DALTON
FULL PAPER

Interaction of zinc(II) with the cyclic octapeptides, cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ and ascidiacyclamide, a cyclic peptide from *Lissoclinum patella*

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The interactions between zinc salts and the naturally occurring cyclic octapeptide ascidiacyclamide in methanol, as well as a synthetic analogue cyclo[Ile(Oxn)-D-Val(Thz)]$_2$, were monitored by ^{1}H NMR and CD spectroscopy. Three zinc complexes were identified, their relative amounts depending on the nature of the anion (perchlorate, triflate or chloride) and the presence or absence of base. Binding constants for two of the zinc species were calculated from CD or ^{1}H NMR spectra, $[Zn(L - H)]^+$ ($K_{Zn(L-H)} = [Zn(L - H)^+]/[Zn^{2+}][(L - H)^-] = 10^{7 \pm 2}$ M^{-1}; 95% methanol/5% water, 298.0 K, $NEt_3/HClO_4$ buffer 0.04 M) and $[ZnLCl]^+$ ($K_{ZnClL} = [ZnClL^+]/[Zn^{2+}][Cl^-][L] = 10^{7.2 \pm 0.1}$ M^{-2}; d_3-methanol, 301 K).

Introduction

Cyclic peptides having the 24-azacrown-8 macrocyclic structure have been isolated from the aplousobranch ascidian *Lissoclinum patella*.[1–4] Many examples of these cyclic peptides have been isolated and characterised, a common structural feature being the presence of oxazoline and thiazole rings. The patellamides (Chart 1) which exhibit these features are also differentiated by the presence of various substituents and by differences in the amino acids comprising the structure. A common and intriguing feature of these cyclic peptides is that their structures define a macrocyclic cavity and we,[5–7] and others,[8] have sought to exploit the macrocyclic properties of these compounds by studying their complexation reactions. That the marine environment from which these macrocyclic peptides originate contains inorganic salts suitable for complexation is unquestioned[8] although the role of these ligands in metal ion complexation and transport within their environment has not been fully elucidated.

The solid state structures of ascidiacyclamide and patellamide A have been reported to exhibit a saddle shaped arrangement[9–13] whereas patellamide D exhibits a twisted figure of eight configuration.[14] The saddle shaped arrangement is retained in the structure of the copper(II) complex of ascidiacyclamide[5] and the same structure was predicted for the copper(II) complex of patellamide D.[6] The two copper(II) ions in $[Cu_2(ascid)_2(\mu\text{-}CO_3)(H_2O)_2]\cdot 2H_2O$ are bonded through thiazole and oxazoline nitrogens and through a deprotonated isoleucine residue, each metal atom being five coordinate with the remaining coordination sites occupied by a water molecule and the bridging carbonate moiety.[5] There is evidence that in solution monomeric and dimeric species (*e.g.* [CuL] and $[Cu_2L]$; L = deprotonated ascidiacyclamide and patellamide D) are present[5–7] and for patellamide A, B and E species such as ML and M_2L [M = Cu(II), Zn(II)] have been predicted.[8] In the case of the complexes with patellamide A, B and E binding constants for the formation of the copper and zinc complexes, in the absence of base, have been reported.[8]

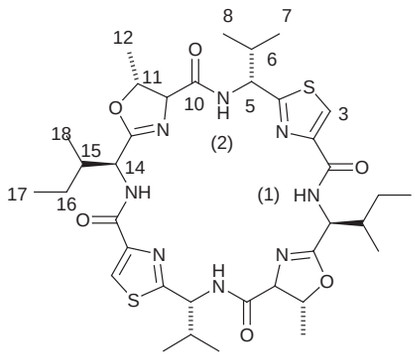
As part of a larger program aimed at investigating (i) the complexation properties of these cyclic peptides, and (ii) the effects on complexation of structural modification to the peptidic backbone,[15] we now report a study of the complexation reactions of two patellamide ligands, ascidiacyclamide (**1**) and an analogue cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ **2** with zinc(II) in methanol. Ascidiacyclamide differs from cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ by the presence of a methyl substituent on each of its oxazoline rings. This study, employing ^{1}H NMR and CD spectroscopy, investigated the role of base and anions in the complexation reactions of zinc(II) with **1** and **2**. The effects on complexation of the structural modifications will be reported in a subsequent publication.[15]

Experimental

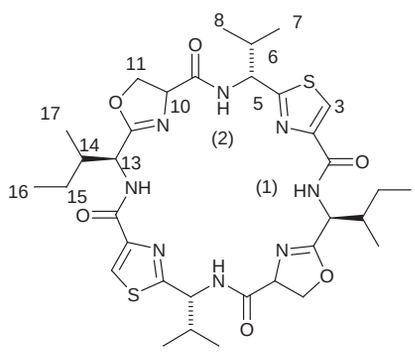
All reagents were of analytical grade and used without further purification (methoxycarbonylsulfamoyl)triethylammonium hydroxide (Burgess Reagent) was from Aldrich. Ascidiacyclamide was isolated and purified from *L. patella* collected from Heron Island on the Great Barrier Reef, Australia.[4] Cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ was prepared as described below. The peptides were found to be pure by HPLC and ^{1}H NMR and the water content was determined from CHN-analysis.

Mass spectra were obtained on a triple quadrupole mass spectrometer (PE SCIEX API III) equipped with an Ionspray (pneumatically assisted electrospray) atmospheric pressure ionisation source (ISMS). Solutions of compounds in 9:1 acetonitrile/0.1% aqueous trifluoroacetic acid for preparative work and methanol for analytical work were injected by syringe infusion pump at μM–pM concentrations and flow rates of 2–5 μl 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 was acquired by scanning quadrupole-1 from *m*/*z* 100–900 with a scan step of 0.1 Dalton and a dwell time of 2 ms.

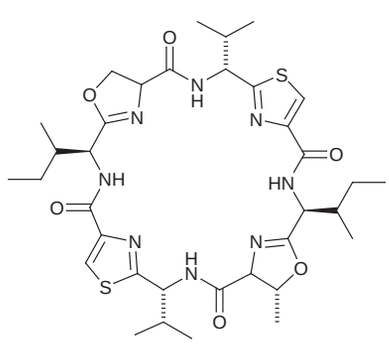
For preparative work, ^{1}H NMR spectra were recorded with either a Varian Gemini 300 or a Bruker ARX 500 spectrometer. Proton assignments were determined by pre-saturation experiments or 2D NMR experiments (DFCOSY, TOCSY). Preparative scale reversed phase HPLC separations were performed with Waters Delta-Pak PrepPak C_{18} 40 mm × 100 mm



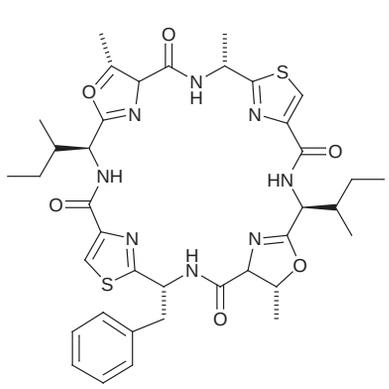
Ascidiacyclamide
(**1**)



cyclo[Ile(Oxn)-D-Val(Thz)]$_2$
(**2**)



Patellamide A



Patellamide D

Chart 1 Cyclic peptides.

cartridges (100 Å); analytical reversed phase HPLC was performed with a Vydac 218TP5415 C18 column using gradient mixtures of water/0.1% TFA (solvent system A) and water 10%/acetonitrile 90%/TFA 0.1% (solvent system B). The purified compounds were analysed by HPLC starting at (65% A, 35% B) using a linear gradient of 2% min^{-1}.

