to the accessibility of the carbonyl oxygens to the incoming calcium ion. Potentially, the carbonyl oxygens adjacent to the thiazole ring are tied up in hydrogen bonds in **4** or are sufficiently shielded from solvent by the side chains to hinder their binding to calcium, whereas in **3** these groups are available to interact with the metal ion.

$$2L + Ca^{2+} \rightleftharpoons [CaL_2]^{2+} \qquad (3)$$

A titration, monitored by ^1H NMR, indicated that addition of calcium perchlorate to a solution of **5** in d_3-acetonitrile resulted in the appearance of a set of new signals for all of the protons in the peptide (Fig. 3). These signals continued to

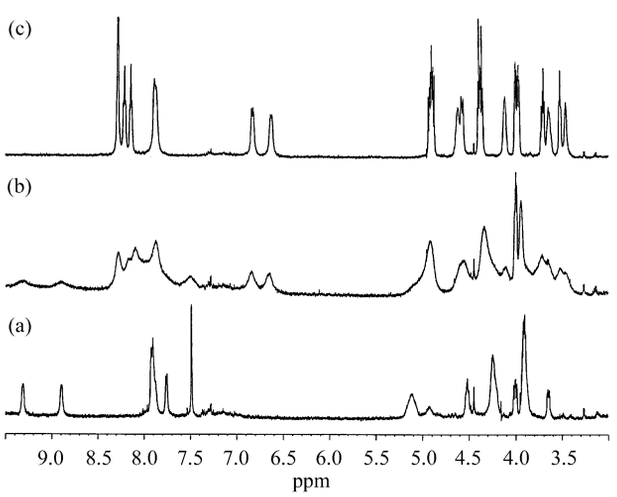


Fig. 3 500 MHz 1D ^1H NMR spectra of **5** (d_3-acetonitrile) with added calcium perchlorate (a) 0, (b) 0.5 and (c) 1.0 equivalents.

increase in intensity with a concomitant decrease in the relative intensity of the original ligand peaks. After addition of one equivalent of calcium, no peaks from the free ligand remained. An upfield shift was observed for the isoleucine amide protons (shifted upfield by 1.43 and 1.02 ppm, respectively) and for the threonine and serine amide proton resonances (1.29 ppm and 0.97 ppm, respectively) upon binding with Ca^{2+}, suggesting that the conformation of the peptide has altered upon complexation. The differences in the free and complexed ligand amide coupling constants for the other residues were 2.6, 2.6, 1.6 and 0.8 Hz for amide protons NH(1), NH(3), NH(4) and NH(6), respectively. The largest change (>4 Hz) in the amide coupling constants occurred for the glycine amide protons, suggesting a change in the torsion angle (HNCαH) of greater than 20°. These changes suggest that the conformation of **5** has undergone a change upon complexation to calcium. The 1 : 1 binding constant for **5** and calcium could not be determined from the NMR data, an apparent consequence of a high binding constant ($\log K > 4$).

The CD spectrum of **5** in acetonitrile consists of a positive CD absorption at 270 nm ($\Delta\varepsilon$ 1.69 M^{-1} cm^{-1}). Addition of calcium led to an increase in the CD signal until a plateau is reached with a maximum wavelength of 266 nm with isosbestic points at approximately 271 nm and 284 nm. Analysis of the titration data[26] suggested a complex model including eqns. (1), (2) and (3) and yielded binding constants of $\log K_{1:1} = 5.7 \pm 0.6$, $\log K_{1:2} = 5.2 \pm 0.6$ and $\log K_{2:1} = 3.6 \pm 1.1$ (Fig. 4).

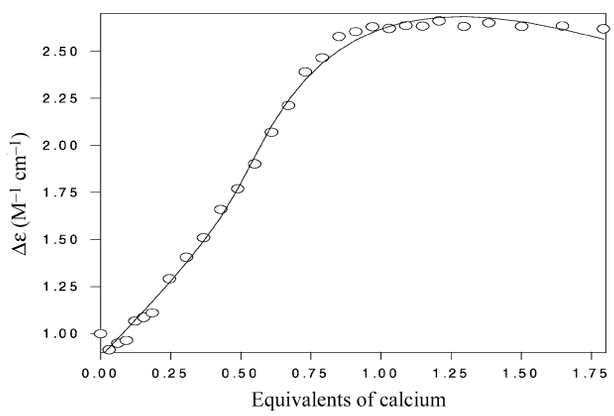


Fig. 4 Calculated (—) and experimental (○) for the CD titration of **5** (0.2763 mM) and calcium perchlorate (0.0166 M) in acetonitrile.

