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Metal binding of *Lissoclinum patella* metabolites. Part 2: Lissoclinamides 9 and 10

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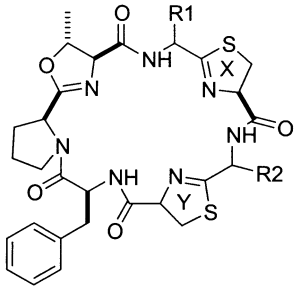
Abstract—Studies on the Thz, Thn and Oxn containing cyclic peptides, lissoclinamides 9 (**9**) and 10 (**10**) isolated from the Indo-Pacific ascidian (seasquirt) *Lissoclinum patella* have delineated their metal binding selectivity. MS and CD competition studies show that lissoclinamide 10 (**10**) shows selectivity for Cu^{2+} in the presence of an excess of Zn^{2+} whereas lissoclinamide 9 (**9**) is less selective for Cu^{2+}. Comparison of the solution state conformations derived from nOe restrained molecular dynamics and additional Monte–Carlo conformational searches suggested binding environments for the Cu^{2+} which confirmed the MS measurements and suggested a reason for the selectivity in the case of lissoclinamides 9 and 10.

1. Introduction

In this paper we shall be concerned with the *Lissoclinum patella* metabolites lissoclinamide 9 and 10, members of the lissoclinamide group of compounds (**1**–**11**), composed of seven amino acid residues containing thiazole (Thz), thiazoline (Thn) and oxazoline (Oxn) ring systems. The isolation and structure determination of lissoclinamides 9 (**9**) and 10 (**10**) was the subject of a previous publication.[1] Several natural and synthetic lissoclinamides were found to be biologically active. Wipf and co-workers showed that the replacement of Thn rings with Oxn rings decreased activity to a greater extent than replacement of Oxn rings with Thn rings.[2] This study further showed that it was not only the individual components of the macrocycle that conferred high activity but rather the overall conformation of the molecules. The structure activity relationship is also demonstrated when comparing lissoclinamides 4 (**4**) and 5 (**5**). These compounds differ only in the oxidation state of a single Thz unit but this difference makes lissoclinamide 5 (**5**) two orders of magnitude less cytotoxic than lissoclinamide 4 (**4**) against bladder carcinoma (T24) cells.[3]

The lissoclinamides resemble 21-azacrown-7 macrocycles but no investigations into their metal binding properties have been reported, unlike for the patellamides for which extensive binding studies have been published in the literature (see preceding paper).



1 Lissoclinamide 1 R1=L-Val R2=D-Ile X=Y=thiazole
2 Lissoclinamide 2 R1=D-Ile R2=D-Ala X=thiazolineY=thiazole
3 Lissoclinamide 3 R1=D-Ile R2=L-Ala X=thiazolineY=thiazole
4 Lissoclinamide 4 R1=L-Val R2=D-Phe X=thiazolineY=thiazole
5 Lissoclinamide 5 R1=L-Val R2=D-Phe X=Y=thiazole
6 Lissoclinamide 6 R1=D-Val R2=D-Phe X=thiazolineY=thiazole
7 Lissoclinamide 7 R1=D-Val R2=D-Phe X=Y=thiazoline
8 Lissoclinamide 8 R1=Val R2=Phe X=thiazolineY=thiazole
11 Ulicyclamide R1=L-Ile R2=D-Ala X=Y=thiazole

