Conformations of cyclic octapeptides and the influence of heterocyclic ring constraints upon calcium binding †

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A comparison is made between the structures and calcium binding properties of four cyclic octapeptides that differ in the number of heterocyclic thiazole and oxazoline ring constraints. The conformations of the naturally occurring cyclic octapeptides ascidiacyclamide **1** and patellamide D **2**, which each contain two oxazoline and two thiazole rings, are compared by ^{1}H NMR spectroscopy with the analogues cyclo(Thr-D-Val(Thz)-Ile)$_2$ **3** with just two thiazoles, and cyclo(Thr-D-Val-αAbu-Ile)$_2$ **4**, with no 5-membered rings. The conformations observed in the solid state for ascidiacyclamide ("saddle") and patellamide D ("twisted figure of eight") were retained in solution, whilst peptide **3** was found to have a "chair" shape and peptide **4** displayed a range of conformations. The solid state structure of **4** revealed that the peptide takes a relatively planar conformation with a number of transannular hydrogen bonds, which are apparently retained in solution. Complexation studies utilising ^{1}H NMR and CD spectroscopy yielded 1 : 1 calcium–peptide binding constants (log K) for the four peptides (2.9 (**1**), 2.8 (**2**), 4.0 (**3**) and 5.5 (**4**)) as well as a 1 : 2 metal–peptide binding constant for **3** (log K = 4.5). The affinity for Ca^{2+} thus decreases with increasing number of 5-membered ring constraints in the macrocycle (**4** > **3** > **2** ≈ **1**).

Introduction

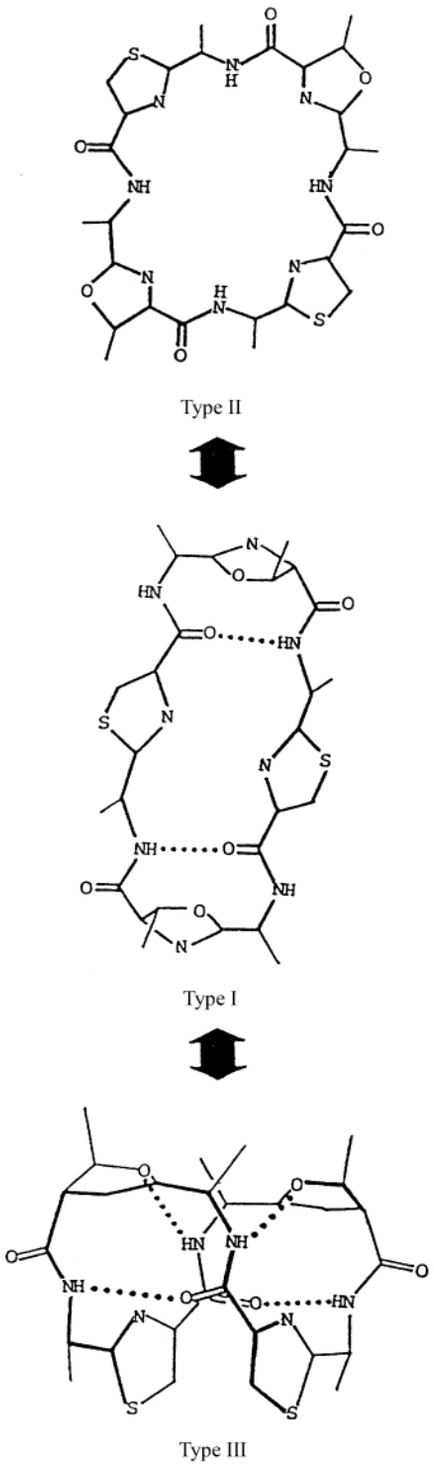
Cyclic peptides have been the subject of extensive investigation due to their involvement in metal binding and transport in biological systems. Examples of naturally occurring cyclic peptides known to bind metal ions include antamanide[1] and valinomycin.[2] Antamanide, a cyclic decapeptide, was isolated from the fungus *Amanita phalloides* and was found to prevent the uptake of the fungal toxin phalloidin. The action of antamanide is dependent upon its specificity for sodium ions in the body. Valinomycin, a cyclic nonapeptide, was found to have antibiotic properties resulting from its ability to bind and transport potassium ions across cell membranes. Analogues of these peptides have been synthesised and their metal complexes investigated.[3–7] The influences of conformationally constraining groups incorporated in the macrocyclic ring have also been investigated.[3,4,8–12] Typically, these constraints have taken the form of proline and/or *N*,*N*′-ethylene bridges.

Our interest in this field stems from the isolation of cyclic peptides from the ascidian (sea squirt) *Lissoclinum patella* from the Great Barrier Reef, Queensland, Australia. Some 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).[13] Type I is an intermediate in the conversion from Type II (described as a "saddle") to Type III (described as a "twisted figure eight"). Ascidiacyclamide (**1**, Chart 1) is an example of a Type II conformation and has been crystallised with a number of different solvates.[13–16] The variations within the crystal structures of these solvates illustrate a considerable degree of flexibility, with the Ile torsion angles (C(O)-N-C_α-(Oxn)) varying from –127 to −160° and Val torsion angles (C(O)-N-C_α-(Thz)) varying from 98 to 134°. The structure of patellamide D (**2**, Chart 1) again highlights the flexibility of the basic peptide backbone. In the crystal,[17] the torsion angles (C(O)-N-C_α-(Oxn)) for the Ile residues are −76 and −80°, whilst the D-Ala and D-Phe torsion angles (C(O)-N-C_α-(Thz)) are 91 and 80°, respectively. The structure is an example of a Type III conformation and is stabilised by intramolecular hydrogen bonds and π-stacking between the thiazoles.

Studies have demonstrated the ability of the patellamides to coordinate to metal ions such as copper(II) and zinc(II).[18–22] A crystal structure of the dinuclear copper μ-carbonato complex of **1** demonstrated that the patellamides can bind two copper ions, each metal being coordinated to the peptide through a thiazole, an oxazoline and a deprotonated Ile amide nitrogen, the coordination geometry of each copper being completed by a bridging carbonate anion and a water molecule.[20] The Type II conformation was retained upon copper binding. Solution studies involving **2** also found formation of a similar metal complex and suggested that the peptide underwent a change from the Type III conformation of the original ligand to a Type II conformation upon copper binding.[18] These studies suggest a potential, although unproven, role for these peptides as metal ion sequestering agents.

In this work we extend the studies conducted into metal complexes of cyclic octapeptides. Detailed herein are the results of the investigation into the influence that these constraining heterocycles have upon the conformations of cyclic octapeptides and the stability of their calcium complexes. Our interest in complexation studies of these cyclic peptides with calcium(II) arises from, in the first instance, the observation of calcium carbonate spicules in the ascidians,[23] and secondly complexation with calcium(II) should not be complicated by deproton-

† Supporting NMR data for compounds **2** and **3** are available as supplementary data from BLDSC (SUPPL. NO. 57684, 4 pp.) or the RSC Library. See Instructions for Authors available *via* the RSC web page (http://www.rsc.org/authors).



Scheme 1 Conformations of the patellamides (taken from ref. 13).

ation equilibria and slow kinetics as observed during previous studies with zinc(II).[22] The cyclic peptides studied include **1** and **2**, as well as cyclo(Thr-D-Val(Thz)-Ile)$_2$ (**3**) and cyclo(Thr-D-Val-αAbu-Ile)$_2$ (**4**), in which the 5-membered ring constraints contained within the macrocycle are successively removed. Threonine was employed to replace the oxazoline ring, whilst α-aminobutyric acid (αAbu), an amino acid isosteric with cysteine, was employed to replace the thiazole ring.

