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Metal binding of *Lissoclinum patella* metabolites. Part 1: Patellamides A, C and ulithiacyclamide A

Linda A. Morris,[a] Marcel Jaspars,[a,*] J. Jantina Kettenes-van den Bosch,[b] Kees Versluis,[b] Albert J. R. Heck,[b] Sharon M. Kelly[c] and Nicholas C. Price[c]

[a]*Department of Chemistry, Marine Natural Products Laboratory, University of Aberdeen, Old Aberdeen AB24 3UE, Scotland, UK*
[b]*Department of Biomolecular Mass Spectrometry, Utrecht Institute for Pharmaceutical Sciences (UIPS) and Bijvoet Center for Biomolecular Research, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[c]*IBLS Division of Biochemistry and Molecular Biology, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK*

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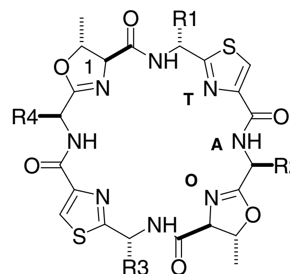
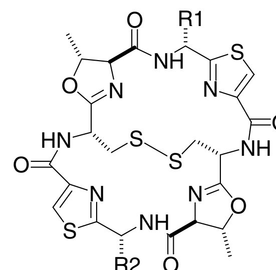
Abstract—Studies on three Thz, and Oxn containing cyclic peptides, patellamide A and C (**1**, **3**) and ulithiacyclamide A (**9**) isolated from the Indo-Pacific ascidian (seasquirt) *Lissoclinum patella* have delineated their metal binding selectivity. Patellamide C (**3**) shows extreme selectivity for Cu^{2+} even in the presence of an excess of Zn^{2+} and shows no binding at all to Co^{2+}, Ni^{2+} and Hg^{2+}. Patellamide A (**1**) is less selective for Cu^{2+}, whereas ulithiacyclamide A (**9**) shows selectivity similar to that of patellamide C (**3**). The selectivity was studied by circular dichroism spectroscopy and mass spectrometry. The CD spectra obtained whilst patellamide C was slowly titrated with Cu^{2+} show one isosbestic point indicating the Cu^{2+} binding involves only two conformations. These studies indicate that Cu^{2+}, not Zn^{2+} is the biologically relevant metal for these compounds and point towards a potential ecological function of these complexes. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

The Indo-Pacific ascidian (seasquirt) *Lissoclinum patella* (Order Enterogona, Family Didemnidae) is known to produce several families of closely related cyclic peptides, many of which have significant biological activity.[1] These peptides have been grouped into structural types according to the number of amino acids (7 or 8) and inclusion of thiazole (Thz), thiazoline (Thn) and oxazoline (Oxn) rings within their structure. The Thz and Thn rings originate from cysteine, the Oxn rings from serine or threonine. The focus here will be on the cyclic peptides containing eight amino acids, and two Thz and two Oxn, such as the patellamides (**1–8**)[2,3,4,5] and the ulithiacyclamides (**9–10**).[6] *L. patella* is host to the photosynthetic prokaryotic symbiont *Prochloron* and it is believed that this organism is responsible for the production of these unusual cyclic peptides.[7] In the case of *L. patella*, extraction of *Prochloron* cells, removed from the host, yields the same or higher amounts of peptides, on a weight for weight basis, than the amounts obtained from the host tissue alone.



(1) Patellamide A R1=R3=D-Val R2=R4=L-Ile Ring 1-noMe
(2) Patellamide B R1=D-Phe R2=L-Leu R3=D-Ala R4=L-Ile
(3) Patellamide C R1=D-Phe R2=L-Val R3=D-Ala R4=L-Ile
(4) Patellamide D R1=D-Phe R2=R4=L-Ile R3=D-Ala
(5) Patellamide E R1=D-Phe R2=R3=L-Val R4=D-Ile
(6) Patellamide F R1=D-Phe R2=R3=Val R4=Ile
(7) Patellamide G R1=D-Phe R2=L-Ile R3=D-Ala R4=L-Leu
(8) Ascidiacyclamide R1=R3=D-Val R2=R4=L-Ile



(9) Ulithiacyclamide A R1=R2=D-Leu
(10) Ulithiacyclamide B R1=D-Ile R2=D-Phe

Keywords: complexation; natural products; circular dichroism; mass spectrometry.

* Corresponding author. Tel.: +44-1224-272895; fax: +44-1224-272921; e-mail: m.jaspars@abdn.ac.uk

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Table 1. Metal content of solvent extracts of *L. patella*

Partition fraction	Mass (mg)	Metals found	Concentration (ppm)
CH_2Cl_2	1420	Zn	2.5
		Fe	1.2
		Cu	2.5
MeOH	5840	None	–
Hexane	2430	Fe	1.4
n-BuOH	710	None	–

The initial interest in these modified cyclic peptides was their potent biological activity. A strong structure–activity relationship has been noted by several workers, with the disulphide bridge in the ulithiacyclamides (e.g. **9**, **10**) making these the most cytotoxic of the compounds isolated from *L. patella*.[6] It is thought that the bridge fixes the conformation of the molecule and that this is the important factor in their high cytotoxicity. The inclusion of an Oxn moiety in a compound was shown by Shioiri and co-workers[8] to give much higher levels of activity than other residues. In addition to their cytotoxic properties, patellamides B (**2**) and C (**3**) have been shown to reduce multi drug resistance (MDR) in vitro of drug resistant lymphoblasts.[9]

The patellamides and ulithiacyclamides resemble 24-azacrown-8 macrocycles and several members of this group have been found to bind Cu^{2+} and Zn^{2+} within their central cavities. A large number of spectroscopic techniques have been used to study the metal complexation, beginning with the circular dichroism (CD) work by Hawkins.[10] His group carried out more extensive work on the copper complexes of ascidiacyclamide (**8**) and patellamide D (**4**) using EPR and magnetic susceptibility techniques and species present were determined by electrospray MS.[11,12] The same workers also determined a crystal structure for the patellamide D (**4**)/Cu_2CO_3 complex in which the CO_3^{2-} bridged the two Cu^{2+} centres. A later CD study by Freeman et al. indicated that the patellamides could bind to Zn^{2+} as well as Cu^{2+}, but that they showed no affinity for the biologically important Mg^{2+} and Ca^{2+}.[13] The Zn^{2+} binding was later studied in detail using ^{1}H NMR, and CD titrations, and the Zn species present were determined by electrospray MS. These studies indicated the importance of kinetics to the binding process.[14] In addition, patellamide derivatives with open Oxn rings have been studied with respect to their binding of potassium[15] and copper.[16]

2. Results and discussion

Our sample of *L. patella* was collected in the Molucca Sea, Sulawesi, Indonesia in 1996, and underwent a solvent extraction followed by a solvent partition to provide water, butanol, methanol, dichloromethane and hexane partition fractions.[17] In order to investigate whether metal chelation by peptides from this species occurred in vivo or only under laboratory conditions, the solvent partition fractions were screened by inductively coupled plasma mass spectrometry (ICP-MS) for their metal content. This analysis revealed that the dichloromethane extract was rich in zinc and copper (see Table 1). The concentration in this extract was roughly 10^4 times greater than that found in the surrounding seawater. The fact that the metals were found in non-polar fractions suggested that they were complexed. The intention was to perform an isolation procedure guided by metal content, but this proved impossible due to the small amounts of isolated material available. We therefore concentrated on investigating the metal binding characteristics of the patellamides and lissoclinamides present in our collection of *L. patella*. The purification procedures yielded patellamide C (**3**) and the new lissoclinamides 9 and 10 whose isolation and structure determination is described elsewhere.[18] In addition, we also included in our studies the pseudosymmetrical patellamide A (**1**) and ulithiacyclamide A (**9**), which were generously donated by Chris Ireland and John Faulkner, respectively.