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

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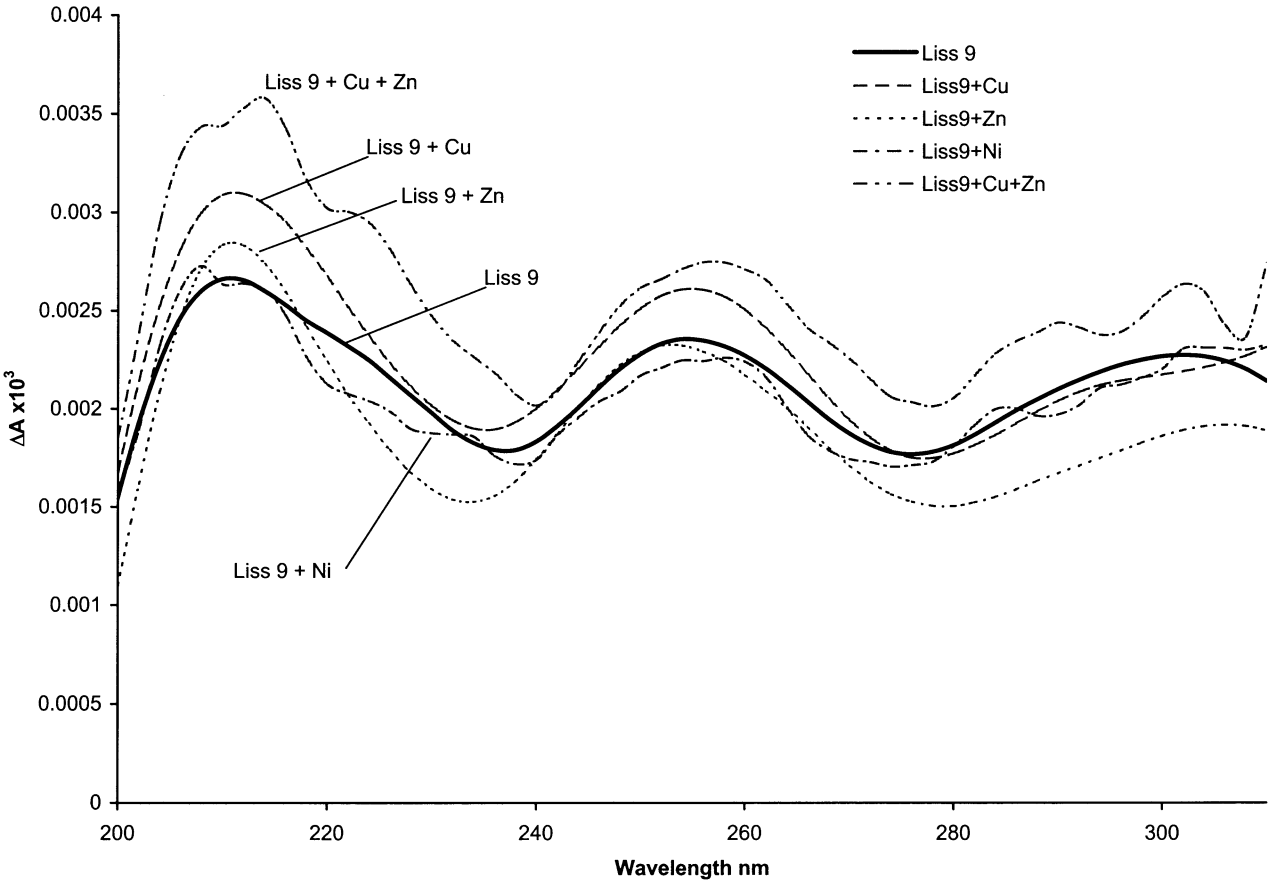
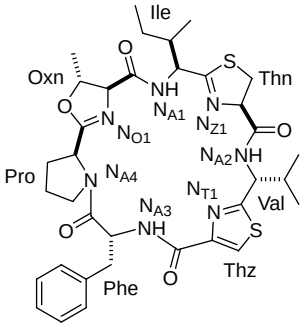
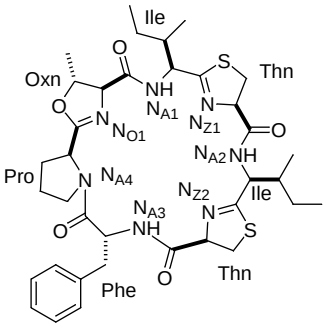


Figure 1. CD spectra of **9** at 0.002 mg/mL in MeOH plus 2 equiv. of copper, zinc, nickel and a mixture of copper and zinc.



9



10

2. Results and discussion

2.1. Circular dichroism studies

The first CD spectra obtained were those of lissoclinamides 9 and 10 (**9**, **10**) in methanolic solution (Figs. 1 and 2, bold traces). The spectra are of much lower intensity than the patellamide spectra and there are no prominent maxima or minima (see preceding paper). Qualitative comparisons to standard protein CD spectra[4] indicate that a weak type II β-turn is present in lissoclinamide 9 (λ_{max}=209, 254 nm) similar to that found in the 'figure of eight' conformations of the patellamides.[5] This β-turn is induced by the Oxn moiety as shown by Wipf et al.[2] in studies on lissoclinamide 7 (**7**) (Table 1). A Monte–Carlo conformational search on lissoclinamide 9 indicates that in the lowest energy form (**9** MC_L, Fig. 3) a hydrogen bond is present between Phe C═O and Ile N–H (N_{A1}–H) and that the ϕ and ψ angles fall in the acceptable range for type II β -turns (Table 1).[6] However in an nOe restrained molecular dynamics structure of lissoclinamide 9 this β-turn is absent (Table 1).[1] Re-examination of the Monte–Carlo molecular modeling data shows that there is a family of structures (**9** MC_H), of which the energy is 8 kJ/mol higher than the global minimum and which shows good overlap with the nOe derived structure in the Pro-Oxn part of the molecule (full backbone RMSD= 0.92 Å; RMSD for Pro–N–Ile–N backbone=0.11 Å). The absence of a β-turn in thermally accessible conformations explains why the intensity of the β-turn signature in the CD spectrum is so low.

The CD spectrum of lissoclinamide 10 shows features consistent with a random coil type structure (Fig. 2, bold trace).[4] The greater flexibility of this molecule, due to the presence of two Thn moieties, is evident in the Monte–Carlo conformational search. The lowest energy structure (**10** MC_L, Fig. 4) does not contain the Phe C═O to Ile N–H (N_{A1}–H) hydrogen bond necessary for a β-turn whereas it is present in a structural family with a slightly higher energy (**10** MC_H, ~1 kJ/mol) (Table 1). Examination of families of conformations within a thermally accessible temperature range shows that tight hydrogen bonded turns can also be formed under the influence of each Thn moiety, although