For analytical work, 1D and 2D NMR spectra were recorded with a Bruker 400 MHz instrument at 301 K or with a Bruker 500 MHz instrument at variable temperature. The CD spectra were recorded with a JASCO J-710 spectrometer equipped with a NESLAB temperature controller maintaining the cell at 25.00 ± 0.02 °C. The spectra were recorded on a 0.1 cm JASCO cell in the wavelength interval 240–300 nm and with a scan speed of 20 or 50 nm min^{-1}.

For the binding constant determinations all metal solutions were diluted with Milli Q water and analysed by ICP-AAS or ICP-MS. For the CD titrations in 95% methanol/5% water 0.1 or 0.2 equivalents of zinc (2 μL of a 0.01 M solution measured with a 10 μL Hamilton syringe) was added to 200 μL of a 1 mM peptide solution. A total of at least 14 spectra were used in binding constant determinations. No supporting electrolyte other than a $NEt_3/HClO_4$ buffer (0.04 M) was used. The program Specfit[16] (a program for global least squares fitting of equilibrium and kinetic systems using factor analysis and Marquardt minimization) was used to extract binding constants from the CD spectra by employing a model including free peptide, free metal and the 1 : 1 metal–peptide complex. ^{1}H NMR titrations were done in d_3- or d_4-methanol on 2 to 6 mM peptide solutions by adding zinc solutions with a 10 μL Hamilton syringe. No supporting electrolyte was used and the solutions were kept at ambient temperature. The binding constant was determined from the relative ratio of peptide and complex, obtained from the ^{1}H NMR data, by a semi-manual iterative procedure using the program KaleidaGraph.[17] The model included the three equilibria (eqns. (8), (9) and (10)) together with the concentration relations $C_L = [L] + [ZnClL]$, $C_{Zn} = [Zn^{2+}] + [ZnCl^+] + [ZnCl_2] + [ZnClL]$ and $C_{Cl} = 2C_{Zn} = [Cl^-] + [ZnCl^+] + 2[ZnCl_2] + [ZnClL]$. The binding constant was obtained from an average of three values calculated from final peptide:complex ratios of 7 : 4, 6 : 5 and 5 : 11.

Synthesis of cyclo[Ile(Oxn)-D-Val(Thz)]$_2$

Abbreviations: DIPEA = diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; BOP = [benzotriazol-1-yloxy-tris(dimethylamino)phosphonium] hexafluorophosphate; TFA = trifluoroacetic acid; KOBt = the potassium salt of hydroxybenzotriazole; Thz = thiazole; Oxn = oxazoline.

Boc-Ile-Ser-D-Val(Thz)-OEt. Boc-D-Val(Thz)-OEt[18] (2.9 g, 8.85 mmol) was stirred in TFA (30 ml) for 30 minutes. The solution was evaporated *in vacuo* and the oily residue neutralised with 5% $NaHCO_3$ (200 ml) and extracted with dichloromethane (2 × 50 ml). The organic extract was dried over Na_2SO_4, filtered and evaporated to give H-D-Val(Thz)-OEt as an oil. H-D-Val(Thz)-OEt was coupled to Boc-Ser-OH (2.5 g, 10 mmol) by dissolving both in DMF (50 ml) together with KOBt (1.7 g, 10 mmol) and BOP reagent (4.42 g, 10 mmol). After stirring at room temperature for 1 hour dimethylaminopropylamine (1.3 ml, 10 mmol) was added and the resulting mixture evaporated to dryness and redissolved in ethyl acetate (100 ml). The solution was washed with 10% $NaHSO_4$ (2 × 50 ml), 5% $NaHCO_3$ (2 × 50 ml) and brine (2 × 50 ml), dried over Na_2SO_4, filtered and evaporated to give Boc-Ser-D-Val(Thz)-OEt as an oil. The oil was deprotected with TFA and coupled to Boc-Ile-OH (2.40 g, 10 mmol) by dissolving both in DMF (30 ml) together with BOP (4.42 g, 10 mmol) and DIPEA (4.3 ml, 25 mmol) and stirring the mixture at room temperature for 1.5 hours. Dimethylaminopropylamine (1 ml, 8 mmol) was then added and the solution evaporated. The oily residue was redissolved in ethyl acetate (200 ml) and the solution was washed with 10% $NaHSO_4$, 5% $NaHCO_3$ and brine solutions, dried over Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography on silica gel using ethyl acetate as eluent to give the title compound as an oil (yield: 3.2 g, 69% based on initial Boc-D-Val(Thz)-OEt). ^{1}H NMR ($CDCl_3$) δ 8.08, s, 1H, Thz-H; 7.51, d, 1H, $J_{ValNH\text{-}Val\alpha}$ = 8.7 Hz, Val-NH;

7.32, d, 1H, $J_{SerNH\text{-}Ser\alpha}$ = 7.0 Hz, Ser-NH; 5.28, dd, 1H, $J_{ValNH\text{-}Val\alpha}$ = 8.7 Hz, $J_{Val\alpha\text{-}Val\beta}$ = 5.4 Hz, Val-αH; 5.05, d, 1H, $J_{IleNH\text{-}Ile\alpha}$ = 5.9 Hz, Ile-NH; 4.46, m, 1H, Ser-α; 4.38, q, 2H, $J_{CH_2\text{–}CH_3}$ = 7.0 Hz, OEt-CH_2; 4.16, m, 1H, Ser-βH; 4.03, m, 1H, Ile-αH; 3.72, m, 1H, Ser-βH; 3.36, s, 1H, Ser-OH; 2.42, m, 1H, Val-β; 2.00, m, 1H, Ile-βH; 1.50–1.59, m, 1H, Ile-γ(CH_2); 1.38, m, 3H, OEt-CH_3; 1.38, m, 9H, Boc-(CH_3); 1.10–1.18, m, 1H, Ile-γ(CH_2); 1.04, d, 3H, $J_{Val\beta\text{-}Val\gamma}$ = 6.6 Hz, Val-γ(CH_3); 0.85–1.01, m, 9H, Val-γ(CH_3), Ile-γ(CH_3), Ile-δ(CH_3). HPLC; retention time rt = 14.8 min. ISMS: M + H = 529.2.