3. Circular dichroism spectroscopy

The first CD spectra obtained were those of non-metal bound patellamide A and C and ulithiacyclamide (**1**, **3**, **9**) in methanolic solution. Previous structural studies have defined the different conformations adopted by the patellamides (Fig. 1).[3,5,19,20] Symmetrically substituted patellamides (e.g. **1**, **8**) adopt the type I 'square' structure both in the solid state and in solution. For the asymmetrically substituted patellamides (**2**–**7**) the predominant conformation in the solid state is the 'figure of eight' type III, held closed by four hydrogen bonds across the macrocyclic ring, with the two hydrogen bonds from NH to C=O forming two type II β turn mimics (Fig. 1). In solution this structure relaxes to give the type II figure of eight conformation. The solution conformations I and II each have a characteristic CD spectrum with patellamide A (**1**,

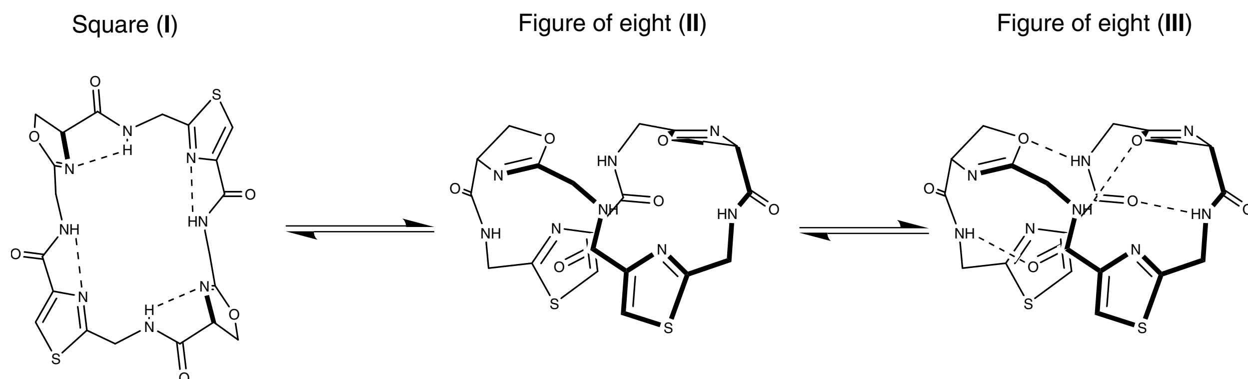

Figure 1. Possible conformations of the *L. patella* metabolites.

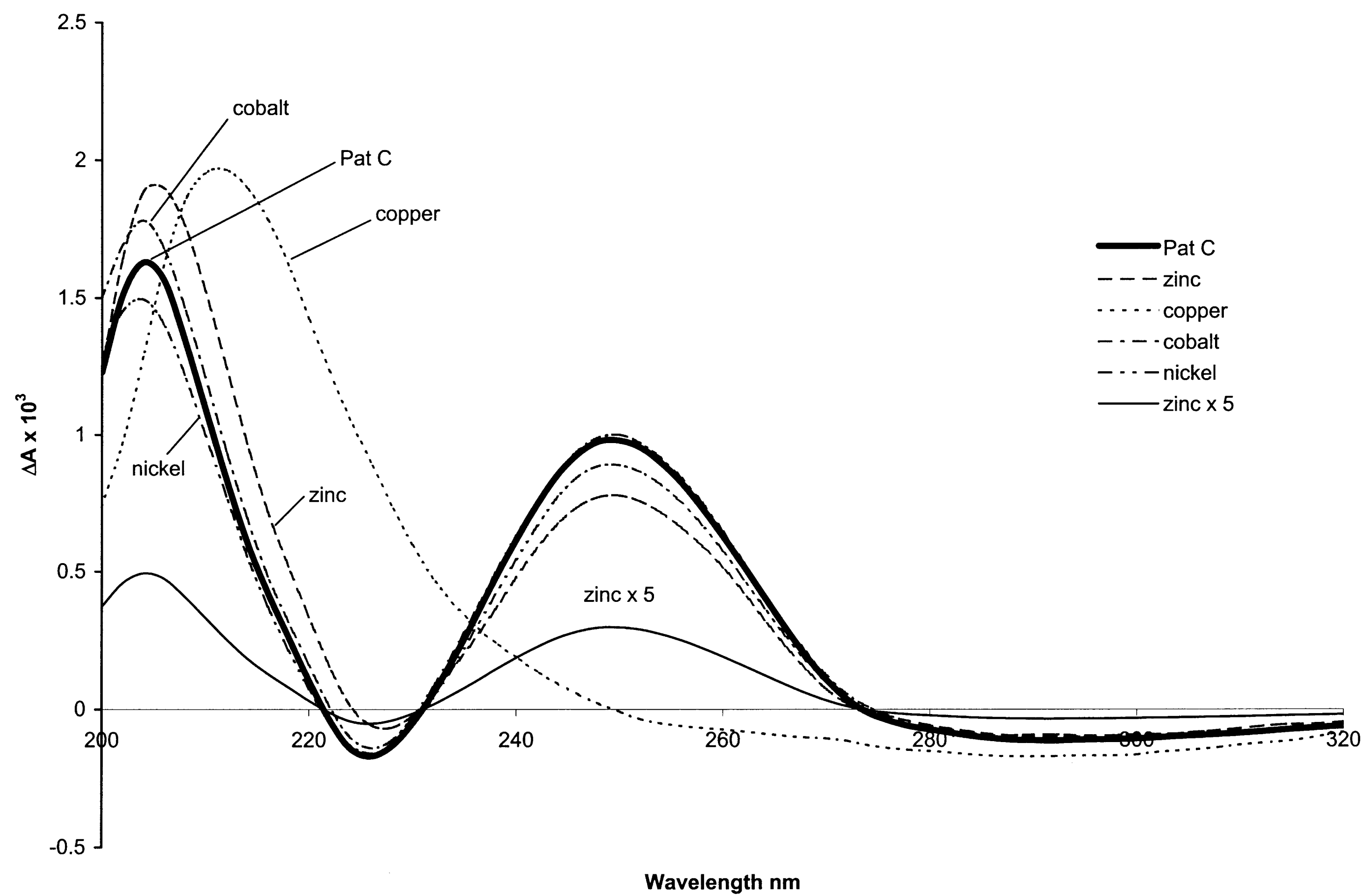

Figure 2. CD spectra of **3** at 0.2 mg/mL in MeOH plus 2 equiv. of each metal and also with 5 equiv. of Zn.

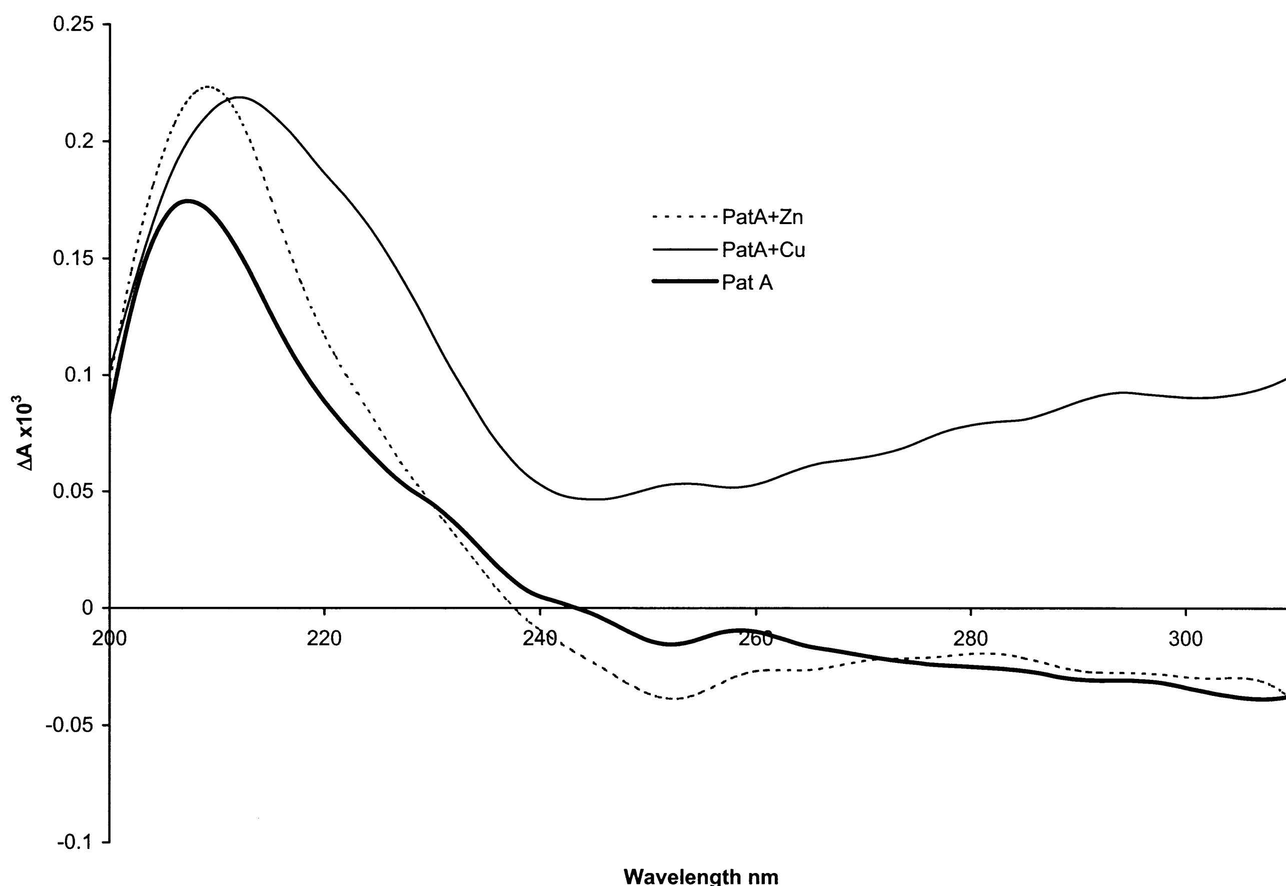

Figure 3. CD spectra of **1** at 0.02 mg/mL in MeOH plus 2 equiv. of copper and zinc.