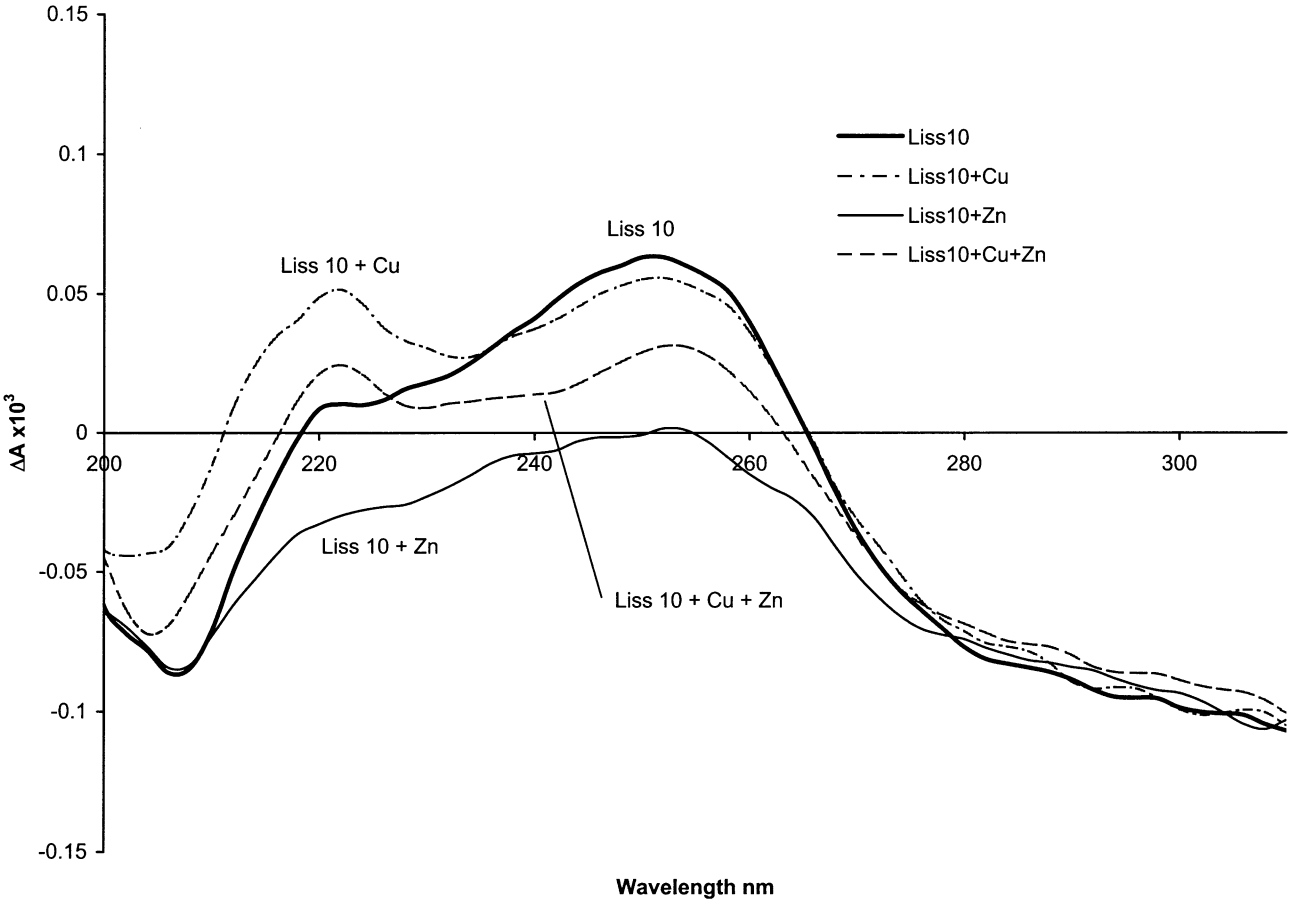


Figure 2. CD spectra of **10** at 0.02 mg/mL in MeOH plus 2 equiv. of copper, zinc and a mixture of copper and zinc.

they do not fit the standard types of turn (e.g. H-bond IleNH (N_{A2}–H) –OxnC═O; $\phi_{Ile}=-30°$; $\psi_{Ile}=109°$; $\phi_{Thn}=137°$; $\psi_{Thn}=-40°$).[6] The absence of a dominant low energy conformation therefore explains why the CD spectrum indicates the presence of a random coil.

Addition of 2 equiv. of $CuCl_2$, $ZnCl_2$ or $NiCl_2$ to a solution of lissoclinamide 9 (**9**) caused little observable change in the CD maxima at 209 and 254 nm indicating that little or no conformational change was taking place (Fig. 1). A small increase in ΔA at λ_{max} 209 and 254 was noted on addition of copper, but the spectral traces for **9**/Zn^{2+} and **9**/Ni^{2+} essentially followed that of native **9**. It was therefore not possible to discern if **9** was binding to Zn^{2+} or Ni^{2+}. The invariance of the CD spectra suggested that the conformation of **9** was very stable. The small change at 209 and 254 nm in a titration of **9** with Cu^{2+}, monitored by CD, was used to estimate binding constants. It was found that the first binding constant $K_1=1.4\pm0.2\times10^4$, similar to that for patellamide C (see preceding paper) and the second binding constant was much weaker, $K_2=125\pm4$.