Experimental

Materials were of analytical grade and were used without further purification. Preparative scale reversed phase (rp) HPLC separations were performed on a Waters Delta-Pak PrepPak C_{18} 40 mm × 100 mm cartridge (100 Å). Linear peptides were purified using gradient mixtures of water–0.1% TFA (solvent system A) and 10% water–90% CH_3CN–0.1% TFA (solvent system B). Peptides were purified using gradient mixtures of water and acetonitrile (100% solvent system A to 100% solvent system B).

Ascidiacyclamide and patellamide D were isolated and purified from *Lissoclinum patella* collected from Heron Island on the Great Barrier Reef, Australia.[24] Peptide **3** was prepared as previously described.[25] The water content of purified peptides was obtained by CHN analysis.

Mass spectra were obtained with a triple quadrupole mass spectrometer (PE SCIEX API III) equipped with an Ionspray (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 focussed 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 Bruker AMX400 spectrometer at 301 K or a Bruker Avance DRX500 at 300 K unless otherwise stated. All coupling constants are expressed in Hz. The proton assignments are related to the numbering scheme in Chart 1.

Circular dichroism (CD) spectra were recorded with a JASCO J-710 spectrophotometer. The measurements were carried out in acetonitrile (spectroscopic grade) using either a 1 cm or 0.1 cm path length cell, from 200–400 nm at 298 K. The cell was maintained at 298 K with a Neslab RTE-111 temperature controller.

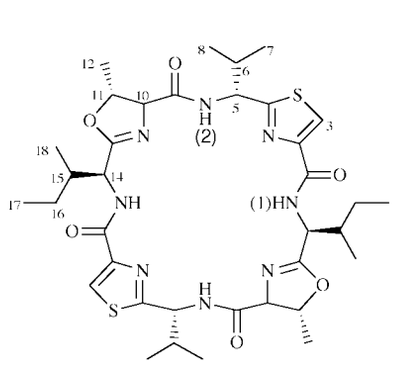
The concentration of calcium ions in the stock solutions was determined by ICP analysis (CSIRO Division of Tropical Agriculture, St. Lucia, Brisbane) after dilution of an aliquot with Milli-Q water.

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

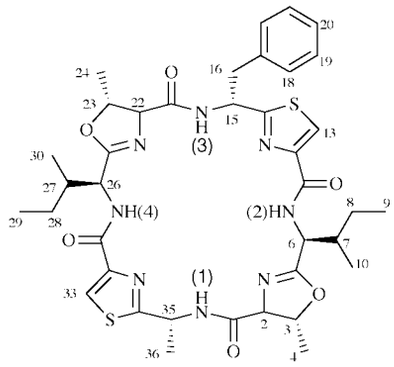
Abbreviations: Boc = *tert*-butyloxycarbonyl; BOP = [benzotriazol-1-yloxytris(dimethylamino)phosphonium] hexafluorophosphate; DIPEA = diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; PAM = phenylacetamidomethyl; TFA = trifluoroacetic acid; Thz = thiazole; Oxn = oxazoline; αAbu = α-aminobutyric acid.

H-(Thr-D-Val-αAbu-Ile)$_2$-OH. Boc-Ile-PAM resin (2.14 g, S. V. (substitutional value) = 0.35 mmol g^{-1}) was deprotected with TFA (5 mL, 2 × 1 min), washed with DMF and a solution of Boc-αAbuOH (0.606 g), HBTU (0.5 M in DMF, 8 mL) and DIPEA (920 mL) was added. The reaction mixture was shaken for 12 min and monitored by the negative ninhydrin test (>99.6% coupling). The resin was washed with DMF. The synthesis was completed by the sequential coupling of Boc-D-Val-OH (4 equiv.), Boc-Thr-OH (4 equiv.), Boc-Ile-OH (4 equiv.), Boc-αAbu-OH (4 equiv.), Boc-D-Val-OH (4 equiv.), Boc-Thr-OH (4 equiv.) using the same procedure. The peptide was cleaved from the resin with HF, precipitated and washed several times with diethyl ether, redissolved in water–acetonitrile (50 : 50) and lyophilised. The crude powder was purified by rp-HPLC to give H-(Thr-D-Val-αAbu-Ile)$_2$-OH as a white powder (200 mg, 32.7% from Boc-Ile-PAM resin). HPLC analysis: (gradient, 30 min), one peak, retention time = 14 min. ISMS: *m*/*z* 815 Da $[M + H]^+$.

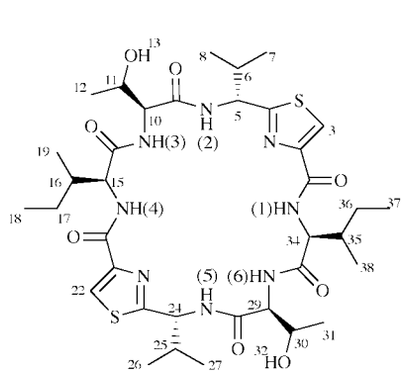
^{1}H NMR (d_3-methanol, 400 MHz, 301 K): δ 8.72 (2H, d, *J* 7, Val-NH, Ile-NH), 8.09 (1H, d, *J* 8, Thr-NH), 8.08 (1H, d, *J* 8, αAbu-NH), 8.06 (1H, d, *J* 9, αAbu-NH), 7.86 (1H, d, *J* 9, Ile-



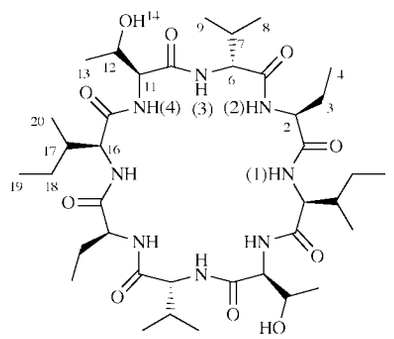
Ascidiacyclamide **1**



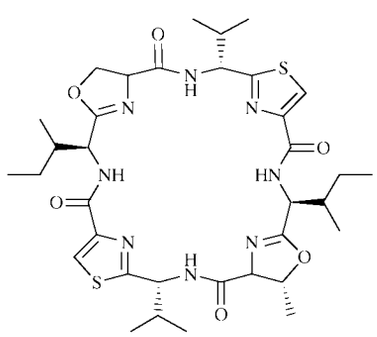
Patellamide D **2**



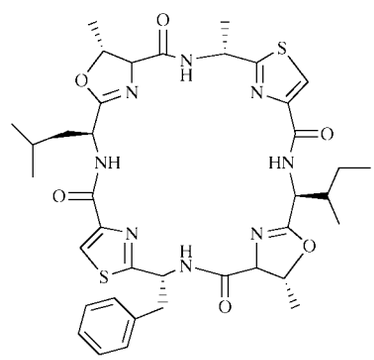
cyclo(Thr-D-Val(Thz)-Ile)$_2$ **3**



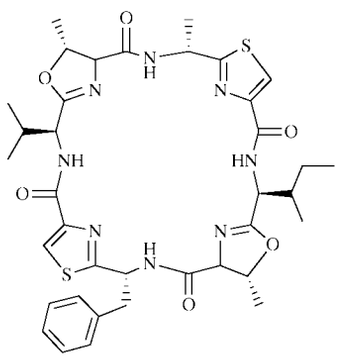
cyclo(Thr-D-Val-αAbu-Ile)$_2$ **4**



Patellamide A



Patellamide B



Patellamide C

Chart 1 Cyclic peptides.