type I) having a maximum at 211 nm and patellamide C (**3**, type II) having maxima at 205 and 250 nm (Figs. 2 and 3, bold trace). Ulithiacyclamide (**9**) whose conformation is held in the square form (I) by a disulphide bridge has CD maxima at 216 and 318 nm (Fig. 4). Typical CD maxima for type II β turns in peptides and proteins are 205 nm (ΔA=0.606×10^{-3}) and ~230 nm (ΔA=0.121×10^{-3}) but the latter can be shifted to 250 nm if there is conjugation (e.g. to Thz) present in the molecule.[21] In the square patellamide structures the 211 nm maximum is due to the peptide bond conjugated to Thz. Random coil values observed in larger peptide and protein CD spectra are 217 nm (ΔA= 0.091×10^{-3}) and 195 nm (ΔA=0.910×10^{-3}). Therefore, the maximum at 250 nm is associated with the figure of eight (II) conformation and the maximum at 211 nm is associated mainly with the square (I) form of the patellamides, providing a useful diagnostic tool for future CD studies.

Preliminary metal binding studies were carried out on patellamide C (**3**) with Zn^{2+} and Cu^{2+}, as we had found them present in the crude extracts of *L. patella*. Ni^{2+} and Co^{2+} were also investigated as they are of a similar size and Ni^{2+} is also known to bind to copper sites in biological systems.[22] We found that changing the anion had no effect on the binding as observed by CD and therefore used the chlorides of each metal. Two equivalents of $CoCl_2$, $NiCl_2$, $CuCl_2$ and $ZnCl_2$ were each added separately to patellamide C (**3**) (Fig. 2). The CD spectrum obtained on addition of copper showed a significant change from the spectrum of the unbound compound with a decrease in ΔA of 1.08×10^{-3} at the λ_{max} at 250 nm and a shift to 211 nm of the other maximum at 205 nm combined with an increase in ΔA of 0.36×10^{-3} (Fig. 2). This suggests that on binding to copper, patellamide C changes conformation to one approximating the square form (I) as the spectrum closely resembles that measured for patellamide A.

There was an observable difference between the **3**/Cu^{2+} spectrum and that of **3**/Zn^{2+} as the latter gave no shift of the 205 nm peak, only an increase in ΔA of 0.291×10^{-3}. In addition there was no collapse of the λ_{max} at 250 nm, merely a decrease in ΔA of 0.267×10^{-3}, suggesting there is little or no conformational change when **3** binds to zinc (Fig. 2). A spectrum was also recorded of the peptide with 5 equiv. of zinc added which showed a large reduction in the amplitude of the maxima at 205 and 250 nm. Spectra of solutions containing **3**/Ni^{2+} and **3**/Co^{2+} were also measured, with addition of Co^{2+} causing no overall change to the shape of the spectrum apart from a small increase of ΔA of 0.097×10^{-3} at the λ_{max} at 205 nm and a decrease in ΔA of 0.072×10^{-3} at the λ_{max} at 250 nm. Ni^{2+} caused small changes, a decrease in ΔA of 0.194×10^{-3} at 205 nm and a decrease in ΔA of 0.072×10^{-3} at 250 nm. This indicated that there was very little or no binding to nickel and possibly a small amount of non-specific binding to cobalt. Solutions containing patellamide C plus 2 equiv. of mercury and also

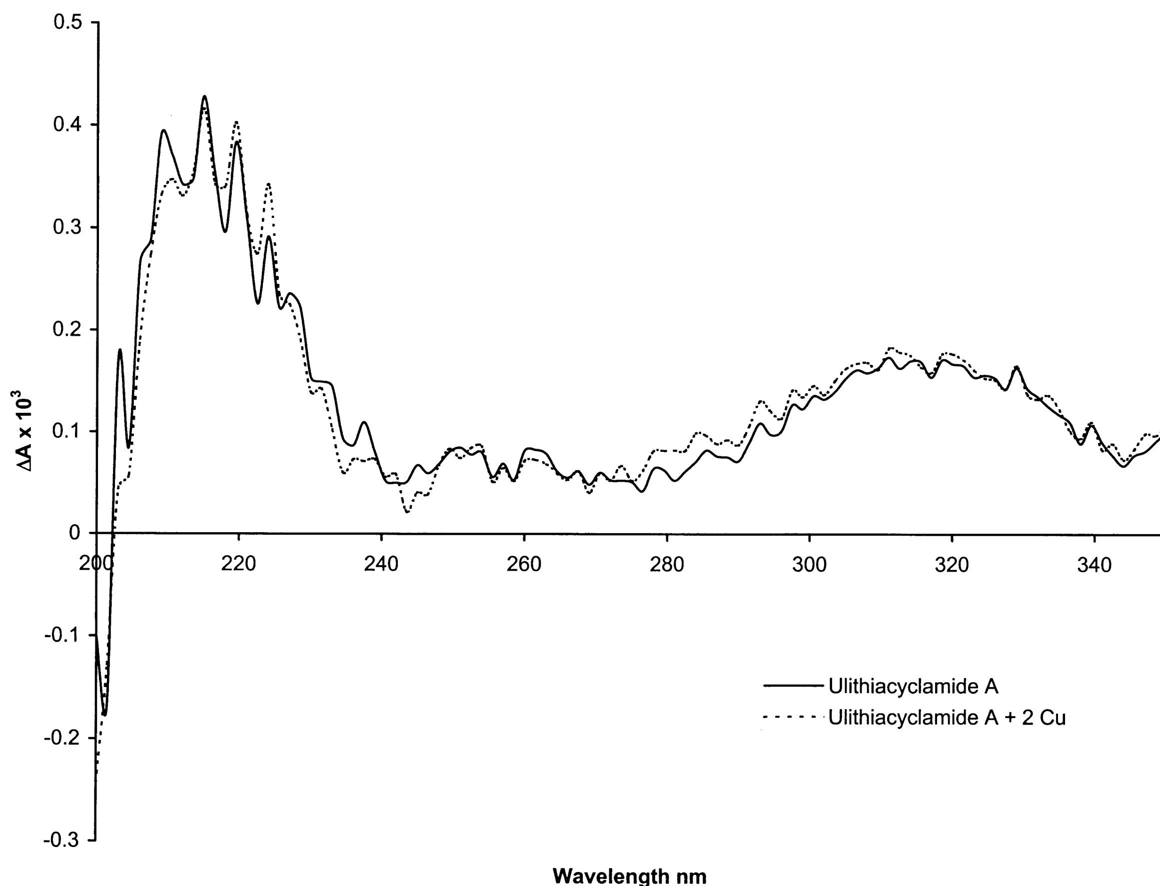

Figure 4. CD spectrum of **9** (0.8 mg/mL in MeOH) and **9** with 2 equiv. of Cu.

with an excess were also measured, but again there was no visible binding effect and any interaction between the metals and the peptide was a non-specific association.

Addition of Zn^{2+} caused no overall change to the spectrum of **1** (Fig. 3) apart from a steady small increase in ΔA at the λ_{max} at 211 nm with increasing zinc concentration, similar to that seen with **3** and Zn^{2+}. After the addition of 1 equiv. the increase in ΔA becomes less marked up to 2 equiv. when the increase stops suggesting the formation of a complex containing two zinc atoms. The first addition of Cu^{2+} to **1** caused a shift of the λ_{max} at 211–213 nm, similar to that seen with addition of copper to patellamide C, and also an increase in the ΔA up to 1 equiv. of Cu (Fig. 3). Further addition of copper then caused a significant broadening of this band up to the addition of 2 equiv. but no change thereafter. Addition of 2 equiv. of Ni^{2+} to a solution of 1 caused no apparent change in the spectrum, indicating either that no binding is taking place or that binding is not associated with any conformational change.

Addition of 2 equiv. of Cu^{2+} to a methanolic solution of ulithiacyclamide A (**9**) caused no change in the CD spectrum (Fig. 4) and CD cannot be used to ascertain metal binding for this compound. Because of the disulphide bridge, the structure of **9** is more rigid than those of the patellamides, and binding to Cu^{2+} will not be accompanied by a conformational change large enough to be noticeable in the CD spectrum.