Boc-Ile$_1$-Ser$_1$-Val$_1$(Thz)-Ile$_2$-Ser$_2$-Val$_2$(Thz)-OEt. Boc-Ile-Ser-D-Val(Thz)-OEt (450 mg, 0.852 mmol) was deprotected with 4 M HCl/dioxane solution (5 ml) for 30 minutes. The solution was evaporated and the residue redissolved in a mixture of CH_2Cl_2 (50 ml) and 5% $NaHCO_3$ (100 ml). The CH_2Cl_2 solution was separated, the water solution was washed with CH_2Cl_2 (1 × 50 ml) and ethyl acetate (1 × 50 ml). The combined organic layers were dried over Na_2SO_4 and evaporated to give H-Ile-Ser-D-Val(Thz)-OEt as an oil. Boc-Ile-Ser-D-Val(Thz)-OEt (450 mg, 0.852 mmol) was dissolved in a mixture of 1 M LiOH (1 ml) and ethanol (5 ml). The reaction mixture was stirred for 6 h at room temperature and acidified to pH ~ 9 with CO_2 (dry ice) and HOBt (14 mg, 0.1 mmol) was added. The solution was filtered, evaporated and redissolved in DMF (10 ml) and BOP reagent (443 mg, 1 mmol) added to H-Ile-Ser-D-Val(Thz)-OEt. The reaction was stirred for 4 h at room temperature and dimethylaminopropylamine (0.1 ml, 0.8 mmol) added. Ethyl acetate (100 ml) was added and the solution washed with 10% $NaHSO_4$ (2 × 50 ml), 5% $NaHCO_3$ (2 × 50 ml) and brine (1 × 50 ml), dried over Na_2SO_4, filtered and evaporated. The residue was dissolved in 15 ml of acetonitrile/water mixture (1 : 1) and purified using preparative HPLC to give the title compound as a white powder (669 mg, 86.3%). ^{1}H NMR (d_6-DMSO, 293 K): δ 8.59, d, 1H, J = 9.2 Hz, Val_2-NH; 8.54, d, 1H, J = 9.2 Hz,Val_1-NH; 8.42, s, 1H, Thz-H; 8.30, d, 1H, J = 8.7 Hz, Ser_2-NH; 8.19, s, 1H, Thz-H; 7.89, d, 1H, J = 8.7 Hz, Ile_2-NH; 7.79, d, 1H, J = 8.5 Hz, Ser_1-NH; 6.79, d, 1H, J = 8.8 Hz, Ile_1-NH; 4.95, m, 4H, Val_1-αH,Val_2-αH, Ser_1-βH, Ser_2-βH; 4.89, m, 3H, Ser_1-βH, Ser_2-αH, Ile_2-αH; 4.29, q, 2H, J = 7.9 Hz, -OEt; 3.84, m, 1H, Ile_1-αH; 3.60, m, 2H, Ser_1-αH, Ser_2-αH; 2.28, m, 2H, Val_1-βH, Val_2-βH; 1.79, m, 1H, Ile_2-βH; 1.68, m, 1H, Ile_1-βH; 1.42, m, 2H, Ile_1-γCH, Ile_2-γCH; 1.34, s, 9H, Boc; 1.29, t, 3H, J = 7.9 Hz, -OEt; 1.06, m, 2H, Ile_1-γCH, Ile_2-γCH; 0.94–0.80, m, 12H, Val_1-γCH_3, Val_2-γCH_3; 0.80–0.73, m, 12H, Ile_1-γCH_3, Ile_2-γCH_3, Ile_1-δCH_3, Ile_2-δCH_3. HPLC; rt = 17.1 min. ISMS: M + H = 911.7.

Cyclo-[-Ile-Ser-D-Val(Thz)-]$_2$. Boc-[Ile-Ser-D-Val(Thz)-]$_2$-OEt (650 mg, 0.714 mmol) was dissolved in 4 M HCl/dioxane solution (10 ml) for 30 min at room temperature. The solution was evaporated and the residue was redissolved in a mixture of 1 M LiOH (2 ml) and ethanol (10 ml). The reaction mixture was stirred for 6 h at room temperature, then acidified to pH ~ 9 with CO_2 (dry ice) and HOBt (14 mg, 0.1 mmol) was added. The solution was filtered, evaporated and redissolved in DMF (20 ml) and the solution added *via* syringe pump to a solution of BOP reagent (885 mg, 2 mmol) in DMF (200 ml) over 5 h. The reaction mixture was stirred overnight and evaporated. The residue was dissolved in 15 ml of acetonitrile/water mixture (1 : 1) and purified using preparative HPLC to give the title compound as a white powder (209 mg, 38.4%). ^{1}H NMR (d_6-acetone) δ 9.52, d, 1H, J = 5.5 Hz, Ile-NH; 8.67, d, 1H, J = 6.0 Hz, Ser-NH; 7.79, d, 1H, J = 9.5 Hz, Val-NH; 7.47, s, 1H, Thz-H; 5.34, m, 1H, Val-αH; 4.47, m, 1H, Ser-αH; 4.12, m, 1H, Ser-βH; 3.99, m, 1H, Ser-βH; 3.93, dd, $J_{Ile\alpha\text{-}IleNH}$ = 5.5 Hz, $J_{Ile\alpha\text{-}Ile\beta}$ = 6.0 Hz, 1H, Ile-αH; 2.42, m, 1H, Val-β; 2.39, m, 1H, Ile-β; 1.81, m, 1H, Ile-γCH_2; 1.29, m, 1H, Ile-γCH_2; 1.14, d, 3H, $J_{Ile\beta\text{-}Ile\gamma}$ = 7 Hz, Ile-γCH_3; 1.07, d, 3H, $J_{Val\beta\text{-}Val\gamma}$ = 6.5 Hz, Val-γCH_3; 1.03, d, 3H, $J_{val\beta\text{-}Val\gamma}$ = 6.5 Hz, Val-γCH_3; 0.89, t, 3H, $J_{Ile\delta\text{-}Ile\gamma}$ = 7.3 Hz, Ile-δCH_3. ISMS: M + H = 765.4. HPLC; rt = 13.0 min.