NH), 7.70 (1H, d, *J* 8, Val-NH), 4.34 (1H, m, Ile-αH), 4.30 (2H, m, αAbu-αH, Val-αH), 4.28 (1H, m, Thr-βH), 4.18 (1H, m, Ile-αH), 4.16 (1H, m, Thr-αH), 4.10 (1H, m, Thr-βH), 3.97 (1H, m, Val-αH), 3.77 (1H, m, Thr-αH), 2.15 (1H, m, Val-βH), 2.04 (2H, m, Ile-βH, αAbu-αH), 1.97 (1H, m, Val-βH), 1.86 (1H, m, Ile-βH), 1.70–1.50 (6H, m, αAbu-βH, αAbu-βH, Ile-γCH_2, Ile-γCH_2), 1.30 (3H, d, *J* 6, Thr-γCH_3), 1.25 (2H, m, αAbu-βH, αAbu-βH), 1.15 (3H, d, *J* 6, Thr-γCH_3), 1.04 (3H, d, *J* 7, Val-γCH_3), 0.99 (6H, m, Val-γCH_3, αAbu-γCH_3), 0.93 (12H, m, Ile-γCH_3, Ile-δCH_3, Val-γCH_3, Val-γCH_3), 0.89 (9H, m, Ile-γCH_3, Ile-δCH_3, αAbu-γCH_3).

^{13}C NMR (d_3-methanol, 400 MHz, 301 K): δ 177.2, 176.9, 176.3, 176.2, 176.0, 175.2, 175.1, 175.0, 172.2, 70.7, 69.8, 64.6, 63.0, 62.7, 36.0, 34.1, 33.3, 28.6, 28.4, 25.2, 25.1, 22.9, 22.5, 22.4, 22.2, 21.8, 20.8, 18.6, 18.5, 14.3, 13.4.

Cyclo(Thr-D-Val-αAbu-Ile)$_2$ (4). A solution of H-(Thr-D-Val-αAbu-Ile)$_2$-OH (50 mg, 61 μmol), BOP (82 mg, 185 μmol), DIPEA (104 μL, 598 μmol) in DMF (100 mL) was stirred at room temperature for one hour. The solvent was removed *in vacuo* and the residue was purified by HPLC (gradient, 60 min). The appropriate fractions were combined and lyophilised to give Cyclo(Thr-D-Val-αAbu-Ile)$_2$ as a white powder (19.9 mg, 40.7%). ISMS: *m*/*z* 797.6 Da [M + H]$^+$.

^{1}H NMR (d_6-DMSO, 400 MHz, 301 K): δ 7.93 (2H, br d, *J* 9, NH(3)), 7.82 (2H, br m, NH(4)), 7.68 (2H, br d, *J* 7, NH(1)), 7.67 (2H, br m, NH(2)), 5.00 (2H, d, *J* 4, H14), 4.28 (2H, m, H16), 4.22 (2H, m, H6), 4.11 (2H, m, H2), 4.07 (2H, m, H11), 3.88 (2H, m, H12), 2.23 (2H, m, H7), 1.75 (4H, m, H18), 1.73 (4H, m, H3), 1.59 (2H, m, H17), 1.06 (6H, d, *J* 6, H13), 0.85 (18H, m, H8, H9, H4), 0.79 (12H, m, H19, H20).

^{13}C NMR (d_6-DMSO, 400 MHz, 301 K): δ 171.6, 171.0, 171.0, 170.1, 66.1, 59.7, 57.5, 57.5, 54.7, 36.7, 29.3, 25.6, 24.5, 19.7, 19.4, 17.1, 15.0, 10.9, 10.6.

The dihydrate was crystallised by slow evaporation of a saturated solution of the peptide in acetone (Found: C, 54.6; H, 9.03; N, 13.3. $C_{38}H_{72}N_8O_{12}$ requires C, 54.8; H, 8.71; N, 13.5%).

Binding constant determination

In a typical CD titration, solutions of cyclic peptides in acetonitrile with concentrations ranging from 0.5–1 mM were titrated with a solution of calcium perchlorate tetrahydrate in 95% acetonitrile–water. Titrations were continued until

Table 1 Binding constants for calcium–peptide complexes in acetonitrile[a]

Peptide	Method	log $K_{1:1}$ (log $K_{1:2}$)
1	NMR[b]	2.9 ± 0.4
1	NMR[b,c]	2.6 ± 0.4
2	NMR[b]	2.8 ± 0.4
3	CD[d]	4.0 ± 0.4 (4.5 ± 0.4)
4	CD[e]	5.5 ± 0.3
4	CD[d,e]	5.5 ± 0.4

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

Table 2 Crystal data for cyclo(Thr-D-Val-αAbu-Ile)$_2$·2H$_2$O (**4**)

Crystal system	Orthorhombic
Space group	$P2_12_12_1$
a/Å	17.646(2)
b/Å	23.396(3)
c/Å	11.5285(9)
V/Å^3	4759.3(7)
Formula weight	831.02
D_{calc}/g cm^{-3}	1.160
Empirical formula	$C_{38}H_{70}N_8O_{12}$
Z	4
Absorption coefficient/cm^{-1}	0.72
Transmission coefficient	0.996–0.796
Reflections measured	3629
Unique reflections	3629
Observed reflections	2058
T/°C	21
λ/Å	1.5418
$R(F_o)$[a]	0.047
R_w[a]	0.044

[a] $R = \Sigma(||F_o| - |F_c||)/\Sigma|F_o|$, $R_w = (\Sigma w(|F_o| - |F_c|)^2/\Sigma wF_o^2)^{\frac{1}{2}}$.

approximately 1.1 equivalents of Ca^{2+} had been added. At least 14 spectra were used to calculate binding constants. Data were analysed using Specfit,[26] a global least squares fitting program for equilibrium and kinetic systems using factor analysis and Marquardt minimization. Duplicate titrations were performed.

For titrations monitored by ^{1}H NMR, a solution of peptide in d_3-acetonitrile (*ca.* 0.5 mM) was titrated with a solution of calcium perchlorate tetrahydrate in d_3-acetonitrile. Typically, at least 1.2 equivalents of Ca^{2+} were added and at least eight data points were used for the calculation of binding constants. Data were analysed by following the change in the chemical shifts of proton resonances as a function of metal concentration and then using a non-linear curve fitting algorithm to fit the data.[27] Only signals from protons which gave a large shift in chemical shift ($\Delta\delta > 0.2$ ppm) over the whole titration were used in the analysis. For **1**, the H3 and H14 signals were monitored and used for the binding constant determination. For **2**, the shifts of the signals due to H6, H15, H23, H26, H35 and H36 were used. Duplicate titrations were performed.

In order to account for the effect of changing ionic strength during calcium perchlorate additions, titrations were performed involving peptides **1** and **4** in the presence of 0.1 M sodium perchlorate and tetraethylammonium perchlorate, respectively (Table 1). From these experiments, it was found that within experimental error the 1 : 1 binding constant for **1** and **4** were not affected by the presence of background electrolyte.