CD was used to monitor the titrations of **1** and **3** with Cu^{2+} and Zn^{2+}, allowing us to calculate the binding constants for

Table 2. Binding constants of patellamides A and C

Peptide	*K* values by CD	*K* values by CD (Freeman et al.[13])	Relative *K* values by MS	Species used for MS calculation
1+Cu	$K_1=2\pm0.3\times10^4$	$K_1=2.0\times10^4$	$K_1=3.3\times10^4$	$[M-H+Cu]^+$
1+Cu_2	$K_2=780\pm12$	–	$K_2=1.0\times10^4$	$[M-2H+Cu_2Cl]^+$
3+Cu	$K_1=6.8\pm0.1\times10^4$	–	$K_1=1.2\times10^4$	$[M-H+Cu]^+$
3+Cu_2	Not observed	–	$K_2=6.0\times10^3$	$[M-3H+Cu_2+(C_3H_6O_3)]^+$
1+Zn	$K_1=3\pm0.4\times10^4$	$K_1=3.0\times10^4$	$K_1=2.8\times10^3$	$[M-H+Zn]^+$
1+Zn_2	$K_2=1000\pm16$	$K_2=16$	$K_2=3.9\times10^3$	$[M-H+Zn_2Cl_2]^+$
3+Zn	$K_1=1.8\pm0.2\times10^4$	–	$K_1=2.4\times10^3$	$[M-H+Zn]^+$
3+Zn_2	$K_2=806\pm8$	–	$K_2=2.6\times10^3$	$[M-H+Zn_2Cl_2]^+$

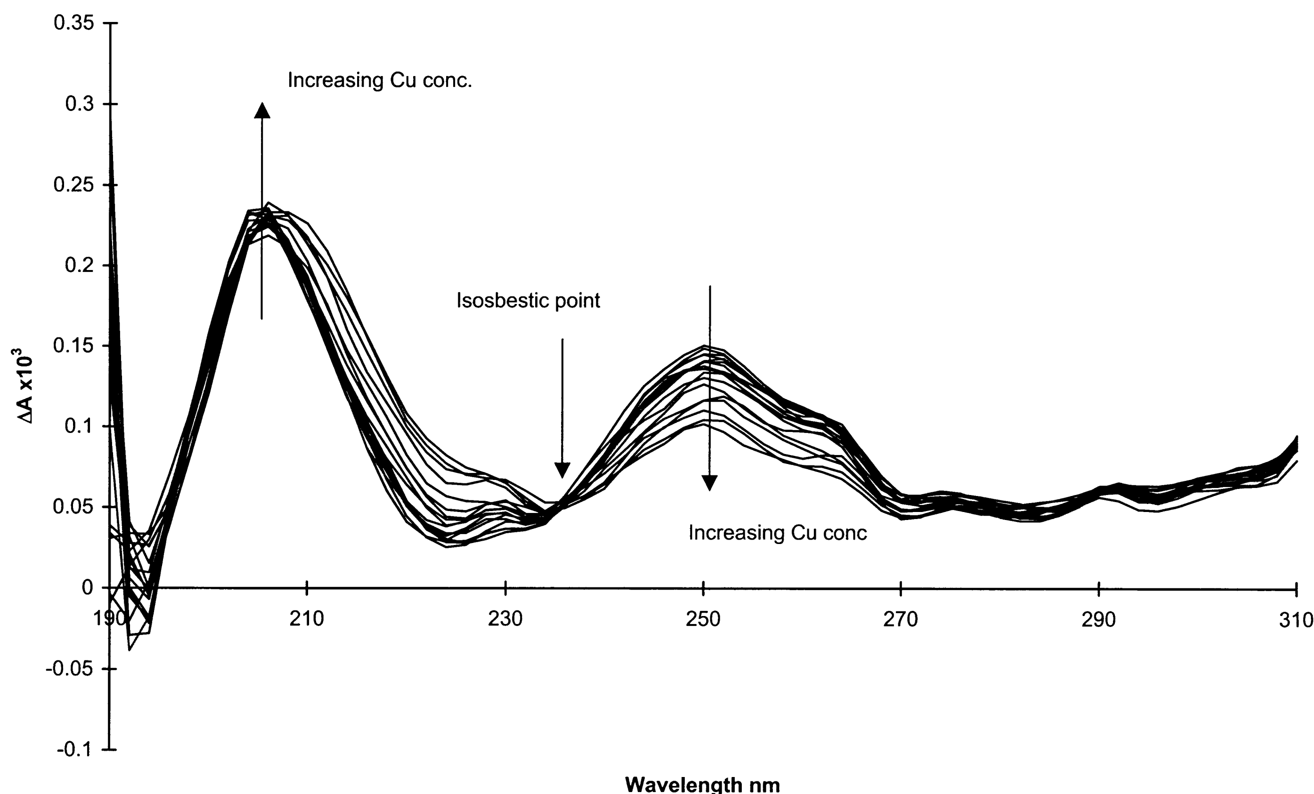

Figure 5. Slow titration of **3** (0.02 mg/mL in MeOH) with Cu^{2+} followed by CD spectroscopy showing an isosbestic point at 234 nm.

the process by previously described methods.[13] Our data for **1** and **3** along with the previously published information for **1** are given in Table 2. Binding constants for **1** agreed well for the primary binding site but diverged for the secondary binding site (Table 2). The existence of a strong secondary binding event is corroborated by MS titration data (see below). That a secondary binding event was not observed in the CD titration of **3** with Cu^{2+} might be due to there being no significant conformational change on binding a second equivalent of the metal, i.e. the second binding site is already formed by the binding of the first equivalent of the metal.[23] Plotting the first part of the **3**/Cu^{2+} CD titration showed the existence of a single isosbestic point at 234 nm, indicating that the binding of Cu^{2+} to **3** involved only two different conformational states (Fig. 5). This is in contrast to the multiple binding states observed by van den Brenk et al. in their ESR studies of the patellamide D/Cu^{2+} complexation in the presence of base.[12]

The fact that K_1 for Cu^{2+} and Zn^{2+} binding with **1** and **3** were the same order of magnitude suggested that patellamides A and C would show little preference for either metal. To determine whether **3** would selectively bind to Cu^{2+} or Zn^{2+} a competitive binding study was carried out between Cu^{2+} and Zn^{2+} with **3** (Fig. 6). The initial experiment involved the addition of 2 equiv. of Cu^{2+} to patellamide C (**3**), after which 2 equiv. of Zn^{2+} were added. The CD spectrum of **3**/Cu^{2+}/Zn^{2+} was essentially identical to that of **3**/Cu^{2+} except for a small change in ΔA at the λ_{max} of 211 nm. A further 2 equiv. of Zn^{2+} were added, and no further change was observed in the CD spectrum. This indicated that the binding is selective for Cu^{2+} in the presence of an excess of Zn^{2+}. Even taking into account the binding constant differential, an observable degree of binding to Zn^{2+} would be expected, and this should manifest itself in an increase of ΔA at λ_{max} 250 nm. In addition, the **3**/Cu^{2+}/Zn^{2+} and **3**/Cu^{2+} spectra have their other λ_{max} at 211 nm and not at 207 nm as observed for **3**/Zn^{2+}. To determine whether the order of addition was important the competition experiment was carried out by adding 2 equiv. of Zn^{2+} to patellamide C after which 2 equiv. of Cu^{2+} were added. The resulting dramatic change is shown in Fig. 6, and the **3**/Zn^{2+}/Cu^{2+} CD spectrum is very similar to that of **3**/Cu^{2+}. This again indicates the strong selective binding for Cu^{2+}. An initial proposal was that the selectivity was kinetic in nature, but re-recording the CD spectrum of the **3**/Cu^{2+}/Zn^{2+} sample after three months showed that no change had taken place. This indicates that no binding of Zn^{2+} to **3** can take place in the presence of Cu^{2+}, and this is confirmed by MS studies, as no mixed Cu^{2+}/Zn^{2+} complexes are observed (see below). In a separate CD experiment, addition of Ni^{2+} to **3** caused a small change in the CD spectrum but upon addition of copper the typical **3**/Cu^{2+} spectrum was obtained. Addition of Ni^{2+} to **3**/Cu^{2+} caused no change. Any binding with nickel is therefore non-specific and does not compete with the binding of copper. A further competition experiment was carried out using **3**/Cu^{2+} and an excess of EDTA, which indicated the EDTA could not displace the Cu^{2+} from **3** despite the formation constant for copper-EDTA being 6×10^{18}, many orders of magnitude greater than that measured for **3**/Cu^{2+}. An equimolar mixture of **3** and EDTA added to 1 equiv. of Cu^{2+} in MeOH resulted only in a conformational change consistent