Addition of 2 equiv. of $ZnCl_2$ to a solution of lissoclinamide 10 (**10**) decreased the intensity of the CD spectrum but did not change its shape relative to that of native **10**, suggesting again that little change of conformation takes place (Fig. 2). However, addition of 2 equiv. of $CuCl_2$ to a solution of **10** increased ΔA at the 220 nm maximum whilst leaving the 255 nm maximum unaffected. This indicates that the addition of Cu^{2+} alters the conformation of lissoclinamide **10**. Lack of material effectively ruled out estimating binding constants for **10** through a CD monitored titration.

Behaviour similar to that of patellamide A (see preceding paper) was observed on adding a mixture of Cu^{2+} and Zn^{2+} to **9** (Fig. 1). Only a very small change was observed, and as

Table 1. Type II β turns in lissoclinamides

	ϕ_{Pro} (°)	ψ_{Pro} (°)	ϕ_{Oxn} (°)	ψ_{Oxn} (°)	H-bond (Å)	E_{MM} (kJ/mol)
Ideal	−60.0	120.0	80.0	0.0	1.8–2.5	
Lissoclinamide 9 NOE	−79.2	10.0	110.8	16.6	4.5	
Lissoclinamide 9 MC_L	−72.8	104.5	97.2	−3.7	2.0	156.5
Lissoclinamide 9 MC_H	−76.5	11.0	117.5	18.6	4.8	164.2
Lissoclinamide 10 MC_L	−73.8	104.2	97.0	42.0	2.8	155.7
Lissoclinamide 10 MC_H	−71.6	112.2	99.5	0.9	2.1	156.8
Lissoclinamide 7 XRD	−59.3	127.0	110.9	−24.7	2.2	

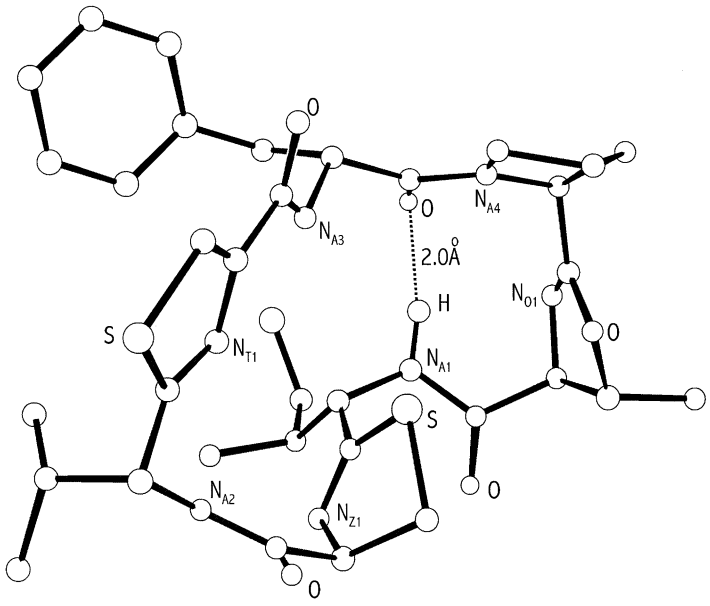


Figure 3. Global minimum **9** MC_L from molecular mechanics Monte–Carlo conformational analysis of **9** showing β-turn H-bond.

the CD spectra of **9**, **9**/Cu^{2+} and **9**/Zn^{2+} were similar in appearance no firm conclusions could be drawn. Addition of a mixture of Cu^{2+} and Zn^{2+} to lissoclinamide 10 (**10**) did indicate that some preferential binding to Cu^{2+} was taking place (Fig. 2). The shape of the resulting CD curve was very similar to that of **10**/Cu^{2+}, suggesting that **10** preferentially binds to Cu^{2+} in the presence of Zn^{2+}, a fact which was later confirmed by mass spectrometry.

3. Mass spectrometry

As **9** and **10** had been found to bind both copper and zinc in the circular dichroism study, it was decided to further investigate these complexes by mass spectrometry in order to ascertain their exact nature and which, if any, counter-ions were incorporated into the complexes. Accurate mass measurements and analysis of isotope ratio patterns were used to assign the components of a complex. We first obtained accurate mass and fragmentation data for uncomplexed **9** and **10**.[1] The copper and zinc complexes of each were then studied in detail, including their fragmentation patterns. In addition, MS was used to monitor some competition experiments.

3.1. Mass spectrometry of Cu and Zn complexes

The main species occurring in the ion trap mass spectra of lissoclinamide 9 and 10 with 2 equiv. of Cu^{2+} or Zn^{2+} are presented in Table 2. The mass spectra of lissoclinamide solutions containing Cu are simple, with few species