Table 1 ^{1}H NMR chemical shifts for **2**, **3** and **5** (CD_3OH)

Proton(s)	**2**	**3**	**5**
3	7.93 (s)	7.94 (s)	7.97 (s), 8.06 (s)
5	5.17 (dd)	4.9 (d)	5.1
6	2.38 (m)	2.6 (m)	2.65, 3.00 (m)
7	1.06 (d)	1.21 (d)	1.12, 1.14 (d)
8	1.15 (d)	0.8–0.9 (d)	0.85, 0.88 (d)
10	—	—	—
11	—	—	—
13	—	—	4.9
14	2.00 (m)	1.75 (m)	1.65 (m), 1.9 (m)
15	1.4 (m), 1.2 (m)	1.4 (m), 1.2 (m)	1.1 (m), 1.2 (m)
16	0.80 (t)	0.8–0.9 (t)	0.70 (t), 0.82 (t)
17	0.82 (d)	0.21 (d)	0.34 (d), 0.34 (d)
N(1)	7.99 (J = 7.8 ± 0.4)	—	8.79
N(2)	7.53 (J = 10.1 ± 0.3)	—	8.76, 8.83

Cyclo[-Ile(Oxn)-D-Val(Thz)-]$_2$. Cyclo[-Ile-Ser-D-Val(Thz)-]$_2$ (200 mg, 0.262 mmol) and Burgess Reagent (120 mg, 0.5 mmol) were dissolved in dry THF (5 ml). The reaction mixture was heated and stirred for 5 h at reflux temperature and evaporated. The residue was dissolved in 15 ml of acetonitrile/water mixture (1 : 1) and purified using preparative HPLC to give the title compound as a white powder (25 mg, 13.2%). ^{1}H 500 MHz NMR ($CDCl_3$) δ 8.02, s, 2H, Thz-H; 8.02, d, 2H, Ile-NH; 7.30, d, 2H, $J_{ValNH\text{-}Val\alpha}$ = 10 Hz, Val-NH; 5.21, m, 2H, Val-α; 4.82, m, 2H, Oxn-H; 4.74, m, 2H, Ile-α; 4.66, m, 2H, Oxn-H; 4.58, m, 2H, Oxn-H; 2.29–2.34, m, 2H, Val-β; 1.95, m, 2H, Ile-β; 1.30, m, 2H, Ile-γCH_2; 1.17, m, 2H, Ile-γCH_2; 1.13, d, 6H, $J_{Val\beta\text{-}Val\gamma}$ = 6.7 Hz, Val-γCH_3; 1.05, d, 6H, $J_{Val\beta\text{-}Val\gamma}$ = 6.6 Hz, Val-γCH_3; 0.80, d, 6H, $J_{Ile\beta\text{-}Ile\gamma}$ = 6.7 Hz, Ile-γCH_3; 0.73, t, 6H, $J_{Ile\gamma\text{-}Ile\delta}$ = 7.2 Hz, Ile-δCH_3. HPLC; rt = 17.4 min. ISMS: M + H = 729.2.

Results and discussion

NMR characterisation of cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ 2

The ^{1}H NMR spectrum of **2** in d_3-methanol was assigned on the basis of 2D COSY and TOCSY NMR spectra (Table 1). Both amide protons of **2** had small temperature coefficients (Ile NH 0.2 ppb/deg, Val NH 1.1 ppb/deg) indicating relatively little interaction of these protons with the solvent.[19] The proton/deuterium exchange rate of both amide protons of **2** was very slow, *i.e.* in d_3-methanol with 12% d_4-methanol no proton exchange could be detected over a month. This implies that the NH groups have very limited exposure to the solvent.[19] However, the proton/deuterium exchange rate of the Ile N(1)H protons in d_4-methanol is faster (within 5 minutes) than that of the Val N(2)H proton (within 10 minutes). Similarly, the proton/deuterium exchange rate in **1** in d_3-acetonitrile with 3% d_4-methanol is faster for the Ile N(1)H proton ($t_{1/2}$ ~ 14 days) than the Val N(2)H proton ($t_{1/2}$ > one month).

Since small temperature coefficients as well as slow proton exchange are observed for both amide protons of **2** it seems likely that the peptide takes one of the conformations found for the other patellamides,[9,14] type II and type III (Chart 2). In both of these two conformations the amide protons are shielded from the solvent. In type II the amide protons are buried within the cavity of the saddle, whilst in type III all amide protons take part in intramolecular hydrogen bonding. Thus, slow proton exchange as well as small temperature coefficients are expected for both conformations and cannot therefore be used to distinguish between them.

It has been found from solid state structures of **1** that in the saddle shape conformation, type II, the peptide is rather flexible in that the C(O)–N–C_α–(Thia/Oxn) torsion angles vary for

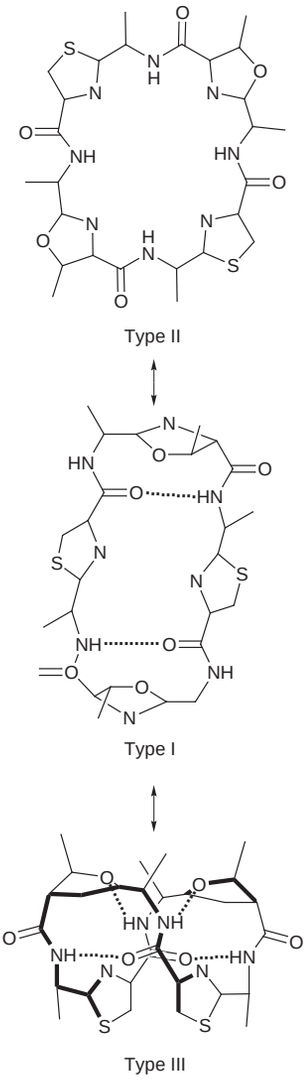


Chart 2 Conformations of the patellamides (taken from ref. 10).

different solvates of the peptide.[10] Thus, the Ile torsion angle varies from −127 to −160° and the Val torsion angle varies from 98 to 134°.[9–12] Translating these torsion angles into $^3J_{HNC\alpha H}$ coupling constants using the possible range of parameters in the Bystrov–Karplus equation[20] yields $^3J_{HNC\alpha H}$ (Ile) of 6.8 to 11.6 Hz, and $^3J_{HNC\alpha H}$(Val) 8.5 to 11.5 Hz. Patellamide D takes the type III conformation in the solid state and translating torsion angles from this structure[14] into coupling constants yields $^3J_{HNC\alpha H}$(Ile) 5.4 to 7.8 Hz and $^3J_{HNC\alpha H}$(Val) 7.3 to 9.8 Hz. The observed coupling constants for **2** (Table 1, $^3J_{HNC\alpha H}$(Ile) 7.8 ± 0.4 Hz and $^3J_{HNC\alpha H}$(Val) 10.1 ± 0.3 Hz) fall within either of these ranges, thus, the coupling constants for the patellamides do not provide information on the conformation in solution.