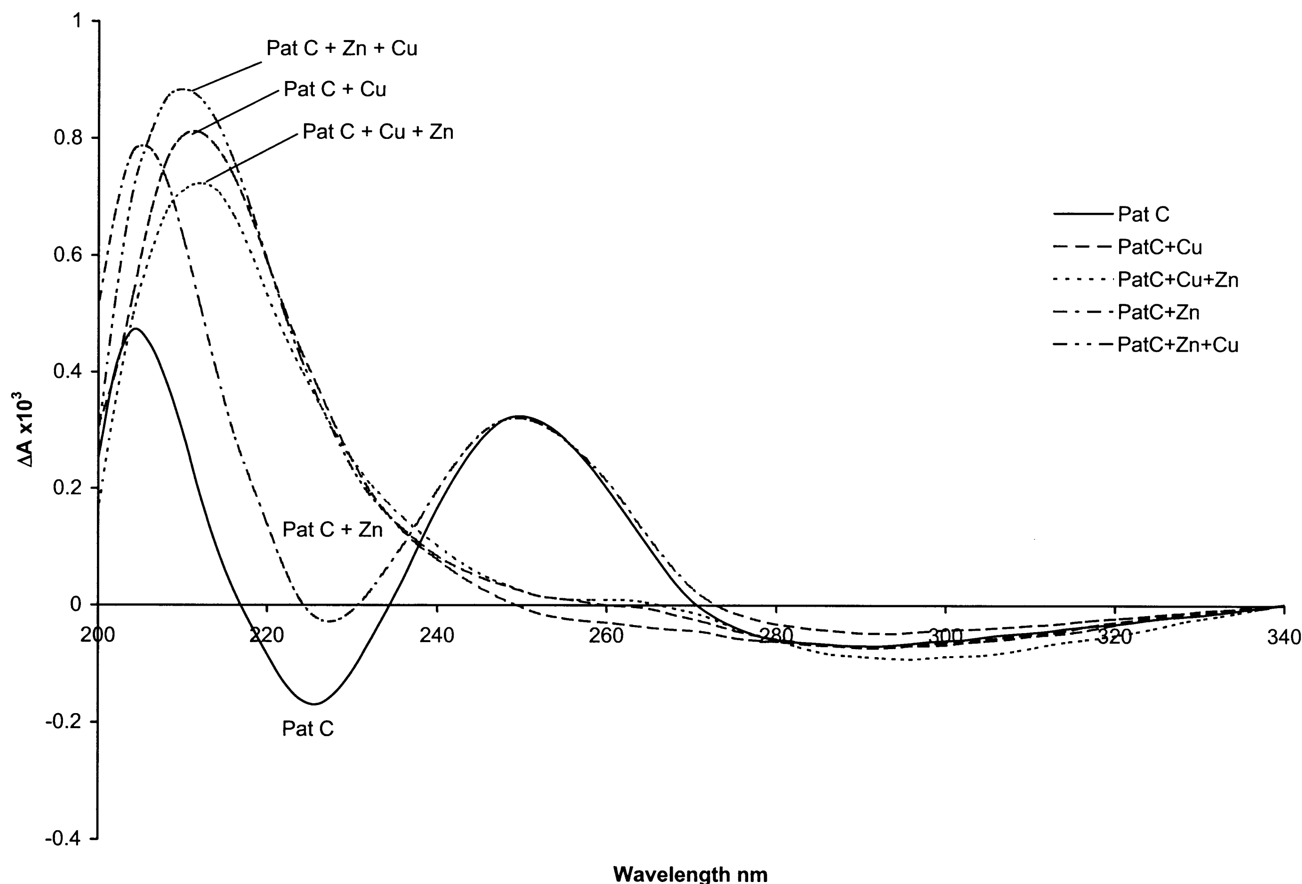

Figure 6. Zinc and copper in competition for patellamide C (**3** at 0.02 mg/mL in methanol).

with **3**/Cu^{2+} being formed as witnessed by CD (i.e. total collapse of the maximum at 250 nm).

The competition spectra of patellamide A with Cu^{2+} and Zn^{2+} are somewhat more ambiguous (Fig. 7). Addition of Cu^{2+} to a solution of **1**/Zn^{2+} caused no major change in the spectrum and similarly addition of Zn^{2+} to **1**/Cu^{2+} caused little change (Fig. 7). This would suggest no preferential binding of either metal takes place. A third experiment was carried out by adding a mixture containing 2 equiv. of Cu^{2+} and 2 equiv. of Zn^{2+} to a solution of **1**. The resulting spectrum appeared to be a mixture of the **1**/Zn^{2+} and **1**/Cu^{2+} spectra with a large increase in the λ_{max} at 209 nm and also distinct broadening, suggesting the presence of a mixture of species (Fig. 7).

4. Mass spectrometry

As **1**, **3** and **9** had been found to bind both copper and zinc in the CD study it was decided to further investigate these complexes by MS in order to ascertain their exact nature and which, if any, counter-ions were incorporated into the complexes. As for CD, all measurements were carried out in methanol solution. Accurate mass measurements and analysis of isotope ratio patterns were used to assign the components of a complex. The copper complexes of ascidiacyclamide (**8**) and patellamide D (**4**) were previously studied using electrospray mass spectrometry by van den Brenk et al.[11,12] Both **4** and **8** were shown to complex two copper atoms by deprotonation of amide nitrogens. Dicopper complexes of **4** with OH^-, CO_2 and CO_3^{2-} were also observed. We first obtained accurate mass and fragmentation data for uncomplexed patellamide A (**1**), C (**3**) and ulithiacyclamide A (**9**). The copper and zinc complexes of each were then studied in detail, including their fragmentation patterns, and this was followed by monitoring some competition and titration experiments by mass spectrometry.

4.1. MS of Cu complexes

The main species occurring in the ion trap mass spectra of **1**, **3** and **9** with 2 equiv. of Cu^{2+} or Zn^{2+} are presented in Table 3. Generally, the mass spectra of Cu-containing samples are less complicated than those of Zn-containing samples.

The first mass spectrum obtained was that of **3** in methanol with 2 equiv. Cu^{2+} (as the chloride) added. The main signals are due to singly charged species, and the base peak is the mono–copper complex at *m/z* 824. There was no evidence for any significant occurrence of doubly charged species and all signals for Cl-containing adducts were minor. In some spectra, a large signal at *m/z* 975 was observed and identified as $[PatC\text{-}3H+Cu_2+C_3H_6O_3]^+$ by accurate mass measurement of both the molecular ion and the fragment ion at $[M-90]^+$. Contrary to the results obtained by van den Brenk et al. we did not observe CO_2 or CO_3^{2-} adducts.[11,12]

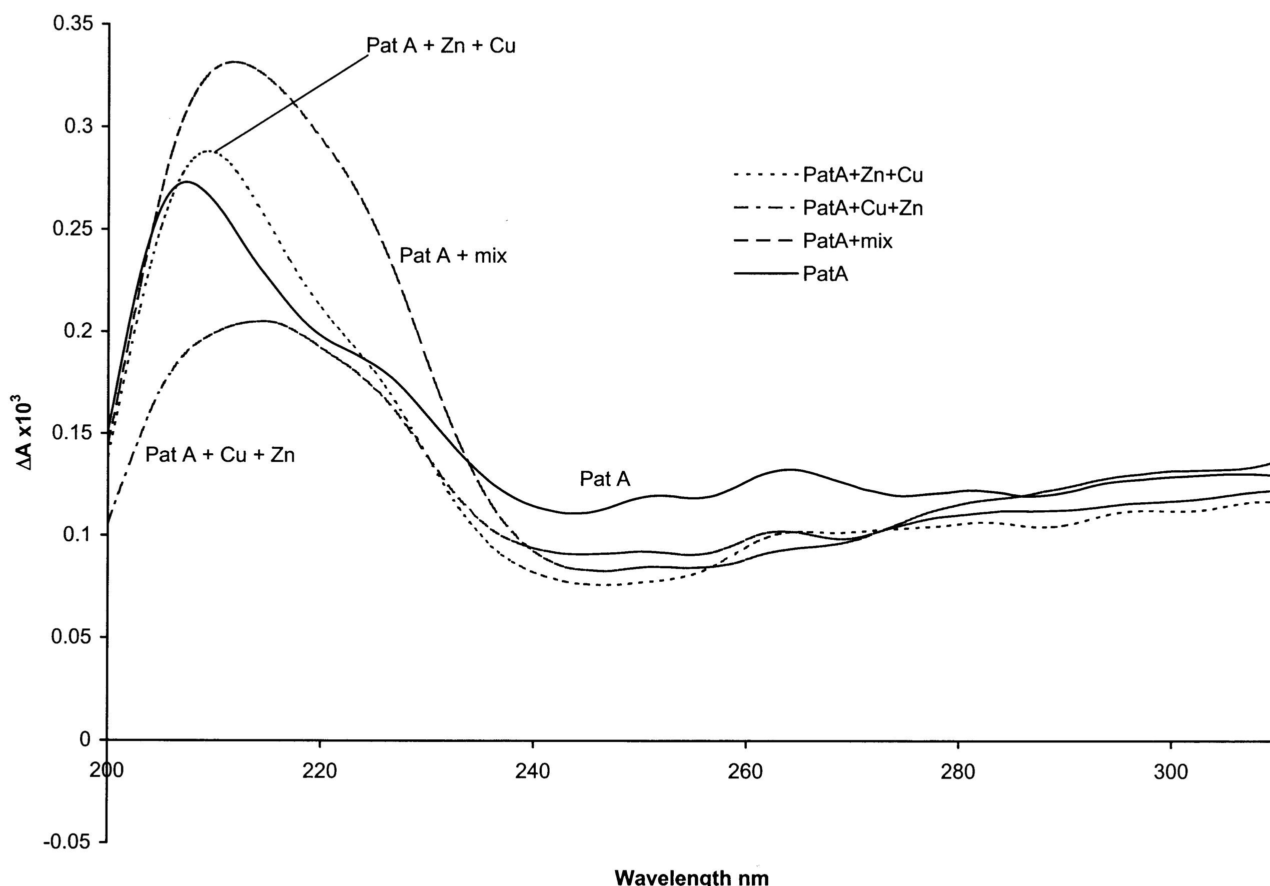

Figure 7. Zinc and copper in competition for patellamide A (**1** at 0.02 mg/mL in methanol).