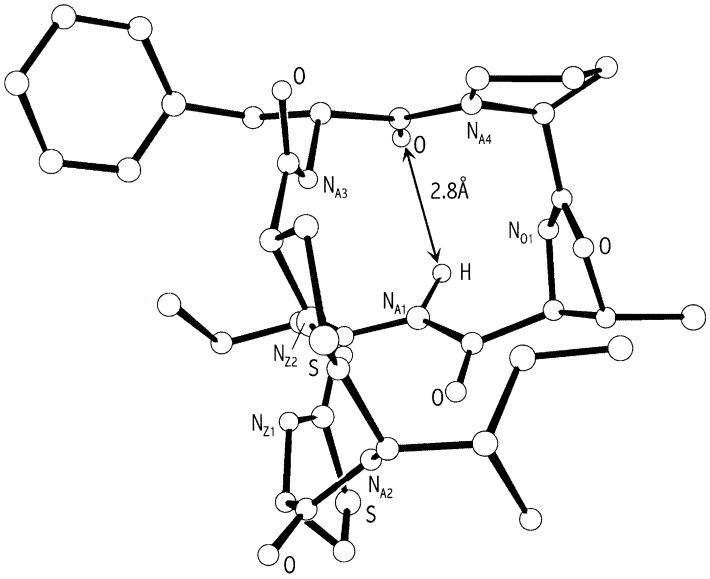


Figure 4. Global minimum **10** MC_L from molecular mechanics Monte–Carlo conformational analysis of **10** in the same orientation as **9** in Fig. 3 showing the increased distance between N_{A1}–*H* and Phe–C═*O*.

Table 2. Main species detected in the MS of Lissoclinamide 9 and Lissoclinamide 10 plus 2 equiv. Cu^{2+} or 2 equiv. Zn^{2+} solution. The relative intensities are from selective ion monitoring traces. Base peaks (100%) are in **bold** type

	m/z, Δ (%)[a]			
Species	**9**+Cu	**9**+Zn	**10**+Cu	**10**+Zn
Singly charged species				
$[M+H]^+$	708.3001	708.3001	724.3341	724.3341
	2.7 (40)	2.7 (35)	1.7 (50)	1.7 (25)
$[M+H+H_2O]^+$		**726**		
Cu containing species				
$[M-H+Cu]^+$	769.2132		**785.2444**	
	2.7 (90)		0.0	
$[M-H+Cu+H_2O]$	787 (75)		803 (10)	
$[M-H+Cu+MeOH]^+$	801 (65)			
$[M+CuCl]^+$	805 (45)			
$[M-2H+Cu_2Cl]^+$	866 (55)			
$[M-2H+Cu_2Cl+H_2O]^+$	884 (75)			
$[M-2H+Cu_2Cl+CO_2]^+$			926[b,c] (50)	
Zn containing species				
$[M-H+Zn]^+$		770 (25)		786 (15)
$[M+ZnCl]^+$		806 (25)		823 (10)
Doubly charged species				
$[M-2H+2Cu]^{2+}$	415.5 (60)			
$[M+Zn]^{2+}$		385.7 (35)		**393.5**

[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.
[b] Determined by isotope ratio model for $C_{37}H_{47}N_7O_7S_2Cu_2Cl$ exp: 926 (90); 927 (50); 928 (100); 929 (50); 930 (50); 931 (25); 932 (12). calc: 926 (72); 927 (35); 928 (100); 929 (42); 930 (51); 931 (21); 932 (10).
[c] Present in a sample containing both Cu^{2+} and Zn^{2+}.