A 2D NOESY NMR spectrum of **2** in d_3-methanol yielded a number of non-sequential cross peaks (3–5, 3–8, N2–7, N2–8, N2–16, N2–17, N1–16, N1–17, 13–16 and 13–17) which implies type II conformation of the peptide. In particular the N2–17 cross peak points towards type II; the equivalent distance in **1** is 3 Å[9–12] and in patellamide D it is >5 Å,[14] thus a type II conformation seems most likely and would be in agreement with the result of a study by CD spectroscopy on the structurally similar peptide patellamide A where it was found that at room temperature the peptide took the saddle shape, type II conformation.[8]

Zn^{2+}–peptide interactions

Zinc binding to the cyclic octapeptides **1** and **2** was followed by CD and ^{1}H NMR spectroscopy. In d_3-acetonitrile broadening

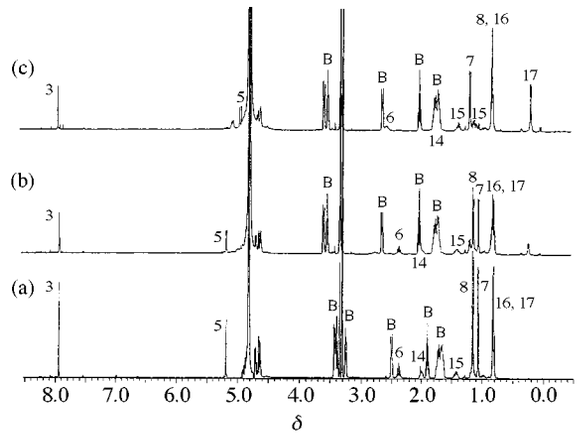


Fig. 1 ^{1}H NMR spectra in d_4-methanol of: (a) a solution containing cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ (1.94 mg, 0.00248 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (1 μl, 0.0067 mmol) in 0.7 ml; (b) added zinc triflate solution (0.00306 mmol); (c) added zinc triflate (a total of 0.0051 mmol). [B = signals arising from 1,8-diazabicyclo[5.4.0]undec-7-ene; numbers refer to assignments of the resonances of peptide **2** in (a) and (b) and of complex **3** in (c) (see Table 1)].

and changes in the chemical shifts of the NMR signals, as well as the development of new broad signals, were observed when a zinc solution was added to a peptide solution and this solvent was therefore not suitable for a study of zinc peptide interactions. In d_3-methanol it was found that the zinc ion was not substitutionally labile on the NMR time scale. Similar slow exchange behavior has been reported for calcium complexes of bicyclic peptides.[21,22] With the peptides **1** and **2**, as the zinc concentration increased a new set of signals appeared and the original peptide signals disappeared. Therefore, d_3-methanol was chosen for a detailed investigation of zinc interactions with the peptides. It was found that up to three zinc complexes could be observed, their relative amounts depending on the type of anion and the presence of base. In the following text we will focus on the conditions under which we observe simple reaction stoichiometry, that is when using triflate or perchlorate salts in the presence of base, as well as when using the chloride salt in the absence of base. Having chloride ion as well as base present leads to very complicated systems which will also be described briefly.

In all studies with the peptides **1** and **2**, we have not observed any differences in their behaviour. Therefore, in the following text we assume that the reactivities of **1** and **2** are identical. However, we do point out on which peptide a specific experiment was done.

Peptide interactions with zinc triflate or perchlorate

In the absence of base, adding zinc triflate or zinc perchlorate to a solution of **2** in d_3-methanol (up to 8 equivalents of zinc to peptide) gave no change in the ^{1}H NMR spectrum. However, in the presence of a base, complex formation was observed and could be followed by ^{1}H NMR without precipitation of $Zn(OH)_2$ when 1,8-diazabicyclo[5.4.0]undec-7-ene was used as base (3 equivalents to the peptide). Titration with up to one equivalent of zinc triflate led to shifts in the base signals but no changes were observed for the peptide signals. The final spectrum after addition of a total of 2 equivalents of zinc triflate showed the presence of one new species, complex **3**, and no signals from the free peptide remained (Fig. 1). We interpret this as the base interacting more strongly with zinc than does the peptide; however, after saturating the base the second equivalent of zinc reacts with the peptide forming a 1:1 complex. Using **1** in place of **2** gives similar changes upon zinc addition.

Investigation of the zinc complexation to the peptide in the presence of triethylamine by CD-spectroscopy showed a gradual spectral change (Fig. 2). The plot (at 265 nm) of θ

Table 2 Electrospray mass spectral data for cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ and its zinc complexes in methanol

Species	*m/z* Exptl.[a]	Calc.[b]
Cyclo[Ile(Oxn)-D-Val(Thz)]$_2$		
LH^+	729.2	729.9
$L(H_2O)H^+$	747.6	747.9
$L(CF_3SO_3H_2)^+$	879.3	879.9
Mononuclear zinc cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ complexes		
$Zn(L - H)^+$ [c]	791.2	791.3
$ZnL(CF_3SO_3)^+$ [c]	941.3	941.3
$Zn(L - 2H)Na^+$	813.4	814.3
$Zn(L - H)(H_2O)(CH_3OH)^+$	843.5	841.3
$ZnLCl^+$ [c]	827.3	827.8
Dinuclear zinc cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ complexes		
$Zn_2(L - 2H)Cl(H_2O)^+$	908.3	908.2

[a] Cone voltage 40 V. [b] Calculated mass over charge ratios were determined using naturally abundant isotopes. [c] Cone voltage 120 V.

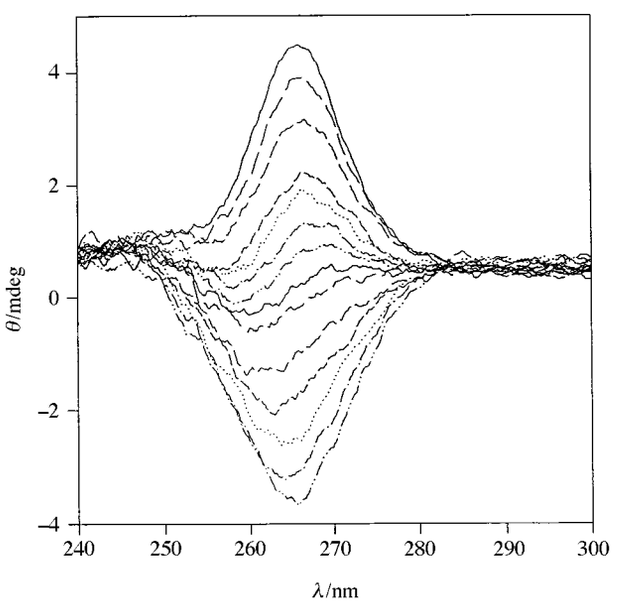


Fig. 2 CD titration of a 95% methanolic solution containing cyclo-[Ile(Oxn)-D-Val(Thz)]$_2$ (1.00 mM, 0.2 μmol) and triethylamine/perchloric acid buffer, $[NEt_3] = 0.04$ M, $[HClO_4] = 0.02$ M, added (from positive to negative Cotton effect) 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.20, 0.24, 0.28, 0.32 and 0.40 μmol zinc perchlorate.