However, our spectra were obtained in MeOH without any addition of acid or base whereas van den Brenk et al. added triethylamine to their solutions. The $C_3H_6O_3$ adduct might be dimethyl carbonate, produced by the reaction between MeOH and CO_3^{2-} obtained from atmospheric CO_2. This then suggests a catalytic function for the dicopper complexes, which will be discussed in Section 5.

The appearance of a dicopper species was unexpected as its formation had not been detected by CD. This suggests that only the binding of the first Cu^{2+} caused any measurable change in the conformation of the molecule and, once this change has occurred, the second Cu^{2+} may be complexed with no further conformational change.[23]

When copper acetate was used instead of copper chloride, *m/z* 975 was the base peak, whereas its abundance was only 10% when copper chloride was used. Only minor peaks are observed for other copper species. This suggests that the formation of the *m/z* 975 species is greatly enhanced in the presence of acetate. In a three-month old solution of patellamide C and copper chloride, the only species present was the dicopper complex at *m/z* 975, indicating that this is the preferred complex.

The mass spectrum of **1**/Cu^{2+} was dominated by the signal at *m/z* 804 for $[PatA{-}H{+}Cu]^+$. No carbonate adducts for **1**/2Cu^{2+} were observed; such adducts were reported for the dicopper complex of ascidiacyclamide (**8**) in the presence of base.[11]

The mass spectrum of **9**/Cu^{2+} has a base peak at *m/z* 921 for the dicopper complex $[Uli\text{-}2H{+}Cu_2Cl]^+$ and a slightly smaller peak at *m/z* 824 corresponding to $[Uli\text{-}H{+}Cu]^+$ showing that the formation of the dicopper complex is highly favored.

4.1.1. MS^n of Cu complexes. MS^n gave insight into how Cu^{2+} is bound to **3**. MS^2 of both $[PatC{+}H]^+$ (*m/z* 763) and $[PatC{+}Na]^+$ (*m/z* 785) showed fragmentation of the macrocycle (loss of 28, 44 and 83/85), followed by side chain fragmentation (loss of 29, 57 and 91) in MS^3. In contrast, MS^2 of *m/z* 824, $[PatC\text{-}H{+}Cu]^+$, gives first side chain fragmentation (loss of 15, 29, 43 and 57). The high abundance of the M−43 and M−57 fragment ions suggests that the first Cu has replaced Val and Ile amide H, which is consistent with the published X-ray structure.[11] For this copper complex, MS^3 on M−43 shows loss of 27 (HCN), 28 (CO) and 44 through fragmentation of the macrocycle. MS^3 on M−57 shows loss of 28, 44 and 83/85 again through fragmentation of the macrocycle. Binding to Cu obviously strengthens the ring structure, and facilitates loss of the side chains. $[PatC\text{-}3H{+}Cu_2{+}C_3H_6O_3]^+$, *m/z* 975 behaves differently again. It first loses the $C_3H_6O_3$ moiety (90), followed in MS^3 by predominantly loss of 91, 43 and 83; M−57 is less abundant. This suggests that in the dicopper complex

Table 3. Main species detected in the MS of **1**, **3**, **9** plus 2 equiv. Cu^{2+} or 2 equiv. Zn^{2+} solution. Base peaks (100%) are in bold type

Species	*m/z*, Δ ()[a]					
	1+Cu	**1**+Zn	**3**+Cu	**3**+Zn	**9**+Cu	**9**+Zn
Singly charged species						
$[M+H]^+$	743.3372 1.3 (5)	743.3372 1.3 (5)	763.3059 1.1 (10)	763.3059 1.1 (20)	763.2222 3.4 (30)	763.2222 3.4 (45)
$[M+Na]^+$			785 (20)	785 (20)		
$[M+H+H_2O]^+$				782 (15)		
$[M+H+MeOH]^+$				795 (20)		
$[M+H+HCl]^+$	779.3154 7.0 (5)					
Cu containing species						
$[M-H+Cu]^+$	**804.2512** 1.2		**824.2198**		824 (90)	
$[M-H+Cu+H_2O]$					842 (50)	
$[M+CuCl]^+$	842.2277 (^{65}Cu) 3.0 (45)		860.1961 1.0 (24)		860 (45)	
$[M-H+CuCl+Na]^+$			882 (10)			
$[M-2H+Cu_2Cl]^+$	901.1440 3.4 (5)				**921**	
$[M-3H+Cu_2+C_3H_6O_3]^+$			975.1767 12.5 (10)			
Zn containing species						
$[M-H+Zn]^+$		805 (50)				825 (17)
$[M+ZnCl]^+$		**841**		**863.1932** (^{66}Zn) 1.2		**861**
$[M+H+ZnCl_2]^+$		877 (30)				898 (10)
$[M-H+Zn_2Cl_2]^+$		939 (15)		959 (50)		960 (25)
Doubly charged species						
$[M+Zn]^{2+}$	403 (15)			413.1091 9.9 (75)		
$[M+Zn+HCl]^{2+}$	421 (20)					
$[M-H+Zn_2Cl]^{2+}$	452 (24)					
$[M-H+Zn_2Cl+H_2O]^{2+}$	461 (20)					
$[M+Zn_2Cl_2]^{2+}$	470 (10)					
$[M-H+Zn_3Cl_3]^{2+}$	519 (15)					

[a] Δ in milli-amu from calculated mass, relative intensity (%); accurate masses were measured with a quadrupole/time-of-flight instrument; all other masses with an ion trap instrument in selective ion monitoring mode. Only relative intensities >10% are included, except when accurate masses were measured.

also the Phe NH is involved. In all cases, the copper atoms showed a very strong binding by patellamide C. No loss of copper was observed, even in MS^4.

For patellamide A, fragmentation of *m/z* 901 $[PatA\text{-}2H+Cu_2Cl]^+$ leads to loss of HCl (MS^2), followed by ring fragmentation in MS^3. Fragmentation of *m/z* 804, $[PatA\text{-}H+Cu]^+$, was similar to that of the corresponding PatC species in that portions of the side chains were lost prior to ring opening. $[M-29]^+$ and $[M-57]^+$ show higher abundances than $[M-15]^+$ and $[M-43]^+$, which may indicate that the Cu is located at the Ile nitrogen. This confirms the identity of the binding site found from the X-ray crystal structure of ascidacyclamide/Cu_2CO_3 structure,[11] which consists of the thiazole (T), oxazoline (O) and intervening amide (A) nitrogens, as indicated on the patellamide structures **1**–**8**.

4.1.2. MS of the Zn complexes. In contrast to the mass spectra of the Cu complexes, the mass spectra of the Zn complexes of **3**, but in particular **1**, generally showed far more doubly charged ions (Table 3). For **1**/Zn^{2+}, the variety is greater than reported previously (Table 3)[14], and with an excess of Zn^{2+} the mass spectrum shows the presence of multiply charged ions from about *m/z* 300 downward. Obviously, Zn preferentially binds without abstraction of hydrogen. For **3**, binding to Zn^{2+} is less specific than it is to Cu^{2+}, as was seen in the CD competition study. The lack of specific binding can be partly put down to the inability of Zn^{2+} to abstract an amide hydrogen in the absence of base, which Cu^{2+} is capable of doing.[24] The Zn complex also shows more H_2O and MeOH adducts, which were all but absent in the Cu complex. Also Cl-containing adducts are far more prominent than in the Cu complex, $[PatC+ZnCl]^+$ at *m/z* 863 being sometimes the base peak.

After addition of 2 equiv. of Zn^{2+} to **1**, a significant proportion of the peptide remained uncomplexed, either reflecting slow binding, as proposed by Grøndahl et al.[14] for (**8**), or non-specific binding that was unstable in the MS environment. An excess of zinc was then added (4 equiv.) to force formation of more zinc bound species. Apart from many multiply charged species, adducts with more than two Zn and HCl were present as well, whilst water adducts were less abundant, reflecting the less specific binding of zinc by patellamide A.