Crystal structure determination of cyclo(Thr-D-Val-αAbu-Ile)$_2$·2H$_2$O (4)

Cell constants were determined by a least-squares fit to the setting parameters of 25 independent reflections, measured and refined on a Rigaku AFC7R diffractometer. The crystallographic data are summarised in Table 2. Data reduction and application of Lorentz, polarisation and semi-empirical psi scan absorption corrections were carried out using teXsan.[28] The structure was solved by direct methods using SHELXS-86[29] and refined using full-matrix least-squares methods with teXsan. Hydrogen atoms were included at calculated sites with thermal parameters derived from the parent atoms. Non-hydrogen atoms were refined anisotropically. Scattering factors were taken from International Tables.[30] Anomalous dispersion effects were included in F_c;[31] the values for $\Delta f'$ and $\Delta f''$ were those of Creagh and McAuley.[32] The values for the mass attenuation coefficients are those of Creagh and Hubbell.[33] All other calculations were performed using the teXsan[28] crystallographic software package of Molecular Structure Corporation.‡

Despite the use of a 2 sigma cut-off in the refinement, the reflection to parameter ratio (*ca.* 4) is less than ideal. However, the refinement is stable with all shifts less than 0.01 sigma and all anisotropic thermal parameters are real and sensible. Isotropic refinement with the carbon atoms resulted in an increase in the R value to 0.066 and a 20–25% increase in the standard deviations of the positional parameters.

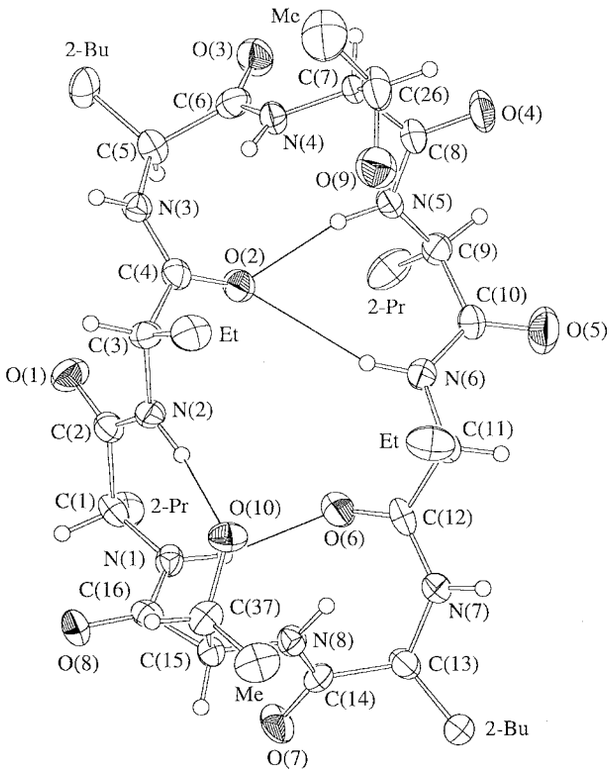


Fig. 1 ORTEP drawing of the peptide in cyclo(Thr-D-Val-αAbu-Ile)$_2$·2H$_2$O. Extended side chains are omitted for clarity; Me denotes the β-carbon of a threonine side chain, 2-Bu denotes the β-carbon of an isoleucine side chain, 2-Pr denotes the β-carbon of a valine side chain, and Et denotes the β-carbon of an α-aminobutyric acid side chain. Hydrogen bonds are indicated.

Results and discussion

Solid state structure of 4

Peptide **4** was synthesised to produce an analogue of **1** lacking oxazoline and thiazole rings. The oxazoline ring was replaced with its precursor, threonine, whilst the thiazole rings were replaced with α-aminobutyric acid (αAbu).

An ORTEP drawing of the structure of **4**·2H$_2$O is presented in Fig. 1. The bond distances and angles formed by atoms of

‡ CCDC reference number 188/198. See http://www.rsc.org/suppdata/p2/a9/a906090a for crystallographic files in .cif format.

Table 3 Macrocyclic backbone bond lengths (Å) and angles (°) for cyclo(Thr-D-Val-αAbu-Ile)$_2$·2H$_2$O (**4**)

N(1)–C(1)	1.46(1)	N(5)–C(9)	1.44(1)
C(1)–C(2)	1.54(1)	C(9)–C(10)	1.52(1)
C(2)–N(2)	1.331(9)	C(10)–N(6)	1.327(9)
N(2)–C(3)	1.465(9)	N(6)–C(11)	1.45(1)
C(3)–C(4)	1.52(1)	C(11)–C(12)	1.52(1)
C(4)–N(3)	1.313(9)	C(12)–N(7)	1.36(1)
N(3)–C(5)	1.447(9)	N(7)–C(13)	1.464(9)
C(5)–C(6)	1.56(1)	C(13)–C(14)	1.53(1)
C(6)–N(4)	1.32(1)	C(14)–N(8)	1.343(1)
N(4)–C(7)	1.442(9)	N(8)–C(15)	1.463(9)
C(7)–C(8)	1.53(1)	C(15)–C(16)	1.51(1)
C(8)–N(5)	1.34(1)	C(16)–N(1)	1.345(9)
N(1)–C(1)–C(2)	111.3(7)	C(9)–C(10)–N(6)	117.3(7)
N(2)–C(3)–C(4)	108.7(6)	C(11)–C(12)–N(7)	114.2(7)
N(3)–C(5)–C(6)	113.1(7)	C(13)–C(14)–N(8)	117.2(7)
N(4)–C(7)–C(8)	112.1(7)	C(15)–C(16)–N(1)	118.6(7)
N(5)–C(9)–C(10)	112.8(7)	C(2)–N(2)–C(3)	118.7(6)
N(6)–C(11)–C(12)	109.9(7)	C(4)–N(3)–C(5)	122.6(6)
N(7)–C(13)–C(14)	113.9(7)	C(6)–N(4)–C(7)	121.4(7)
N(8)–C(15)–C(16)	114.0(6)	C(8)–N(5)–C(9)	125.1(6)
C(1)–C(2)–N(2)	117.3(7)	C(10)–N(6)–C(11)	123.8(7)
C(3)–C(4)–N(3)	116.1(7)	C(12)–N(7)–C(13)	123.5(7)
C(5)–C(6)–N(4)	118.3(7)	C(14)–N(8)–C(15)	122.4(7)
C(7)–C(8)–N(5)	118.8(7)	C(16)–N(1)–C(1)	124.0(6)

Table 4 Torsion angles (C(O)–N–C_α–C(O)) and amide coupling constants for **2** and **4**

Residue	Angle from crystal structure/°	$J_{HNC\alpha H}$/ Hz	Angle from coupling constant[a] (d_3-acetonitrile)/°
Peptide **2**			
Ile	−76.4[b]	7.5	−80→−85, −152→−165
Ile	−80.2[b]	7.5	−80→−85, −152→−165
D-Ala	91.0[b]	9.0	87→100, 145→155
D-Phe	83.8[b]	9.5	90→105, 140→153
Peptide **4**			
D-Val	99.0(8), 101.3(9)	8.5	86→93, 150→160
αAbu	−60.5(9), −128.0(9)	8.0	−82→−90, −153→−160
Ile	−55(1), −71.7(9)	5.0	−65→−72, −170→−175
Thr	−71.1(9), −75(1)	6.5	−73→−80, −162→−168

[a] Derived from Bystrov, see ref. 40. [b] From Schmitz *et al.*, see ref. 17.

the macrocyclic ring (Table 3) show no unusual values when compared to other cyclic octapeptides.[34–36] All amide bonds were found to have the *trans* configuration. The molecule was found to deviate significantly from the expected C_2-symmetry with the major deviation occurring around the α-aminobutyric acid residues as seen in the difference between the two C(O)–N–C_α–(O) torsion angles, which vary by 68° (Table 4).