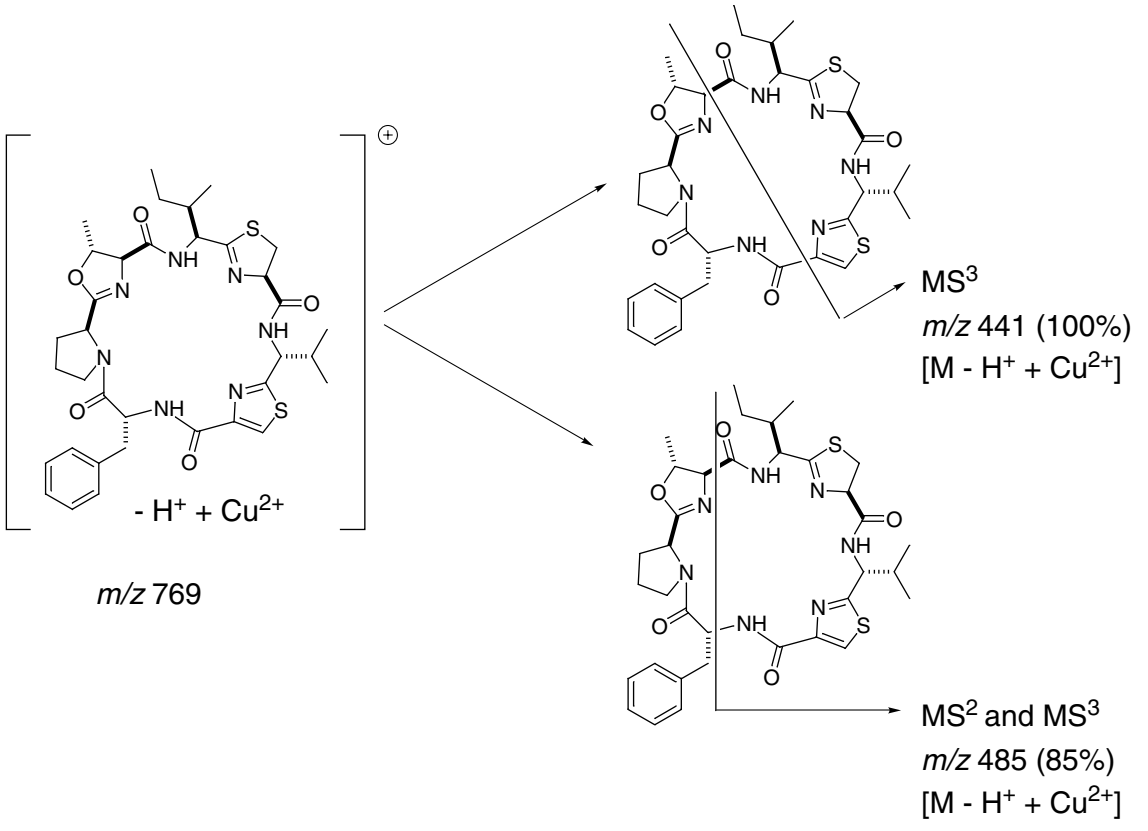


Figure 5. MS^n fragmentations of $[\mathbf{9}\text{-}H+Cu]^+$ which led to the identification of the Cu^{2+} binding site in **9**.

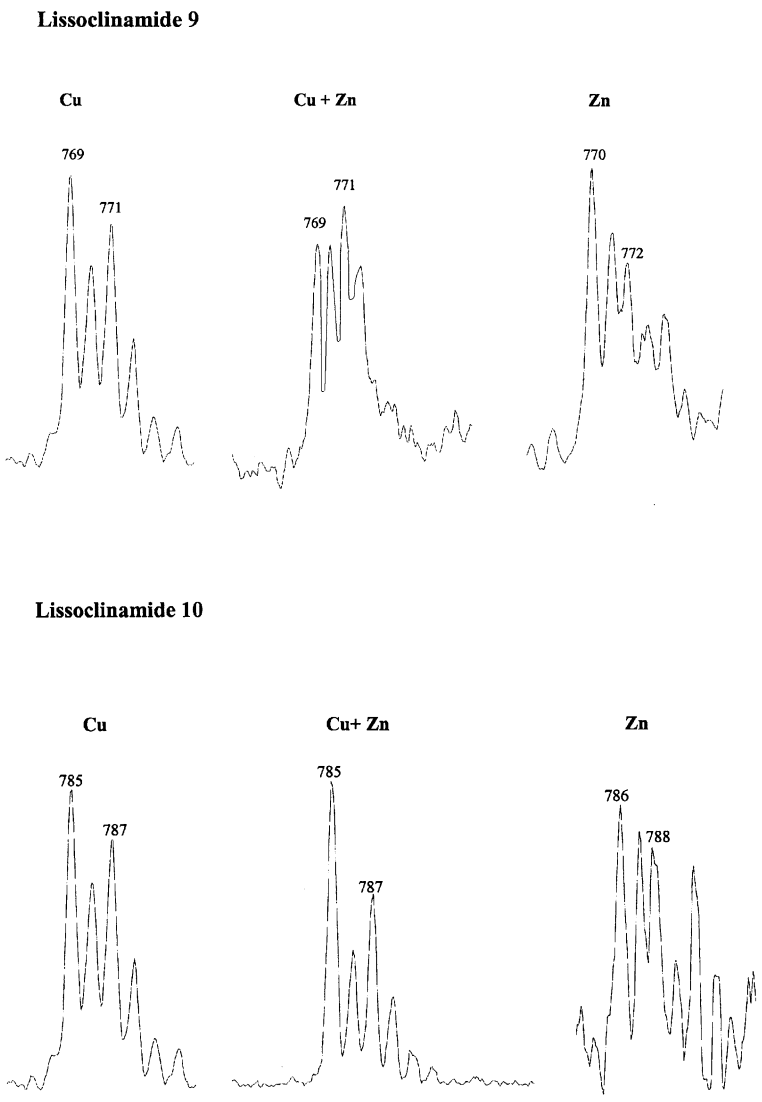


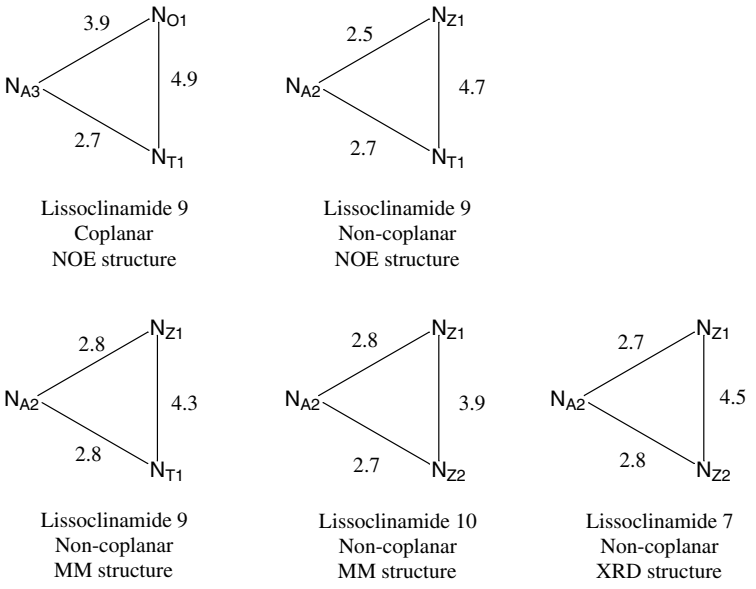
Figure 6. Selected ion monitoring spectra of $[M-H+X]^+$ (M=lissoclinamide; X=metal ion) after addition of Cu^{2+}, $Cu^{2+}+Zn^{2+}$ or Zn^{2+}.