Ulithiacyclamide (**9**) shows a relatively simple MS when complexed with Zn^{2+} (Table 3), with only zinc and zinc/chloride complexes appearing and fewer water and methanol adducts than the zinc complexes of **1** and **3**.

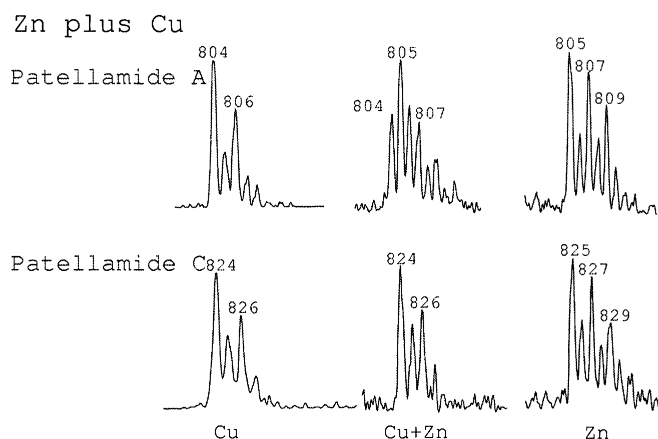

Figure 8. Selected ion monitoring spectra of $[M-H+X]^+$ (M=patellamide; X=metal ion) after addition of Cu^{2+}, Cu^{2+} + Zn^{2+} or Zn^{2+}.

4.1.3. MS^n of the Zn complexes. MS^2 of *m/z* 413 $[PatC+Zn]^{2+}$ was similar to that of the singly charged Na adduct: loss of 28 and 85, and some 43 (instead of 44 for the Na adduct); up to MS^4 mainly fragmentation of the ring was observed, but Zn is retained. The singly charged *m/z* 825 ($[PatC-H+Zn]^{2+}$) showed loss of both a ring fragment (85) and a side chain (57); for the HCl adduct of both **1** (*m/z* 841) and **3** (*m/z* 861) initial loss of HCl in MS^2 was followed by ring fragmentation in MS^3.

4.1.4. MS competition and titration experiments. These were carried out in a similar manner to those performed using CD. Zinc was added to copper bound patellamide and copper to zinc bound patellamide and the resulting solutions analyzed. A mixed solution of copper and zinc was also added to unbound peptide in some cases to ensure that observed selectivity of binding was real and not dependant on which metal was introduced first to the peptide. The selected ion monitoring traces for the $[M-H+X]^+$ ions for the various solutions are shown in Fig. 8.

As was seen in the CD competition study copper displaced zinc from patellamide C and when a mixture of the two metals was added only copper species were formed. When copper was titrated into a solution containing **3** with 2 equiv. of Zn^{2+}, addition of 0.25 equiv. of Cu^{2+} already displaced the Zn; *m/z* 824 and 975 became dominant and the multiply charged Zn species disappear from the spectra. A further experiment was carried out with a solution of **3** with 2 equiv. of Cu^{2+} in which monocopper and dicopper species at *m/z* 824 and 975, respectively, were present (Table 3). Formic acid was added to this solution to a final concentration of 1%, causing the dicopper species at *m/z* 975 to disappear, but not the monocopper species at *m/z* 824.

Competition experiments with patellamide A produced mixed Cu/Zn complexes in each case and multiply charged species persisted even after adding 2 equiv. of Cu^{2+} to a solution of **1** containing 2 equiv. of Zn^{2+}. These results agreed with those obtained by CD where a hybrid copper/zinc spectrum was obtained in the presence of both metals. Further evidence came from isotope patterns and HRMS of a solution of patellamide A to which a mixture of copper and zinc had been added which showed the isotope pattern for a Cu/Zn complex and a peak at 902.1476 which differs only Δ 8 ppm from the value calculated for $[PatA\text{-}2H+CuZnCl]^+$.

Ulithiacyclamide (**9**) behaved very much like patellamide C (**3**) during competition studies. Addition of 2 equiv. of an equimolar mixture of Cu^{2+}/Zn^{2+} to **9** in methanol resulted in the formation of only copper species, *m/z* 824 (55%) $[Uli\text{-}H+Cu]^+$; *m/z* 860 (100%) $[Uli+CuCl]^+$ and *m/z* 921 (65%) $[Uli\text{-}2H+Cu_2Cl]^+$. Within a few minutes, the *m/z* 921 dicopper complex becomes the dominant species.

As an additional experiment, a 1:1 mixture of patellamides A and C was made in methanol and to this was added 1 equiv. of Cu^{2+}. The resulting spectrum showed as the 100% peak $[PatA\text{-}H+Cu]^+$ at *m/z* 805. The patellamide C was mainly in the form of $[PatC+Na]^+$ at *m/z* 785. This would suggest that patellamide A can out-compete patellamide C for copper, but further experiments would be necessary to confirm this. This is in contrast to the binding constants measured by CD, but consistent with those obtained by MS (Table 2).

Mass spectrometric titration experiments were undertaken in a similar manner to those described by Brady and Sanders for measuring relative binding affinities of metals with steroid derivatives by ESI-MS.[25] Titrations with zinc and copper solutions were carried out on patellamides C and A by sequential addition of 0.25 equiv. of the metal solutions to the peptides (conc. 0.021 mg/mL in MeOH). Once fully complexed zinc species were obtained for each peptide, copper solution was then titrated into these at a rate of 0.25 equiv. to again observe the competition effects.

For the Cu^{2+} and Zn^{2+} titrations of **1** and **3**, the concentration of metalated species relative to $[M+H]^+$ was plotted against the concentration of the metal to give relative binding affinities. A problem with this method is the differential ionisation efficiency of the different complexes and therefore results from this method must be regarded as approximate. Mass spectrometric studies above have shown that the cyclic peptides will be divided over various complexes (Table 3), and therefore species chosen for monitoring were those present throughout the whole range of the titration. For titration of **3** with Cu^{2+} the main species are

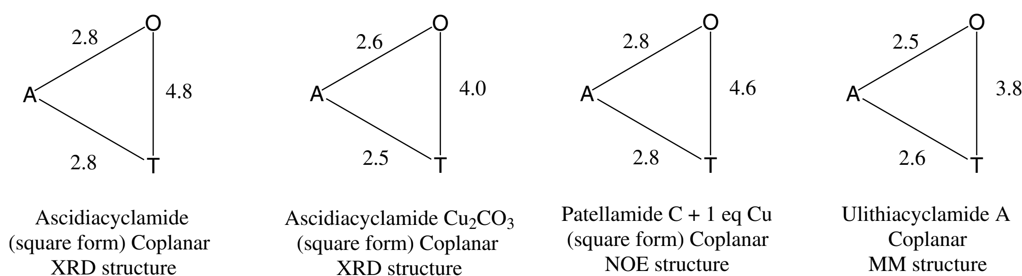

Figure 9. Distances in Å between and relative orientation of nitrogen atoms available for copper binding in **1**, **3**.

$[PatC+H]^+$ and $[PatC\text{-}H+Cu]^+$ making this type of calculation a valid method for determining relative binding affinities. A relative binding affinity for dicopper complex of **3** at *m*/*z* 975 was also measured. This is in contrast to the CD method, as no K_2 could be ascertained due to absence of conformational change upon binding the second equivalent of Cu^{2+}. The calculations for Zn^{2+} binding affinities are much less reliable as the Zn^{2+} is distributed across many species, perhaps giving rise to the unexpected observation that K_1 and K_2 are of the same magnitude. The relative binding constants for zinc and copper calculated for patellamides A (**1**) and C (**3**) from the MS titrations are given in Table 2.

From plots of the relative intensities of the metal complexes formed it could be seen that the formation of many zinc species was independent of the concentration of metal salt added. This was the case for all the doubly charged zinc complexes of patellamide C, which after an initial fast increase in their intensities stabilised at around 1 equiv. of zinc added and remained more or less constant. This was also the case with zinc complexes of patellamide A with only the intensities of the $[PatA+H+ZnCl_2]^+$ and $[PatA\text{-}H+Zn_2Cl_2(H_2O)_2]^+$ showing a linear dependence on concentration.

The formation of $[PatA\text{-}2H+Cu_2Cl]^+$ became constant after the addition of 1 equiv. of copper suggesting that although two coppers could be bound by patellamide A this complex was much less stable than $[PatA\text{-}H+Cu]^+$ or $[PatC\text{-}3H+Cu_2+C_3H_6O_3]^+$. The formation of $[PatA\text{-}H+Cu]^+$ was very clear with the intensity of that species reaching a maximum after the addition of 1 equiv. of the metal and remaining constant thereafter.

The appearance of the $[PatC\text{-}3H+Cu_2+(C_3H_6O_3)]^+$ complex of patellamide C appeared to be dependent on the concentration of metal solution added, with a steady increase in intensity with increasing metal concentration, but previous measurements of this complex had shown that its formation was very slow.