The structural analysis revealed the presence of transannular hydrogen bonds between the amide hydrogens of the valine residues and the carbonyl oxygens of the α-aminobutyric acid residues (N5–H···O2: 2.92 Å, 165.0° and N1–H···O6: 3.02 Å, 163.2°). The hydrogen bonds result in the formation of β-turns within the cycle with concomitant pinching of the peptide backbone. A weak interaction between one of the α-aminobutyric acid amide hydrogens and the second α-aminobutyric acid carbonyl oxygen (N6–H···O2: 3.43 Å, 159.2°) contributes further to the stabilisation of the observed conformation where the peptide backbone assumes a relatively planar conformation. The only other intramolecular hydrogen bond was between the α-aminobutyric acid amide hydrogen and the hydroxy oxygen on the neighbouring threonine residue (N2–H···O10: 3.25 Å, 156.0°). The N6–H···O2 and the N2–H···O10 interactions contribute to the deviation from C_2-symmetry.

The D-valine side chains were found to occupy axial positions on one side of the molecule, the α-aminobutyric acid and threonine side chains occupy axial positions on the opposite side whilst the isoleucine side chains were found to exist in equatorial positions relative to the plane of the peptide.

Solution conformation studies

Although X-ray structures are known for **1**, **2**[13–17] and now **4**, there is evidence that some cyclic octapeptides can adopt multiple conformations in solution,[37,38] depending upon the solvent being used and the constraints present in the molecules. Thus, before beginning titrations with calcium perchlorate to determine binding constants, it was of interest to ascertain whether the conformations found in the solid state were retained in solution. The conformations of these cyclic octapeptides in d_3-acetonitrile were investigated through the use of 1D and 2D ^{1}H NMR spectroscopy.

For macrocycle **1**, X-ray crystal structures[13–16] show that the peptide backbone adopts a "saddle-shaped" conformation (Type II, Scheme 1) with the thiazole and oxazoline heterocyclic rings occupying the corners of a rectangle. The valine and isoleucine side chains occupy pseudoaxial positions but in opposite directions, thus helping to define the saddle shape. The structure has no intramolecular hydrogen bonds to stabilise the conformation, so the shape observed in the crystal appears to be due solely to the influence of the thiazole and oxazoline rings. In solution, NMR spectra reveal that coupling constants for the amide protons of **1** in d_3-acetonitrile (J = 7.9 and 10.2 Hz) lie within the ranges of both Type II and Type III[22] and thus, do not provide conclusive evidence in favour of either structure. Temperature coefficients for the amide protons were relatively low ($\Delta\delta/\Delta T$ of 1.9 (IleNH) and 0.4 (ValNH) ppb K^{-1}) and deuterium exchange (3% CD_3OD) was very slow ($t_{1/2}$ of 14 days (IleNH) and greater than one month for ValNH). These observations are consistent with either the amide hydrogens being involved in hydrogen bonding or with the amide hydrogens being buried within the cavity of the peptide and hence shielded from the solvent. The 2D NOESY spectrum of **1** in d_3-acetonitrile revealed non-sequential cross peaks between the amide protons NH(1) and NH(2), and between NH(2) and H18 (IleγCH_3). From the crystal structures of **1**,[13–16] the corresponding interproton distances are 3.48 to 3.60 Å, and 3.24 and 3.48 Å, respectively, and would be expected to give rise to cross peaks in the NOESY spectrum. Thus, the results of the NOESY experiment suggest that the peptide adopts the Type II conformation in solution.

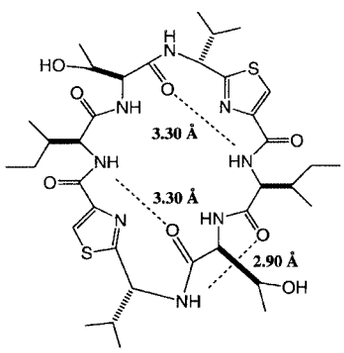
Although differing from **1** only in the replacement of the two valine residues by a phenylalanine and alanine, the crystal structure of **2** reveals a vastly different shape. This different solid state structure is described as a "twisted figure eight" (Type III conformation, Scheme 1).[17] Unlike **1**, intramolecular hydrogen bonds are found to stabilise this structure, with further stability being provided by a π-stacking interaction between the two thiazoles. In acetonitrile solution the NOESY spectrum of **2** contained a number of cross peaks between inter-residue protons (Table S1 †), the most diagnostic of which were those showing coupling between the thiazole protons and transannular β-protons (*i.e.* between H33 and H16, and H13 and H36). Such coupling was not observed in the spectrum of **1** and indeed, would not be expected for Type II conformations, where the distances between the thiazole and transannular β-protons are greater than 9 Å, based on the crystal structures of **1** and patellamide A (Chart 1).[39] Only in the Type III conformation would such a coupling be expected to arise, due to the close approach of the two thiazole rings. Torsion angle data derived from the coupling constants[40] were found to correspond closely to those deduced from the crystal structure (Table 4), with the differences being less than 6.5° and provide further

evidence of peptide **2** having the Type III conformation in solution.

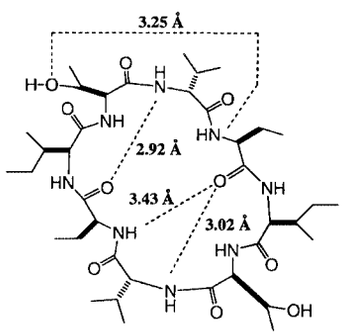
To establish whether the hydrogen bonds observed in the crystal structure of **2** were retained in solution, variable temperature and deuterium exchange experiments were conducted. The variable temperature data showed that all amide protons had very low temperature coefficients ($\Delta\delta/\Delta T < 0.08$ ppb K^{-1}) and the deuterium exchange experiment (5% of d_4-methanol) showed very slow exchange ($t_{1/2} > 1$ day) of the Ile amide protons with solvent, whilst the Ala and Phe amide protons were marginally faster ($t_{1/2} \approx 12$ hours). Both experiments indicated that the hydrogen bonds observed in the crystal structure of **2** were retained in d_3-acetonitrile.

The result of this conformational study for **2** in solution is different from the result of a study on two structurally related cyclic octapeptides, patellamide B and C (Chart 1), in chloroform.[41] Relaxation and NOESY NMR experiments on patellamide B and C gave evidence for a peptide backbone conformation similar to the Type III conformation (Scheme 1). However, based exclusively on variable temperature data ($\Delta\delta/\Delta T = 6–12 \times 10^{-4}$ ppm K^{-1}) it was concluded that the amide hydrogens were exposed to the solvent and it was suggested that these peptides display a new conformation, a "twisted figure eight" lacking hydrogen bonds. However, intramolecular hydrogen bonds in these peptides were excluded solely on the basis of the temperature coefficients and no other experimental data (deuterium exchange or effect of DMSO addition) was obtained on the extent of amide exposure to solvent.[41]

A solution structure determination in d_6-acetone for peptide **3** revealed that this peptide adopts yet another conformation in solution, this being described as a "chair" shape.[25] The thiazole



cyclo(Thr-D-Val(Thz)-Ile)$_2$ **3**



cyclo(Thr-D-Val-αAbu-Ile)$_2$ **4**

rings sit at the apical positions of the structure and transannular hydrogen bonds between the Ile amide protons and Thr carbonyl oxygens produce two β-turns. The asymmetry arises due to a hydrogen bond between a D-Val amide proton (NH(5)) and an Ile-CO (adjacent to NH(3) on C14), thus creating a γ-turn. A comparison was made between the 1D ^{1}H NMR spectra of **3** in d_6-acetone and d_3-acetonitrile (Table S2†). Common to both spectra were up-field shifted isoleucine methyl signals (H37 = 0.64 and H38 = 0.52 ppm for acetonitrile and H37 = 0.64 and H38 = 0.49 ppm for acetone). The large shift in these signals has been attributed to the fact that this residue lies above one of the thiazole rings.