(mdeg) *versus* equivalents of added zinc(II) reached a plateau after approximately two equivalents of zinc perchlorate. Further addition of zinc caused additional changes in the spectrum but no second plateau was observed after adding seven equivalents of zinc. Employing the data up to the addition of two equivalents of zinc for the determination of a binding constant resulted in a model including a 1:1 complex giving a significantly better fit to the data (half as large residuals) than a model including a 2:1 complex.

The mass spectrum of **2** and zinc triflate indicated the presence of a $[ZnL(CF_3SO_3)]^+$ species but no dimeric zinc peptide complex could be detected (Table 2). However, the mass spectrum of a solution of **2** and zinc chloride indicate the presence of both mononuclear and dinuclear complexes containing chloride, *i.e.* $[ZnLCl]^+$ and $[Zn_2(L - 2H)Cl(H_2O)]^+$ (Table 2).

Structure of the zinc–peptide complex 3

The zinc–peptide complex **3** formed in solutions containing cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ (or ascidiacyclamide), base and zinc triflate (or perchlorate). ^{1}H NMR titrations in the presence of the base 1,8-diazabicyclo[5.4.0]undec-7-ene together with CD and mass spectroscopy gave evidence for **3** being a 1:1 (metal:peptide) complex (see above). Since the complex only forms in the presence of base we suggest that the peptide is partly deprotonated. On the basis of proton/deuterium exchange rates of the free peptide as well as comparison with the copper structure,[5] the most likely site for deprotonation is the isoleucine amide nitrogen. The ^{1}H NMR spectrum of complex **3** was assigned on the basis of 2D COSY and TOCSY NMR spectra, and chemical shifts of the peptide **2** and of the zinc complex **3** are given in Table 1.

In **3** the number of signals in the ^{1}H NMR spectra implies that the molecules have C_2 symmetry. In order to form a C_2-symmetric zinc complex in which one zinc is bound to a partly deprotonated peptide, we must assume deprotonation at two identical amide nitrogens to form a mononuclear complex, *i.e.* $[Zn(L - 2H)]$. However, it is hard to justify that zinc chooses to form a C_2-symmetric complex when it requires deprotonation of two amide protons. It is possible that the apparent C_2-symmetry arises from fast exchange or fast molecular motion which, on average, yields a C_2-symmetric molecule. A low temperature ^{1}H NMR study (down to −90 °C) of complex **3** was not able to confirm this as slow exchange or freezing out of one dominant conformation was not achieved.

In conclusion complex **3** is proposed to be a $[Zn(L - H)]^+$ complex in which the zinc ion is coordinated to deprotonated isoleucine amide nitrogen and probably also to one or two other donor atoms from the peptide.

Kinetic effects in zinc–peptide interactions in the presence of base

Although the Zn^{2+} ion is generally considered labile, a close-fitting ligand can alter the reaction rate significantly as has been observed in a macrobicyclic complex.[23] Furthermore, slow kinetics in barium ion binding to cyclic tetrapeptides in 95% methanol have been observed.[24,25]

Formation of complex **3** was monitored by CD spectroscopy. The spectrum of a solution of **1**, triethylamine (4 equivalents to peptide) and zinc perchlorate (1.5 equivalents to peptide) was indicative of partial zinc–peptide complex formation. The solution was monitored over one hour and no change in the spectrum could be observed. Addition of perchloric acid (4 equivalents to peptide) immediately reformed the spectrum of the free peptide. Thus, there seems to be no kinetic effects in the formation of complex **3** and reversibility in the system is evident (eqn. (1)). Therefore we chose to quantify this by a binding constant determination employing CD spectroscopy (see below).

Investigation of the formation of complex **3** by NMR spectroscopy over a longer time period resulted in the emergence of a more complicated picture. When adding one equivalent of zinc triflate to a solution of 1 mM **2** and three equivalents of base (triethylamine or 1,8-diazabicyclo[5.4.0]undec-7-ene), partial formation of complex **3** was observed instantly. However, after three days another species, complex **4**, was present as well (Fig. 3). Looking at the sample over months it was clear that the formation of **4** was indeed a very slow process. Thus, in the presence of base an initial equilibrium is established forming complex **3** (eqn. (1)) followed by an additional slow process forming complex **4** (eqn. (2)).

$$Zn^{2+} + L \overset{\text{fast}}{\rightleftharpoons} [Zn(L - H)]^+ + H^+ \quad (1)$$

$$[Zn(L - H)]^+ \overset{\text{slow}}{\rightleftharpoons} [Zn(L - H)^*]^+ \quad (2)$$

Due to co-precipitation of zinc hydroxide in aged basic solutions it was not possible to extract quantitative information from these NMR experiments and a binding constant for

Table 3 Binding constants for base chloride assisted formation of Zn^{2+}–peptide complexes in 95% methanol

Ligand	Base	Metal salt	log K
1	NEt_3–buffer[a]	$Zn(ClO_4)_2 \cdot 6H_2O$	3.0 ± 0.3[c,d]
2	NEt_3–buffer[a]	$Zn(ClO_4)_2 \cdot 6H_2O$	2.7 ± 0.3[c,d]
2	NEt_3[b]	$Zn(ClO_4)_2 \cdot 6H_2O$	2.9 ± 0.3[c,d]
2	NEt_3[b]	$Zn(CF_3SO_3)_2$	3.4 ± 0.3[c,d]
1	—	$ZnCl_2$	7.2 ± 0.1[e,f]

[a] 0.04 M NEt_3 and 0.02 M $HClO_4$. [b] 4 equivalents. [c] K_{obs} in eqn. (3). [d] Determined from CD titration. [e] K_{ZnCl_L} in eqn. (8), an average of three values calculated from final peptide : complex ratios of 7 : 4, 6 : 5 and 5 : 11. [f] Determined from batch NMR titration in d_3-methanol.

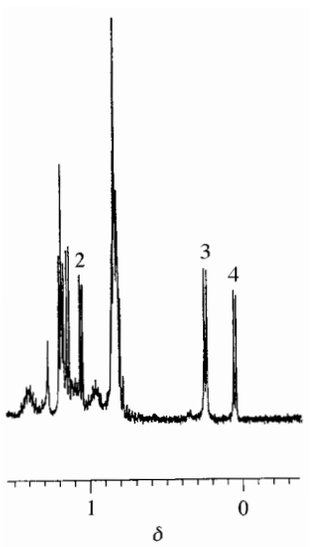


Fig. 3 Methyl region of a ^1H NMR spectrum of cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ (1.14 mg, 0.0016 mmol) in 0.7 ml d_4-methanol with added 1,8-diazabicyclo[5.4.0]undec-7-ene (0.005 mmol) and zinc triflate (0.0019 mmol); spectrum recorded after 4 months. (Numbers refer to resonances assigned to peptide **2**, complex **3** and complex **4**.)