A competition titration was undertaken to confirm the magnitude of the 1 : 1 binding constant.[42] The titration involved the addition of **5** to a solution of calcium perchlorate and **2** (eqn. (4)) in acetonitrile (Fig. 5).

$$CaL(\mathbf{2}) + L(\mathbf{5}) \rightleftharpoons CaL(\mathbf{5}) + L(\mathbf{2}) \qquad (4)$$

By including a ligand with a binding constant for calcium(II) that differs by less than three orders of magnitude from the ligand under investigation, the measured binding constant is reduced to this difference. Hence, if this observed binding constant (the overall binding constant of both ligands for calcium) is determined, the actual binding constant of the ligand under investigation can be determined. **2** was chosen as the competing ligand, as its binding constant with calcium in acetonitrile ($\log K_{1:1} = 2.9$) is suitable. The CD spectrum of a solution of **2** and one equivalent of calcium perchlorate in acetonitrile was recorded after successive additions of **5** in acetonitrile. The resulting spectra contain two isosbestic points, at approximately 252 nm and 279 nm. Modeling of these spectra using Specfit[26] provided a satisfactory fit when a 1 : 1 complex of **5** and calcium was considered and the binding constant for **2** and calcium was fixed at $\log K_{1:1} = 2.9$. The magnitude of the binding constant for [Ca(**5**)] from the Specfit model was found to be $\log K_{1:1} =$

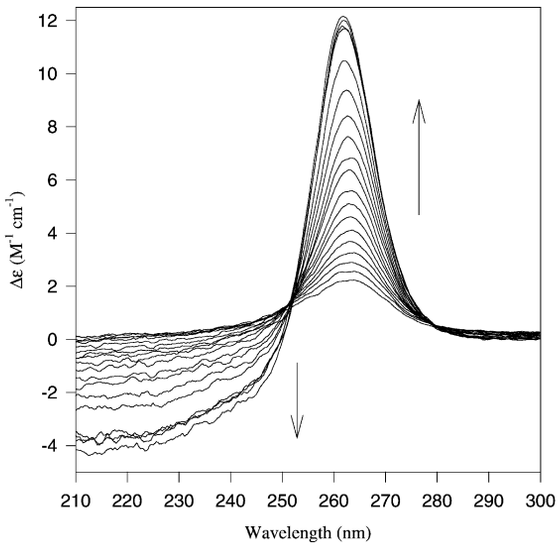


Fig. 5 CD competition titration of an acetonitrile solution containing patellamide D (**2**) (0.1235 mM) and calcium perchlorate (0.1236 mM) with (as indicated by arrows) 0, 0.069, 0.139, 0.208, 0.277, 0.347, 0.416, 0.485, 0.554, 0.624, 0.693, 0.762, 0.832, 0.901, 0.970, 1.040, 1.054, 1.067, 1.081 equivalents of **5** (0.276 mM).

5.7 ± 0.5, a value that agreed with that obtained from the direct titration of calcium and **5** in acetonitrile. However, the 1 : 2 and 2 : 1 metal complexes could not be verified by the NMR or CD competition experiments.

Conclusion

The results of the present study, and our previous work,[21] are summarized in Table 1. Previously we posed the question as to whether the increase in stability of the Ca^{2+} complexes for **1**, **2**, **3** and **6** (Chart 1) arose from the increase in flexibility of the peptide or the increase in the number of carbonyl oxygen donors, or a combination of both.[21] With peptides **4** and **5** described in the present work we have been able to investigate the effect of substitution of L-threonine for D-threonine within an otherwise identical cyclic octapeptide. The difference in magnitude of the observed binding constant is ascribed to the accessibility of the carbonyl oxygens to the incoming calcium ion, due to the different conformations of the peptides.

With peptide **5** we have been able to investigate the effect of increasing the flexibility of the peptide without increasing the number of carbonyl donors. The number of carbonyl donors available on each peptide has only a limited effect on the calcium binding. Thus the number of carbonyl groups available for coordination with the Ca^{2+} ion varied from four to eight, with peptides **1** and **2** having four donors, **3**, **4** and **5** with six donors and **6** with eight. Peptides **5** and **6** which are both flexible exhibit binding constants of the same order ($\log_{10}K$ ~ 5.5) but differ in the number of carbonyls (six and eight, respectively). On the other hand all peptides with six carbonyls (**3**, **4** and **5**) exhibit a range of binding constants ($\log_{10}K$ 2 to 5.7). Within this set (**3**, **4** and **5**), the most flexible peptide has the largest binding constant. Peptides **3** and **4** are both presumably equally rigid and both have six carbonyls but the difference in their capacity to bind Ca^{2+} is most likely based on the different initial conformations of the peptide. It is clear that the most important factor is the peptide flexibility, which allows for a conformation of the peptide capable of binding calcium efficiently.

The magnitude of the cyclic octapeptide–calcium perchlorate binding constants for 1 : 1 complexes in acetonitrile was in the order **6** ~ **5** > **3** > **2** ~ **1** > **4**. This rank order is in agreement with the previous suggestion that the binding of calcium is facilitated by reducing the conformational rigidity of the peptides.[21]

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