A variable temperature experiment with **3** in d_3-acetonitrile showed that both Thr amide protons had larger temperature coefficients (>2.5 ppb K^{-1}) than were observed for the hydrogen bonded amides in **2**, suggesting that they were exposed to solvent or only weakly hydrogen bonded. When d_4-methanol (5% volume) was added to a solution of **3** in d_3-acetonitrile at 300 K, it was found that some of the amide signals overlapped. However, at 320 K, the amide proton signals were resolved and it was evident that the NH(3), NH(6) (Thr amides) and NH(5) (D-Val amide) protons exchanged most rapidly. In conclusion, it appears that the structure of **3** is very similar in d_3-acetonitrile and d_6-acetone, with the peptide adopting the "chair" conformation in both solvents.

Molecular modelling for the peptide cyclo(Thr-D-Val-Cys-Ile)$_2$,[42] an analogue of **4**, suggested that peptides lacking any constraints apart from the macrocyclic ring and hydrogen bonds are very flexible and can potentially adopt a myriad of conformations in solution. The flexibility of **4** was confirmed by comparing the differences in the backbone torsion angles derived from the crystal structure with those calculated from the coupling constants (Table 4). Only torsion angles for two residues (Thr and Ile) agree within experimental error, the remaining angles differing by up to 35°.

The variable temperature experiment for **4** showed that all the amide protons had very small temperature coefficients ($\Delta\delta/\Delta T < 0.004$ ppm K^{-1}). The deuterium exchange rate of the amide protons (5% volume d_4-methanol) over eleven hours was less than 25%. These results were suggestive of the retention of the transannular and inter-residue hydrogen bonds and/or solvent shielding. As a result of the hydrogen bonding network observed in the crystal, it appears that the two isoleucine amide protons are free to interact with the solvent. However, these are potentially shielded from the solvent due to steric effects imposed by the Ile and αAbu side chains, and as a result, are not observed in variable temperature and deuterium exchange experiments. It was concluded that the hydrogen bonds seen in the crystal structure of peptide **4** are probably retained in solution.

Binding constants

Binding constants were determined either by CD or NMR spectroscopy and, for all peptides studied, 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.[3,8,43]

Broadening and shifting of signals in the NMR spectra of **1** and **2** in d_3-acetonitrile were observed upon addition of calcium perchlorate. The binding constants for the respective 1 : 1 complexes were calculated from the change in the chemical shift of a number of protons (Fig. 2), assuming a simple complexation model (eqn. (1)) where L = peptide.

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

$$[CaL]^{2+} + L \overset{K_{1:2}}{\rightleftharpoons} [CaL_2]^{2+} \quad (2)$$

It was found that the magnitudes of the 1 : 1 metal–ligand binding constants (log K) for **1** (2.9 ± 0.4) and **2** (2.8 ± 0.4) (Table 1) were low when compared to other cyclic octapeptides (log K = 4–7).[8] This suggests that cyclic octapeptides containing the backbone structure of the patellamides, *i.e.* two thiazoles and two oxazoline rings, are relatively poor complexing agents towards Ca^{2+}. As well, the calcium binding constants for both peptides were the same within experimental error, suggesting that, when the ligand conformations in solution are considered,

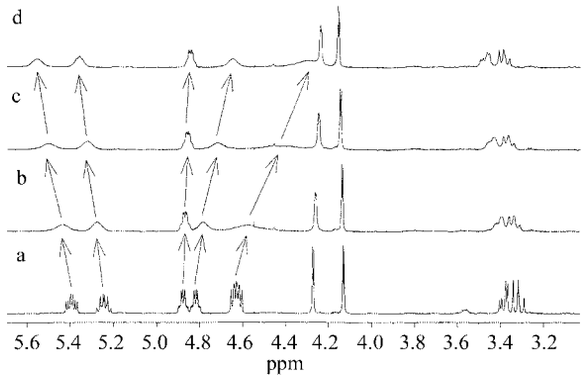


Fig. 2 Selected region of the ^{1}H NMR spectra of (a) peptide **2** in d_3-acetonitrile (0.889 mM); added calcium perchlorate (b) 0.338 equivalents, (c) 0.676 equivalents and (d) 1.127 equivalents.

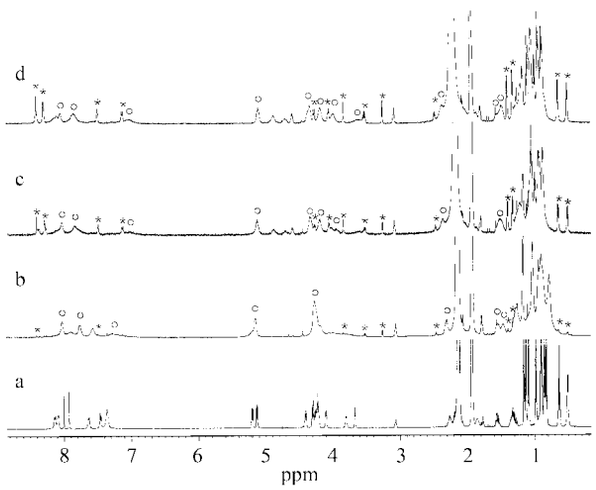


Fig. 3 ^{1}H NMR spectra of (a) peptide **3** in d_3-acetonitrile (1.1 mM); added calcium perchlorate (b) 0.25 equivalents, (c) 0.60 equivalents and (d) 0.95 equivalents. "○" refers to resonances assigned to the 1:2 calcium–peptide complex and free peptide in fast exchange, "*" refers to resonances assigned to the 1:1 calcium–peptide complex.

preorganisation of the donor groups has no effect on the strength of binding to Ca^{2+}.

A titration, monitored by NMR spectroscopy, of peptide **3** with calcium perchlorate demonstrated broadening and shifting of the peptide signals on addition of the metal (Fig. 3). The appearance of new signals in the spectrum was also observed. These observations suggested that two species were formed in this titration. Signals due to the two α-H of valine (H5 and H24) broadened and shifted when up to half an equivalent of Ca^{2+} was added, after which there was no discernable change. This suggests formation of a 1:2 calcium–ligand complex (eqn. (2)) with fast exchange kinetics on the NMR time scale. The second species evident in the NMR titration was assigned to a 1:1 metal–ligand complex (eqn. (1)) exhibiting slow exchange kinetics. Further evidence for the existence of these two complexes was provided from titrations monitored by CD spectroscopy. The CD spectrum of the free ligand **3** in acetonitrile exhibited a very small negative band at 276 nm ($\Delta\varepsilon$ −0.012 cm^2 mmol^{-1}) (Fig. 4). On addition of calcium perchlorate, the intensity of the signal increased, reaching a maximum after half an equivalent of added Ca^{2+} (Fig. 4a) (λ_{max} 279 nm) consistent with formation of a 1:2 metal–ligand complex. Further addition of Ca^{2+} up to one equivalent led to a decrease in the intensity of the CD signal (Fig. 4b) consistent with formation of a 1:1 complex. The binding constant for the 1:1 complex of **3** (log K = 4.0, Table 1) obtained from the CD titration was significantly higher than for **1** and **2**. Calcium–peptide (1:2)