complex **4** could not be obtained. In solutions of **2**, base and zinc triflate the ratio between **2** and complex **3** and **4** indicates that **4** is also a 1 : 1 (metal : peptide) complex. Since it (like **3**) only forms in the presence of base, we suggest that **4** is also a $[Zn(L - H)]^+$ complex in which the zinc ion binds differently to the peptide than in complex **3**. Rearrangements in zinc peptide complexes have been observed for other systems.[26]

Binding constant for the Zn^{2+}–peptide complex 3

The spectral changes associated with zinc addition (up to 2.4 equivalents, Fig. 2) in basic solutions were used to determine the binding constant for complex **3**. Measurements were performed in 95% methanol solutions either containing four equivalents of NEt_3, or in buffered solutions with 0.04 M NEt_3 and 0.02 M $HClO_4$. No supporting electrolyte other than the buffer was used. The results (Table 3) show that NEt_3 solutions and the buffered solutions yield the same value for the binding constant for a 1 : 1 complex. Furthermore, there is no effect of the peptide (ascidiacyclamide **1** *versus* cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ (**2**)) or of the anion (perchlorate *versus* triflate) within experimental error.

Peptide solutions containing the base 1,8-diazabicyclo-[5.4.0]undec-7-ene instead of NEt_3 show similar changes upon addition of zinc. However, the data did not indicate simple formation of a 1 : 1 complex which is probably due to the zinc ion interacting with the base, as was also concluded from the ^1H NMR experiments. The perchlorate salts of lithium, sodium, magnesium, calcium and barium, all metals that prefer oxygen donor ligands, did not give rise to spectral changes when performing titrations similar to the one described above.

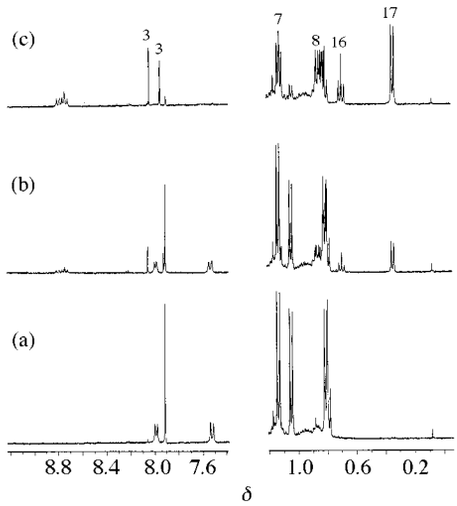


Fig. 4 Selected regions of the ^1H NMR spectra of: (a) a solution containing cyclo[Ile(Oxn)-D-Val(Thz)]$_2$ (1.3 mg, 0.0017 mmol) in 0.7 ml d_3-methanol; (b) added zinc chloride solution (0.009 mmol zinc); (c) added zinc chloride solution (a total of 0.075 mmol). [Numbers refer to assignments of the resonances of complex **5** (see Table 1).]

The calculated binding constant obtained in titrations using zinc perchlorate or zinc triflate (log $K = 3.0 \pm 0.3$) describes the overall equilibrium of eqn. (3) (L is **1** or **2**), in which triethyl-

$$Zn^{2+} + L + NEt_3 \overset{K_{obs}}{\rightleftharpoons} [Zn(L - H)]^+ + HNEt_3^+ \quad (3)$$

amine assists deprotonation of the peptide. We have chosen to describe zinc perchlorate and zinc triflate as being totally dissociated as there is evidence that this is the correct description in a methanol solution when the concentration of the salt is less than 0.2 M.[27] Eqn. (3) can be described as a sum of eqn. (4)–(6).

$$Zn^{2+} + (L - H)^- \overset{K_{Zn(L-H)}}{\rightleftharpoons} [Zn(L - H)]^+ \quad (4)$$

$$HNEt_3^+ \overset{K^H_{NEt_3}}{\rightleftharpoons} NEt_3 + H^+ \quad (5)$$

$$L \overset{K^H_L}{\rightleftharpoons} (L - H)^- + H^+ \quad (6)$$

The binding constant $K_{Zn(L-H)}$ (eqn. (4)) which describes the binding of zinc to the deprotonated peptide is the formation constant for the complex. Unfortunately, the protonation constants for amides are not known, and therefore the formation constant can only be estimated. By assuming $pK^H_L = 15$ (amide deprotonation),[28] and $pK^H_{NEt_3} = 10.9$,[29] we estimate the value to be $K_{Zn(L-H)} = [Zn(L - H)^+]/[Zn^{2+}][(L - H)^-] = 10^{7 \pm 2}\ M^{-1}$. The binding constant for the non-base assisted formation of the complex can be calculated using eqn. (3) and (5) and yields $K = [Zn(L - H)^+][H^+]/[Zn^{2+}][L] = 10^{-7.9 \pm 0.3}\ M^{-1}$. Clearly, the assistance from a moderately strong base ($pK_a > 9$) is needed for the formation of complex **3** in which the zinc ion stabilises the deprotonated peptide.

Peptide interactions with zinc chloride in the absence of base

When zinc chloride is added to a solution of **2** in d_3-methanol changes in the ^1H NMR spectrum are observed (Fig. 4) and similar changes are observed when **1** is used in place of **2**. These changes are assigned to formation of a product, complex **5**, which lacks C_2 symmetry. When a total of 6 equivalents of zinc chloride has been added to the peptide solution approximately 80% of peptide still remains unreacted. When a total of 45 equivalents of zinc chloride has been added 10% remains. Thus, in the course of adding 45 equivalents of zinc chloride a gradual formation of a new product is evident. By CD spectroscopy,

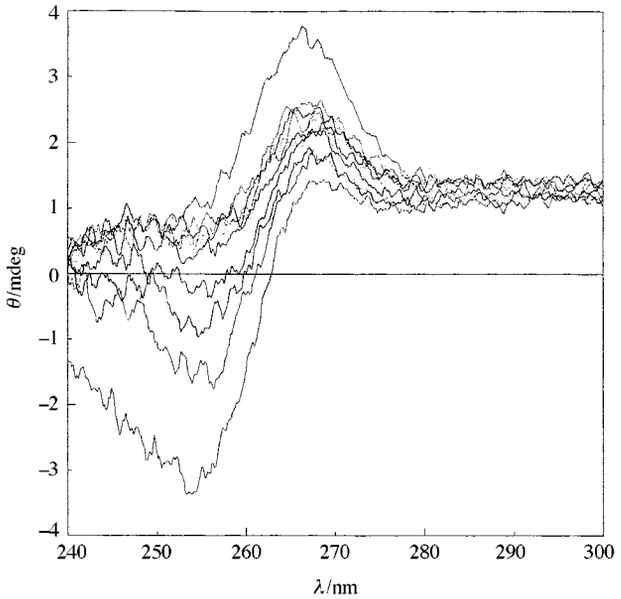


Fig. 5 CD titration of a 95% methanolic solution containing cyclo-[Ile(Oxn)-D-Val(Thz)]$_2$ (1.1 mM, 0.21 μmol) with added (from positive to negative Cotton effect), 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, 4.5, 5.5 and 7.5 equiv. zinc chloride.