present, whereas those of Cu containing lissoclinamide 9 and in particular those of Zn containing samples of both **9** and **10** are complex. In contrast to the mass spectra of the Cu complexes, those of the Zn complexes generally showed more multiply charged ions. Obviously, Zn has a higher preference for binding without the abstraction of hydrogen. The same was observed for patellamides A and C (see preceding paper).

For lissoclinamide 9 (**9**/Cu^{2+}), water, methanol and Cl^- adducts are far more pronounced than for the patellamides (see preceding paper). A few doubly charged ions were present as well, the strongest being at *m/z* 415.5 for $[Liss9\text{-}2H+Cu_2]^{2+}$. The MS of **10**/$Cu^{2+}$ shows $[Liss10\text{-}H+Cu]^+$ and its water adduct, as well as a dicopper complex, in combination with a considerable amount of uncomplexed **10**.

MS^n data of native **9** and **10** have been previously reported.[1] In MS^2 of **9** the main losses (apart from some 18 and 28) are 147 (PheCHNHCO, base peak), 69 (Pro) and 28 (CO). MS^2 of the Cu complex of **9** (*m/z* 769) shows loss of 91 ($PheCH_2$) as the base peak. All other fragmentations, apart from loss of 27/28 (CHN, CO) involve Pro, Oxn and $PheCH_2CH$, but the amide group ($N_{A3}H-C{=}O$) between Phe and Thz appears to remain intact. MS^3 of *m/z* 769 shows two large Cu containing fragments (Fig. 5). This suggests that Cu binds to the part of the molecule containing Ile, Thn, Val, and Thz. In MS^2, the Cu_2Cl complex (*m/z* 866) loses both HCl and CuCl, but the second Cu remains. These results indicate specific binding of one Cu^{2+} by **9** with a second more loosely bound, in agreement with the CD measurements of copper binding by this peptide.

In the MS^2 of native **10**, fragmentation is more pronounced. The base peak occurs for the loss of Pro+CO, but the relative intensity of signals corresponding to, amongst others, loss of 18, 28, 44, 69 (Pro), 85 (Thn) and 147 (PheCHNHCO) are over 50%. In the MS^2 of the Cu complex (*m/z* 785) the main fragmentation is loss of 83 (Oxn) and/or 71 (Pro+2H). Also, some side chain fragmentation (loss of 29, 57 and 91) is observed, which is absent in the MS^2 of **9**. However, the relative intensity of the corresponding fragment ions is below 20%. The fragmentation for the Cu complexes of both **9** and **10** strongly suggest that the binding site for copper is N_{Z1}, N_{A2} and N_{T1} for **9** and N_{Z1},



T = Thiazole nitrogen, A = Amide nitrogen, O = Oxazoline nitrogen, Z = Thiazoline nitrogen

Figure 7. Distances in Å between and relative orientation of nitrogen atoms available for copper binding in **9**, **10**.

N_{A2} and N_{Z2} for **10**. That only one amide nitrogen is involved in binding is attested to by the fact that only one amide hydrogen is abstracted in the binding process, thus ruling out the other possibilities from the MS data for **9** (Fig. 5) of N_{A1}–N_{Z1}–N_{A2} and N_{A2}–N_{T1}–N_{A3}.

The mass spectrum obtained for a mixture of **9** and Zn^{2+} showed little evidence for binding of zinc. The major peaks are presented in Table 2. The water adduct of **9** was of much greater intensity than that seen in the spectrum of the native peptide, but this may be due to water being present in the $ZnCl_2$ used. The Zn complexes tended to fragment in a way similar to the native peptide, losing CO and other ring fragments, such as Thz and Pro.[1]

In the mass spectrum of **10** with Zn^{2+} only minor peaks for singly charged species such as *m/z* 786 $[Liss10\text{-}H+Zn]^+$ and its HCl adduct are observed, however, the peaks for doubly charged species for example that at *m/z* 393.5 $[Liss10+Zn]^{2+}$ are more intense, and there was also a significant amount of unbound peptide present. When fragmented, the $[Liss10+Zn]^{2+}$ species loss part of the Ile sidechain and part of the ring but retained its dipositive charge. As with patellamide C/Cu^{2+} (see preceding paper), **10**/Zn^{2+} showed no loss of the metal on fragmentation. Lissoclinamide **10** seems to bind smaller amounts of zinc compared to copper but in a more specific manner than lissoclinamide **9**.