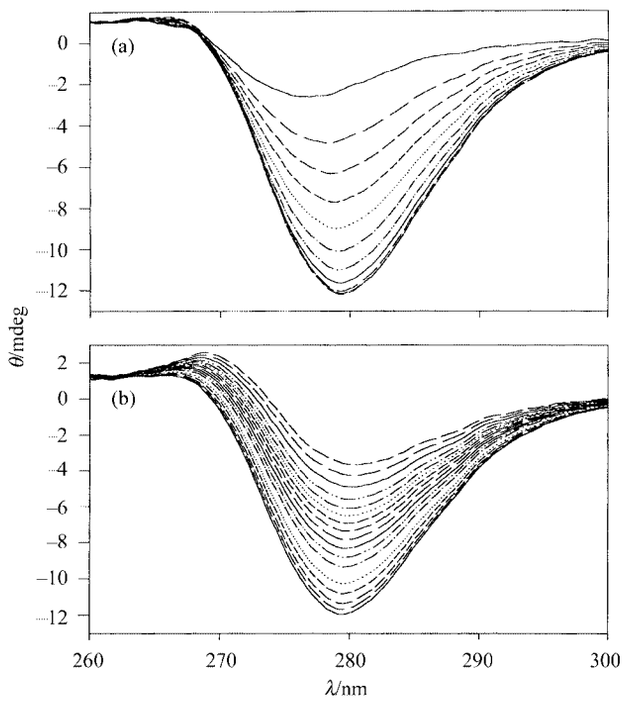


Fig. 4 CD titration of an acetonitrile solution containing peptide **3** (0.469 mM) with added (a) (decreasing θ) 0, 0.075, 0.125, 0.175, 0.224, 0.274, 0.324, 0.374, 0.424, 0.474, 0.524 and (b) (increasing θ) 0.574, 0.624, 0.674, 0.748, 0.823, 0.898, 0.948, 1.023, 1.098, 1.173, 1.247, 1.322, 1.397, 1.472, 1.572, 1.696, 1.821, 1.946 equivalents of calcium perchlorate.

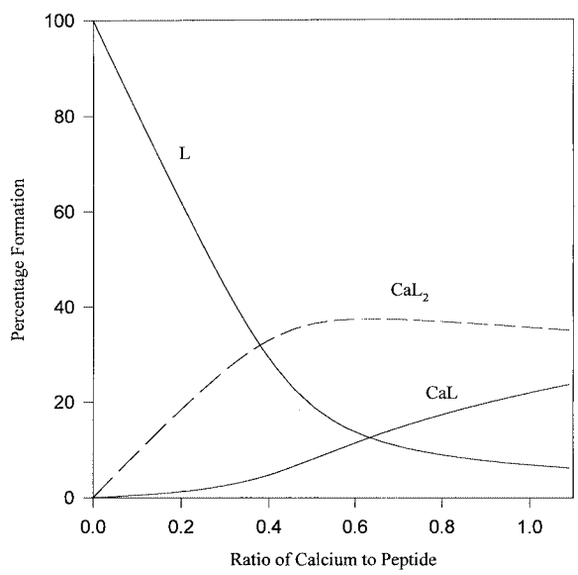


Fig. 5 Speciation plot for calcium complexes of peptide **3**, total concentration of peptide 0.75 mM, log $K_{1:1}$ = 4.0, log $K_{1:2}$ = 4.5, L: free peptide, CaL: 1:1 calcium–peptide complex, CaL_2: 1:2 calcium–peptide complex.

complexes have been observed for other cyclic octapeptides containing proline and/or *N*,*N*′-ethylene bridges.[11,44–46] The magnitudes of these 1:2 binding constants vary from log $K_{1:2}$ = 3 to 4.9, similar to the value for **3** (log $K_{1:2}$ = 4.5, Table 1).

A speciation plot (Fig. 5) calculated for the ligand concentration used in the NMR titration of **3** and the binding constants for the 1:1 and 1:2 metal–ligand complexes for **3** (Table 1) obtained from the CD experiment yields relative ratios of the species in solution and permits comparison with the results of the NMR titration (Fig. 3). Thus, in the NMR spectra the signals for the 1:1 metal–ligand complex start to appear after addition of 0.25 equivalents of Ca^{2+} (Fig. 3b) the speciation

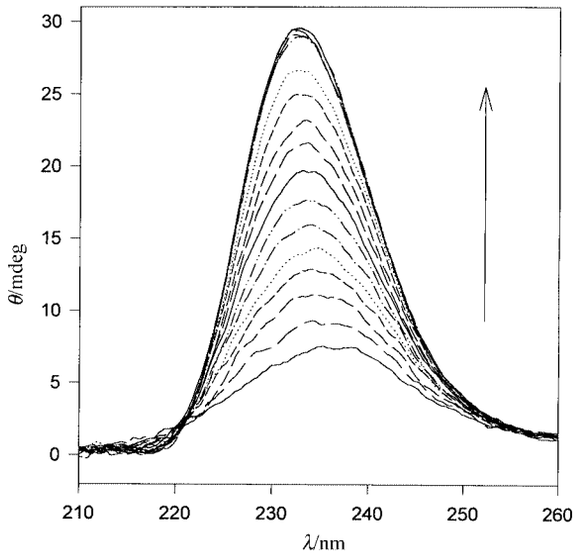


Fig. 6 CD titration of **4** in acetonitrile (0.5 mM) with added (increasing θ) 0, 0.12, 0.22, 0.31, 0.40, 0.50, 0.59, 0.68, 0.77, 0.87, 0.96, 1.05, 1.14, 1.24 equivalents of calcium perchlorate.

plot predicting that this species represents 4% of the total complex present. After approximately 0.5 equivalents of Ca^{2+} has been added no significant change in the chemical shift of the signals corresponding to the 1:2 metal–ligand complex is observed in the NMR spectrum (Fig. 3c–d) in agreement with the speciation plot. Finally, at one equivalent of Ca^{2+} signals for both the 1:1 and 1:2 metal–ligand complexes are present in the NMR spectrum (Fig. 3d), again in agreement with the speciation plot. There is good agreement between the binding constants obtained from the CD titrations and the species that can be observed in the NMR titration.

Addition of calcium perchlorate to peptide **4** in acetonitrile resulted in the appearance of new signals in the ^1H NMR spectrum. After addition of one equivalent of Ca^{2+} no signals from the uncomplexed peptide remained, indicating formation of a 1:1 complex. A combination of high binding constant and slow exchange kinetics on the NMR time scale for this complex prevented determination of the binding constant from the NMR data. Instead, titrations involving peptide **4** and calcium perchlorate were monitored by CD spectroscopy. The peptide CD spectrum displayed a positive band with a maximum at 238 nm (Fig. 6). Addition of calcium perchlorate led to an increase in this positive band until a plateau was reached (λ_{max} 232 nm) corresponding to the addition of one equivalent of metal ion suggesting formation of a 1:1 complex. Addition of more Ca^{2+} led to further changes that were not investigated. The magnitude of the 1:1 binding constant (log $K = 5.5$, Table 1) was significantly higher than for the other peptides studied (**1**, **2** and **3**), but compares favourably with other cyclic octapeptides known to bind Ca^{2+}.[8]

Concluding comments

Other studies involving Ca^{2+} binding to cyclic octapeptides incorporating proline and *N*,*N*′-ethylene bridge constraints did not focus systematically on the effect of the constraints.[8] The present study, which involved successively removing thiazole and oxazoline ring constraints in the cyclic octapeptides studied, represents an investigation into the structural effects on metal binding of such ring constraints. The 1:1 calcium binding constants for these peptides (Table 1) display a systematic trend (**4** > **3** > **2** = **1**) consistent with increased macrocyclic flexibility permitting oxygen donor atoms greater access to the metal ion. However, whether the trend of affinity towards the Ca^{2+} ion can be attributed solely to the increased flexibility is open to question as, in the process of replacing constraining groups within the octapeptide, the number of amide carbonyl groups available for bonding has been effectively doubled. It is not clear at this stage whether the increase in stability of the Ca^{2+} complexes arises from the increase in flexibility of the peptide or the increase in the number of carbonyl oxygen donors, or a combination of both. Further work is in progress to elucidate the separate contributions of these effects and will be reported subsequently.