the spectral changes associated with addition of zinc chloride seem to reach a plateau after addition of 4 equivalents (Fig. 5). Further addition of zinc chloride results in further changes. Applying a simple model similar to that used by Freeman *et al.*[8] (eqn. (7)) for formation of a 1 : 1 complex for the first part of

$$Zn^{2+} + L \xrightleftharpoons{K_{ZnL}} [ZnL]^{2+} \quad (7)$$

this titration yields a binding constant which approximates that reported for patellamide A (log $K = 4.5$).[8] In that work the implication was that the equilibrium is shifted totally to formation of the 1 : 1 complex when 4 equivalents of zinc chloride is added. However, in our work the ^{1}H NMR titration showed that a simple 1 : 1 complex is not totally formed at this point in the titration. It is clear that a different model is needed to explain the observed changes in the CD spectra upon addition of zinc chloride.

Structure of the zinc–peptide complex 5

Complex **5** formed in solutions of **1** or **2** and zinc chloride. The ^{1}H NMR spectrum of **5** in d$_3$-methanol shows only three amide signals, thus one amide signal is missing. From 2D TOCSY NMR experiments two valine and one isoleucine amide protons could be observed. One explanation for the absence of one amide signal is that deprotonation at an amide nitrogen has occurred as a consequence of zinc binding to the amide nitrogen in a metal assisted deprotonation reaction. However, if that was the case, one would expect this complex to dominate in basic solution which is not the case as complex **3** (which has a very low binding constant) is dominating at low metal to peptide ratios (see later). Another explanation for the absence of one of the amide signals in the ^{1}H NMR spectrum is that zinc is binding to the neutral amide linkage, thereby enhancing the proton exchange rate of the amide proton leading to broadening of the signal. Enhanced proton exchange rates upon zinc binding has been observed for other peptides.[30] Binding a metal ion to a neutral amide linkage can occur through the amide oxygen of the amide tautomer (**a** in Chart 3) or the amide nitrogen of the imidol tautomer (**b** in Chart 3).[31] Of the two potential coordination sites the carbonyl oxygen is considered the most likely on the basis of protonation, infrared and crystallographic studies[28] and to the best of our knowledge there is no

a b

Chart 3 Metal binding modes to a neutral amide linkage.

crystallographic evidence for a zinc ion coordinated to the nitrogen of the imidol tautomer of a neutral amide linkage. The ^{1}H NMR spectra of complexes **3** and **5** are very similar, both containing highly shielded Ile methyl groups (Table 1), thus suggesting that the peptide conformation is similar for the two complexes, but not necessarily that the zinc ion is coordinated to the peptide in a similar manner. On this basis we propose that the zinc ion in complex **5** is coordinated to the isoleucine amide oxygen of the neutral amide linkage and it is likely that the metal also binds to one or two other donor atoms of the peptide. Since complex **5** only can be detected in the presence of chloride ion it is assumed that chloride is also coordinated to zinc in the complex.

Kinetic effects in zinc–peptide interactions in the absence of base

In our studies we found that zinc binding, particularly in the absence of base and presence of chloride ion, is a slow process. For example, adding 20 equivalents of zinc chloride to a solution of **2** and leaving it for three days changes the extent of reaction from 54 to 65%. After an additional week, no further reaction had taken place. Due to the slow kinetics we decided to obtain a binding constant for complex **5** by monitoring the ^{1}H NMR spectra of equilibrated solutions of peptide **1** and zinc chloride in different ratios.

Binding constant for the zinc–peptide–chloride complex 5

Solutions of **1** and zinc chloride in d$_3$-methanol were kept at ambient temperature and the spectra recorded frequently over two weeks to ensure that equilibrium had been reached. The relative intensities of the thiazole signals (obtained from the thiazole integrals) provided the relative ratio of starting material **1** and complex **5** which were used to calculate the binding constant.[32] The model used to calculate a binding constant for complex **5** took into account that zinc chloride is not dissociated in methanol under the conditions studied (log $K_1 = 3.9$ and log $K_2 = 4.2$; eqn. (9) and (10)).[33] Considering each of the

$$Zn^{2+} + L + Cl^- \xrightleftharpoons{K_{ZnClL}} [ZnClL]^+ \quad (8)$$

$$Zn^{2+} + Cl^- \xrightleftharpoons{K_1} ZnCl^+ \quad (9)$$

$$ZnCl^+ + Cl^- \xrightleftharpoons{K_2} ZnCl_2 \quad (10)$$

four complexes (Zn_xCl_yL, x and $y = 1$ or 2) as possible candidates for complex **5**, it was found that a complex with the stoichiometric formula ZnClL gave the best fit to the data (agreement between binding constants at different points in the titration was more than 5 times better than any other considered stoichiometric formula). Thus, the model included eqn. (8) to (10) and yielded a binding constant of $K_{ZnClL} = [ZnClL^+]/[Zn^{2+}][Cl^-][L] = 10^{7.2 \pm 0.1}$ M^{-2} (Table 3). Despite the high binding constant for complex **5** a large excess of zinc chloride is needed to completely form the complex because of the competing equilibria (eqn. (9) and (10)) forming the $ZnCl^+$ and $ZnCl_2$ complexes.

Peptide interactions with zinc chloride in the presence of base

In ^{1}H NMR titrations of **2** with zinc chloride in the presence of base (triethylamine or 1,8-diazabicyclo[5.4.0]undec-7-ene) the three complexes **3**, **4** and **5** could all be observed. The ratio

between the three complexes changed throughout the titration; complex **3** was initially the major product but after two equivalents of zinc chloride had been added complex **5** dominated. In solutions which had been left for several days, the ratio between the complexes had changed further. Obviously the product distribution at any time in the titration is controlled by thermodynamic as well as kinetic factors. Due to the complexity of this system these titrations were not taken any further.

Conclusion

It was possible to obtain reliable binding constants for the $[Zn(L - H)]^+$ complex (in the presence of base) and for the $[ZnClL]^+$ complex (in the absence of base). In our study of the patellamides **1** and **2** we have shown the importance of taking into account kinetic factors. Also we found that no simple ZnL^{2+} complex formed which illustrates the importance of examining the species of the reaction mixture before calculating binding constants. The binding constant for a zinc patellamide A complex ($K = 10^{4.5}$) was determined previously assuming a simple ZnL^{2+} complex, thus, employing the model described by eqn. (7).[8] Although similar constants can be calculated for the structurally similar **1** and **2** (Chart 1), we have shown by NMR that the model described by eqn. (7) does not adequately describe the species in solution, instead a model described by eqn. (8)–(10) was employed for the system.

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