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Structure Determination and MS^n Analysis of Two New Lissoclinamides Isolated from the Indo–Pacific Ascidian *Lissoclinum patella*: NOE Restrained Molecular Dynamics Confirms the Absolute Stereochemistry Derived by Degradative Methods

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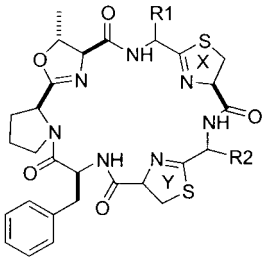
Abstract—Two new lissoclinamides, lissoclinamides 9 and 10 were isolated from an Indonesian collection of the ascidian *Lissoclinum patella* along with the known patellamide C. The structures of the lissoclinamides were determined by a combination of 2D NMR, selective 1D TOCSY, MS and MS^n techniques. The assignment of absolute stereochemistry was achieved by the hydrolysis of lissoclinamides 9 and 10 followed by chiral TLC. In the case of lissoclinamide 9, NOE restrained molecular dynamics studies were also performed confirming the proposed stereochemistry.

Introduction

Study of the ascidian *Lissoclinum patella* (Order Enterogona, Family Didemnidae) has previously yielded several families of closely related cyclic peptides, many of which have significant biological activity.[1] These peptides have been grouped into four main structural types—ulithiacyclamides, patellamides, lissoclinamides and tawicyclamides—according to the number of amino acids and inclusion of thiazole (Thz), thiazoline (Thn) and oxazoline (Oxn) rings within their structure. The lissoclinamides (**1**–**8**) and ulicyclamide (**11**)[2–4] are all derived from a cyclic heptapeptide in which a threonine has been cyclised to an oxazoline and two cysteines have been cyclised to give a thiazole or thiazoline. The linear sequence of amino acid residues is Xxx(Thn or Thz)-Yyy(Thn or Thz)-Cys-Phe-Pro(Oxn) where Pro(Oxn) indicates an oxazoline ring moiety formed from a proline and a threonine as in **9** and **10**. Xxx(Thn or Thz) and Yyy(Thn or Thz) are thiazoline (Thn) or thiazole (Thz) containing variable D or L amino acids, where Xxx or Yyy are Val, Ala, Phe or Ile (e.g. see **9**, **10**). The oxazoline- and thiazole-containing octapeptide patellamides are the most abundant compounds found in *L. patella* by weight.[5–9]



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: lissoclinamide; patellamide C; stereochemistry.
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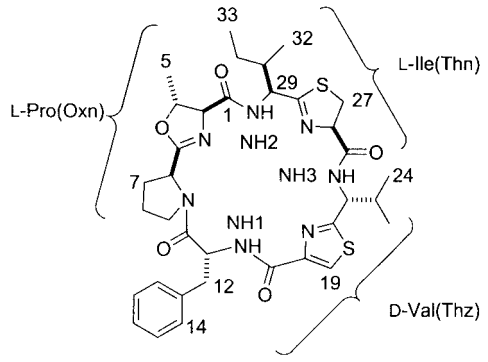

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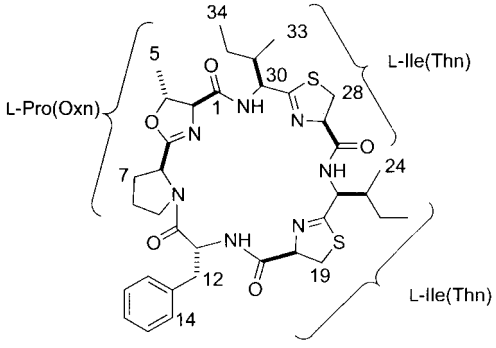

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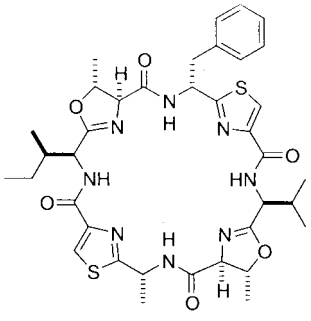

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These cyclic peptides have shown significant bioactivity in a variety of screening regimes and a strong structure–activity relationship has been noted by several workers. The inclusion of an oxazoline moiety in a compound was shown by Shioiri and co-workers[10] to give much higher levels of activity than other residue types. However, a comparison of natural and synthetic lissoclinamides by Wipf and co-workers showed that the replacement of thiazoline rings with oxazolines decreased activity to a greater extent than replacement of oxazoline rings with thiazolines.[11] 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 this molecule. This 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 thiazole unit but this difference makes lissoclinamide 5 (**5**) two orders of magnitude less cytotoxic than lissoclinamide 4 (**4**) against bladder carcinoma (T24) cells.[12] In addition to their cytotoxic properties, patellamides B and C (**12**) have been shown to reduce multi-drug resistance (MDR) in vitro of drug resistant lymphoblasts.[7]

The peptides from *L. patella* are also of interest for their metal-binding properties. In structure they resemble 21-azacrown-7 and 24-azacrown-8 macrocycles and several members of the patellamide group have been found to bind copper and zinc atoms within their central cavities.[13,14] The metal-binding properties of the lissoclinamides and patellamides will be the subject of a forthcoming publication.