5. Conclusions

In this section, the main points arising from each of the studies above will be discussed and related to different aspects of the metal-binding properties of **1**, **3** and **9**. The studies by CD on **3** showed it bound Cu^{2+}, and this binding of copper resulted in a change from the figure of eight (II) to the square (I) conformation.

Binding of a second equivalent of copper by **3**, seen in the MS, was not detected by CD presumably indicating no conformational change was associated with this binding. Patellamide A (**1**), which adopts mainly the square form (I) in solution, was also shown to bind Cu^{2+}, but in doing so only small conformational changes occurred as shown by CD. This can be related to the X-ray crystal structure of ascidiacyclamide which distorts only slightly from the square form to accommodate two copper atoms.[11] Only small amounts of 1:2 **1**/Cu^{2+} complexes were detected by MS. The dicopper complex of the structurally similar **8** was only formed after addition of large amounts of base (4 equiv.),[11] and perhaps patellamide A also requires base to form complexes containing $2Cu^{2+}$. Ulithiacyclamide (**9**), however, readily forms dicopper complexes without any change in its conformation, with the dicopper complex being the preferred one.

The inter-atomic distances and relative orientations of the copper binding sites within **1**, **3** and **9** were available from previous work,[23] and compared to the sites present in the X-ray crystal structure of the dicopper complex of **8** (Fig. 9).[11] The copper binding site is made up of the Thz (T), amide (A) and Oxn (O) nitrogens, which we have named the 'TAO' motif. In the X-ray crystal structure of the Cu_2CO_3 complex of ascidiacyclamide[11] the T–A and O–A distances are both roughly 2.5 Å, whereas the O–T distance is longer at 4.0 Å. If this is taken as the ideal copper geometry for the TAO motif, which we have confirmed using ab initio electronic structure calculations,[23] then comparisons can be made with the geometries of **1**, **3** and **9**. With the crystal structure of ascidiacyclamide (**8**) as model for **1** then the main difference is an increase in length of the O–T distance (Fig. 9). The NOE structure[23] of **3**/Cu^{2+} shows that the uncomplexed TAO site has a slightly shorter O–T distance than **8**. The most remarkable geometry is that of the very rigid ulithiacyclamide (**9**) which seems to have evolved the ideal TAO copper-binding site (Fig. 9).

Binding sites for copper in patellamide C were also deduced from the MS fragmentation of the 1:1 copper complex. Patellamides A and C were shown to form complexes with zinc in both the CD and MS studies. The formation of multiply charged, transient and aggregate species with

zinc would suggest that zinc complexes are largely non-specific associations with various counter-ions included to satisfy the charge balance requirements. Looking at the square conformation of the patellamide backbone and looking for possible tetrahedral sites for zinc, we can see that three nitrogen atoms might be suitably oriented to provide this. This may explain the lack of specificity between Cu^{2+} and Zn^{2+} observed for square **1** in methanol, in both the CD and MS studies. The observation, by MS, of a mixed **1**/Cu^{2+}/Zn^{2+} complex (Fig. 8) confirms this lack of selectivity. In the figure of eight form of **3** only two nitrogens are suitably positioned for the formation of tetrahedral complexes. This would lead to the less strong binding of Zn^{2+} by **3**, as observed. Without added base, copper can more easily deprotonate amides than zinc, so we could predict that copper would out-compete zinc for peptide ligands.

The observed selectivity might be explained in terms of the Irving–Williams series of complex stability for divalent ions bound to conformationally flexible ligands.[27] The series would lead us to expect selectivity for Cu^{2+} over Zn^{2+} as observed as well as preferential binding to Cu^{2+} over Ni^{2+}. Pseudosymmetrical **1** is non-selective for Cu^{2+}, even though the Irving–Williams series would lead us to expect selectivity, whereas **3** is selective for Cu^{2+}. Since the binding of Cu^{2+} to **3** involves a large conformational change, it may be that this is the driving force behind the selectivity. The order of the Irving–Williams series can be changed by altering the binding geometry of the ligand, i.e. the use of conformationally rigid ligands. Therefore, an explanation for this selectivity must be the different binding environments favored by the two metals. Cu^{2+} is known to prefer a square planar or square pyramidal environment and this is provided by the TAO motif when the patellamides adopt the square form. Zn^{2+} favours a tetrahedral binding environment which is not available in either conformation of **3**, and although binding of zinc does take place it is probably less ideal than that with copper so this could be an explanation for the observation that zinc is able to be displaced from **3** on the addition of copper. Nickel and copper are sometimes able to occupy the same binding sites.[22] Ni^{2+} complexes prefer an octahedral environment with symmetric bonds.[26] Within the macrocyclic cavity of patellamides the sixth site is blocked and only square pyramidal co-ordination is possible, and this might be why Ni^{2+} is unable to bind.

Grøndahl et al.[14] found that binding of zinc to ascidiacyclamide (**8**) was a very slow process. If this is the case with all of the peptides and zinc it would also help explain the observed selectivity for copper over zinc. However, when solutions of **3** and a mixture of Cu^{2+} and Zn^{2+} were measured after three months no change had occurred and only the copper complex was in evidence.

A deciding factor in the specificity of binding of Cu^{2+} to **3** compared to **1** may be the difference in their respective side chains. The presence of the phenylalanine may distort the planarity of the square conformation of patellamide C, suggesting that this distortion changes the geometry of the macrocycle sufficiently to provide the ideal binding site for copper in the TAO motif.

The above study indicates that copper is the biologically relevant metal for these cyclic peptides. The presence of two Cu^{2+} centres within 3.6–4.5 Å of each other[11] in these complexes suggests a biological function for them in the activation of dioxygen, such as in haemocyanin and tyrosinase.[28,29,30] In methanolic solution dicopper complexes are known to form dimethylcarbonate (MeO–(C=O)–OMe) from atmospheric carbon dioxide, as was observed for patellamide C (**3**). The rate of formation of dimethylcarbonate is maximum when the Cu···Cu distance is ~4 Å.[31] This suggests that the *L. patella* metabolites may have evolved as small dicopper enzymes with potential functions as catalases, peroxidases, oxidases or oxygenases. It is intriguing to contemplate that the disulphide bridge in ulithiacyclamide may be directly involved in a redox process with the Cu centres which are in very close proximity.

6. Experimental

6.1. General

Compounds **1**, **3** and **9** were isolated or donated and their spectra were assigned as described previously.[18]

6.2. Circular dichroism spectroscopy

CD spectra were acquired either at the BBSRC CD Facility at Stirling, UK or at the Faculty of Pharmacy, Universiteit Utrecht, Utrecht, The Netherlands. All spectra were recorded between 200 and 320 nm at a speed of 10 nm/min, resolution 0.2 nm, response 2 s and sensitivity 20 mdeg. All solutions were prepared in spectroscopic grade methanol. Metal solutions (0.1 M) were prepared from their chloride salts and then further diluted to give 0.5 and 1.0 mM stock solutions. Compounds **1**, **3** were prepared at 0.002 mg/mL in methanol. The copper and zinc salt solutions were titrated separately into 11 mL of the solution of peptide using 25 μL portions of the stock solutions (0.04 equiv.) and a full spectrum recorded after each addition. The spectra were corrected for dilution using the program available on the instrument. The absorbances at 250 and 211 nm after each addition of copper, and at 205, 211 and 250 nm for each addition of zinc were plotted separately to produce a titration curve for each of these metals. This was carried out using copper over the concentration range 0–3 equiv. Unbound peptide was also titrated with zinc solution in the same manner up to 5 equiv. and absorbance at 205 and 250 nm recorded after each addition.

6.3. Mass spectrometry

All mass spectrometry was carried out at Department of Biomolecular Mass Spectrometry, Utrecht University, Utrecht, The Netherlands. All samples were dissolved in HPLC grade methanol. Accurate mass measurements were carried out with a quadrupole-time-of-flight instrument (Q-TOF, Micromass) with nano-electrospray ionisation, at a resolution of 5000. An oligipeptide (phenylalanine)$_n$ was used as lock mass and n was chosen in such a way that the lock mass was higher than the accurate mass to be

measured. All other MS experiments were carried out with an ion trap instrument (LC-Q, Thermoquest/Finnigan) equipped with a nanoflow electrospray probe. The spray voltage was 0.9 kV and the capillary temperature 80°C. For the titration experiments the instrument was optimised for $[M+H]^+$, and the settings were not changed during the titration experiments. MS titrations were monitored by measuring the formation of the metalated species as well as the loss of uncomplexed peptides, using both the full spectrum and the selected ion mode of the instrument in parallel, as the ionisation efficiency of these species is very different. For MS^n experiments the relative collision energy was adjusted in such a way that the relative intensity of the parent ion was still approximately 30%.

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