Addition of both Cu^{2+} and Zn^{2+} to **9** and **10** seems to show mixtures of Zn^{2+} and Cu^{2+} complexes for **9**, with several multiply charged species remaining, but for **10** the mono Cu^{2+} complex at *m/z*785 is the main species (Fig. 6). Interestingly, the spectrum of **10** with Cu^{2+} and Zn^{2+} also shows a peak at *m/z* 926, which could be the CO_2 adduct of **10**+Cu_2Cl (Table 2), similar to that observed for ascidiacyclamide.[7]

4. Structural studies

Ab initio molecular mechanics conformational analyses were performed for **9** and **10** in order to assess the geometry of potential metal-binding sites within the molecule. These sites were thought to involve the Thz/Thn and intervening amide nitrogens. Global minima were located for lissoclinamides 9 and 10 with E_{MM2^*}=156.53 and 155.72 kJ/mol, respectively. For **9** the 19 lowest energy structures fell within an RMSD=0.25 Å (Fig. 3), and for **10**, the 99 lowest energy structures fell within an RMSD of 0.1 Å (Fig. 4), indicating that global minima had been located.

In the nOe restrained dynamics structure of **9**[1], a coplanar Cu^{2+} binding motif made up of N_{T1}, N_{A3} and N_{O1} (cf. the 'TAO' motif in the patellamide, see preceding paper) can be observed but the distances for binding to Cu^{2+} are non-ideal (Fig. 7). MS studies already indicated that N_{O1} was probably not involved in Cu^{2+} binding. MS fragmentation studies, allowed the identification of Cu^{2+} binding sites in **9** and **10**, and these were then studied in the global energy minima obtained from the Monte–Carlo conformational searches. The MS of **9** suggested that a binding site might present itself between the Thz N_{T1}, intervening amide N_{A2} and Thn N_{Z1} nitrogens, the 'TAZ' motif. The distances measured from the nOe and conformational search structures were similar to those in the TAO motif in native ascidiacyclamide (see preceding paper). In **10**, a similar motif, comprising of two Thn nitrogens N_{Z1} and N_{Z2} and the intervening amide nitrogen N_{A2} could be identified (the 'ZAZ' motif) with distances and geometry similar to the

dicopper ascidiacyclamide (see preceding paper). The geometries of the TAZ motif in **9** and the ZAZ motif in **10** were compared to the ascidiacyclamide TAO motif and in **10** was found to be closer to the ideal geometry for binding to Cu^{2+}. The two Thn nitrogens N_{Z1} and N_{Z2} are pointing directly at each other and the centre of the potential binding site. In **9**, the geometry was less optimal and the Thz N_{T1} and Thn N_{Z1} could be seen to be less well directed towards the centre of the binding site. In both the molecules, the binding amide nitrogen N_{A2} was easily accessible from outside the macrocycle. In addition, the presence of two Thn moieties in **10**, increases the flexibility around ZAZ motif and allows it to adopt the ideal binding geometry for Cu^{2+}. In **9**, the substitution of one Thn for a Thz increases rigidity around the TAZ motif, thus not allowing it to adopt an ideal Cu^{2+} binding geometry. At the moment, the identity of the second binding Cu^{2+} site in **9** and **10** is still ambiguous.

In **9**, the β-turn hydrogen bond (Fig. 3) does not involve the amide N_{A2}–*H* of the TAZ motif. In **10** (MC_H) however, a hydrogen bond could be seen to involve the amide N_{A2}–*H* that would have to be removed for full occupation of the ZAZ motif by Cu^{2+}. This hydrogen bond would have the effect of elongating and weakening the N–H bond, therefore making deprotonation easier. This, combined with the Thn geometry would suggest that **10** would be a better ligand for copper than **9** in that less energy would be required for complexation of the metal. The geometry of the Cu^{2+} ZAZ binding site in lissoclinamide 7 (**7**), taken from the crystal structure,[2] is found to be similar to that of **9** (Fig. 7).

In the lissoclinamides it is probable that the inclusion of two thiazoline moieties is necessary for efficient and strong binding to two Cu^{2+}. The added flexibility conferred by these structural units seems to be important for the binding to take place in the first instance, and for it to remain stable after it has occurred. It is possible that during extraction and isolation procedures, thiazolines in thiazoline containing lissoclinamides are oxidised to thiazoles. The formation of a CO_2 adduct of the dicopper complex of **10** again allows speculation of these dicopper complexes in the activation of dioxygen (see preceding paper).

5. General experimental

Compounds **9**, **10** were isolated and their spectra were assigned as described previously.[1]

5.1. 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 **9**, **10** were prepared at 0.02 mg/mL in methanol.

5.2. 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 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%.

5.3. Computational studies

Monte–Carlo conformational searching. The initial structures for compounds **9** and **10** were input by hand using the Macromodel v6.5 graphical user interface.[8] Chirality was assigned at all stereo centres but no further manual manipulation of the structures was undertaken, thus reducing operator bias in the conformational search as far as possible. The Macromodel native Monte–Carlo search algorithm was employed and all structures obtained were optimised using the MM2* force field.[9] To improve the comparability of the structures generated from this search with those obtained experimentally, the generalised Born solvent accessible area (GB/SA) continuum description of $CHCl_3$ was employed.

Investigation of hydrogen bonding. The resulting optimised structures obtained in the Monte–Carlo searches were refined using the semi-empirical PM3 molecular orbital method available in MOPAC v6.0[10] and the output geometry analysed for instances of short (<2.25 Å) inter-residue C═O…H–N distances.

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