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References

1 D. J. Patel, *Biochemistry*, 1973, **12**, 667.
2 M. Pinkerton, L. K. Steinrauf and P. Dawkins, *Biochem. Biophys. Res. Commun.*, 1969, **35**, 512.
3 D. Baron, L. G. Pease and E. R. Blout, *J. Am. Chem. Soc.*, 1977, **99**, 8299.
4 J. P. Degelaen, P. Pham and E. R. Blout, *J. Am. Chem. Soc.*, 1984, **106**, 4882.
5 I. L. Karle, J. Karle, T. Wieland, W. Burgermeister, H. Faulstich and B. Witkop, *Proc. Nat. Acad. Sci. USA*, 1973, **70**, 1836.
6 I. L. Karle, *Biochemistry*, 1974, **13**, 2155.
7 I. L. Karle, *Proc. Nat. Acad. Sci. USA*, 1985, **82**, 7155.
8 H. Miyake and Y. Kojima, *Coord. Chem. Rev.*, 1996, **148**, 301.
9 E. R. Blout, C. M. Deber and V. Madison, *Acc. Chem. Res.*, 1976, **9**, 106.
10 Y.-Y. H. Chiu, L. D. Brown and W. N. Lipscomb, *J. Am. Chem. Soc.*, 1977, **99**, 4799.
11 D. S. Seetharama Jois, K. R. K. Easwaran, M. Bednarek and E. R. Blout, *Biopolymers*, 1992, **32**, 993.
12 M. A. Bednarek, B. E. Campbell, K. R. K. Easwaran and E. R. Blout, *Biopolymers*, 1987, **26**, S11.
13 T. Ishida, Y. In, M. Doi, M. Inoue, Y. Hamada and T. Shioiri, *Biopolymers*, 1992, **32**, 131.
14 Y. In, M. Doi, M. Inoue and T. Ishida, *Acta Crystallogr., Sect. A*, 1994, **50**, 432.
15 Y. In, M. Doi, M. Inoue and T. Ishida, *Acta Crystallogr., Sect. A*, 1994, **50**, 2015.
16 T. Ishida, M. Tanaka, M. Nabae and M. Inoue, *J. Org. Chem.*, 1988, **53**, 107.
17 F. J. Schmitz, M. B. Ksebati, J. S. Chang, J. L. Wang, M. B. Hossain, D. D. van der Helm, M. H. Engel, A. Serban and J. A. Silfer, *J. Org. Chem.*, 1989, **54**, 3463.
18 A. L. van den Brenk, D. P. Fairlie, G. R. Hanson, L. R. Gahan, C. J. Hawkins and A. Jones, *Inorg. Chem.*, 1994, **33**, 2280.
19 A. L. van den Brenk, PhD Thesis, The University of Queensland, 1994.
20 A. L. van den Brenk, K. A. Byriel, D. P. Fairlie, L. R. Gahan, G. R. Hanson, C. J. Hawkins, A. Jones, C. H. L. Kennard, B. Moubaraki and K. S. Murray, *Inorg. Chem.*, 1994, **33**, 3549.
21 D. J. Freeman, G. Pattenden, A. F. Drake and G. Siligardi, *J. Chem. Soc., Perkin Trans. 2*, 1998, 129.
22 L. Grøndahl, N. Sokolenko, G. Abbenante, D. P. Fairlie, G. R. Hanson and L. R. Gahan, *J. Chem. Soc., Dalton Trans.*, 1999, 1227.
23 D. L. Parry, PhD Thesis, The University of Queensland, 1988.
24 B. M. Degnan, C. J. Hawkins, M. F. Lavin, E. J. McCaffrey, D. L. Parry, A. L. van den Brenk and D. J. Watters, *J. Med. Chem.*, 1989, **32**, 1349.
25 G. Abbenante, D. P. Fairlie, L. R. Gahan, G. R. Hanson, G. K. Pierens and A. L. van den Brenk, *J. Am. Chem. Soc.*, 1996, **118**, 10384.
26 R. A. Binstead and A. D. Zuberbühler, SPECFIT, A program for global least squares fitting of equilibria and kinetic systems using factor analysis and Marquardt minimization, Version 2.09, 1993–1995, Spectrum Software Associates, Chapel Hill, NC, USA.
27 C. S. Wilcox and M. D. Cowart, *Tetrahedron Lett.*, 1986, **27**, 5563.
28 *teXan*, *Crystal Structure Analysis Package*, Molecular Structure Corp., The Woodlands, TX, 1985, 1992.
29 G. M. Sheldrick, SHELX-86, *Program for Structure Determination*, University of Gottingen, FRG, 1986.
30 D. T. Cromer and J. T. Waber, *International Tables for X-Ray Crystallography*, Kynoch Press, Birmingham, 1974, vol. 4.

31 J. A. Ibers and W. C. Hamilton, *Acta Crystallogr.*, 1964, **17**, 781.
32 D. C. Creagh and W. J. McAuley, *International Tables for Crystallography*, Kluwer Academic Publishers, Boston, 1992, vol. C.
33 D. C. Creagh and J. H. Hubbell, *International Tables for Crystallography*, Kluwer Academic Publishers, Boston, 1992, vol. C.
34 K. D. Kopple, G. Kartha, K. K. Bhandary and K. Romanowska, *J. Am. Chem. Soc.*, 1985, **107**, 4893.
35 K. D. Kopple, K. K. Bhandary, G. Kartha, Y.-S. Wang and K. N. Parameswarin, *J. Am. Chem. Soc.*, 1986, **108**, 4637.
36 K. D. Kopple, Y.-S. Wang, A. Go Cheng and K. K. Bhandary, *J. Am. Chem. Soc.*, 1988, **110**, 4168.
37 S. Y. Ko and C. Dalvit, *Int. J. Protein Res.*, 1992, **40**, 380.
38 S. Kimura and Y. Imanishi, *Biopolymers*, 1983, **22**, 2207.
39 Y. In, M. Doi, M. Inoue, T. Ishida, Y. Hamada and T. Shioiri, *Chem. Pharm. Bull.*, 1993, **41**, 1686.
40 V. F. Bystrov, *Spin-Spin Coupling and the Conformational States of Peptide Systems*, Pergamon Press, Oxford, UK, 1976, vol. 10.
41 T. Ishida, Y. In, F. Shinozaki, M. Doi, D. Yamamoto, Y. Hamada, T. Shioiri, M. Kamigauchi and M. Sugiura, *J. Org. Chem.*, 1995, **60**, 3944.
42 P. Hunt, R. M. Cusack, L. Grøndahl, G. Abbenante, D. P. Fairlie, L. R. Gahan and G. R. Hanson, unpublished results.
43 C. K. Vishwanath and K. R. K. Easwaran, *Biochemistry*, 1982, **21**, 2612.
44 Y. Kojima, Y. Ikeda, H. Miyake, I. Iwadou, K. Hirotsu, K. Shibata, T. Yamashita, A. Ohsuka and A. Sugihara, *Polym. J.*, 1991, **23**, 1359.
45 B. E. Campbell, K. R. K. Easwaran, G. C. Zanotti, M. A. Staples, E. T. Fossel and E. R. Blout, *Biopolymers*, 1986, **25**, S47.
46 V. Madison, C. M. Deber and E. R. Blout, *J. Am. Chem. Soc.*, 1977, **99**, 4788.

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