This article describes the structure elucidation of two new lissoclinamides (**9**, **10**) using small quantities of material. One of the techniques used, selective 1D TOCSY, has been described in our previous publication on a cyanobacterial metabolite.[15] In addition, we have obtained extensive MS fragmentation data from FAB-MS and ion trap ESI-MS^n experiments which confirm the proposed structures. We determined the absolute stereochemistry of the amino acid residues by acid digestion followed by chiral TLC. In addition, the solution conformations of all four possible stereoisomers containing D- or L-Ile, D- or L-Val and D-Phe of lissoclinamide 9 (**9**) have been determined using NOE restrained molecular dynamics.

Results and Discussion

Structure determination

The sample of *L. patella* was obtained from Halmahera, Indonesia in November 1996 and preserved in a mixture of alcohol and seawater. Exhaustive extraction with MeOH and CH_2Cl_2 followed by solvent partition of the resulting oil gave a CH_2Cl_2 fraction which was chromatographed using size exclusion chromatography and ODS-HPLC to give patellamide C (**12**) as major component (50 mg). In addition two other compounds were isolated in small quantities (9 and 4 mg) which were tentatively identified from their ^1H NMR spectra as lissoclinamides. The key signatures of lissoclinamides in a ^1H NMR spectrum are 3 NH doublets, resonances from 1 or 2 phenylalanine residues and 0, 1 or 2 proton singlets at $\sim\delta$ 8 ppm characteristic of a thiazole ring. For lissoclinamide 9 (**9**) we observed 3 NH doublets, 1 phenylalanine, and 1 thiazole. These indicators were also noted for lissoclinamide 10 (**10**) except the thiazole singlet was missing, perhaps indicating the presence of a thiazoline. The molecular formulae of **9** and **10** were determined to be $C_{35}H_{45}N_7O_5S_2$ and $C_{36}H_{49}N_7O_5S_2$, respectively, by exact mass measurements at high resolution: the $[M+H]^+$ of a known compound was used as the lock mass. Compound **9** has 17 double bond equivalents and **10** has 16 double bond equivalents, which is consistent with the change from a thiazole to thiazoline between **9** and **10**. Apart from this difference in oxidation states, the molecular formulae lissoclinamides 9 and 10 only differ by the mass of a CH_2.

The ^1H NMR resonances of the peptides were assigned from selective 1D TOCSY spectra. Essentially these spectra provide a 1D ^1H NMR subspectrum for each of the residues

Table 1. ^{1}H and ^{13}C shifts and 2D correlations of lissoclinamide 9 (**9**) at 400 and 100 MHz in $CDCl_3$

Atom #	δ ^{13}C ppm	δ ^{1}H ppm (J, Hz)	HMBC C→H	1D-TOCSY H→H
1	172.3 s	–	2, 3, 29, NH2,	
2	76.8 d	4.23 d (5.6)	5	
3	82.1 d	4.77 t (6.9, 5.6)	2, 5	
4	170.7 s	–	2	
5	22.9 q	1.44 d (6.8)	2	
6	57.8 d	4.57 t (6.9, 5.5)	–	7/7′, 8/8′, 9/9′
7	29.9 t	2.24 m, 1.95 m	–	
8	26.6 t	2.07 m, 1.92 m	–	
9	48.5 t	3.74 t (9.6, 8.5), 3.33 m	–	
10	170.4 s	–	6, 9, 12	
11	53.1 d	5.13 m	12/12′	NH1, 12/12′, 14
12	39.5 t	3.06 dd (14, 5.1), 2.94 dd (14, 8)	11	
13	136.7 s	–	12/12′	
14	130.9 d (2C)	7.12 m	12/12′	
15	129.4 d (2C)	7.14 m	16	
16	128.0 d	7.15 m	15	
17	172.3 s	–	11, 19, NH1	
18	149.3 s	–	19	
19	125.3 d	8.0 s	–	
20	160.8 s	–	21, 22, NH3	
21	55.4 d	5.35 dd (8.6, 3)	24	NH3, 22, 23, 24
22	41.7 d	1.94 m	21, 24	
23	17.4 q	0.94 d (7.5)	–	
24	14.6 q	0.75 d (7.5)	–	
25	177.7 s	–	26, 27, NH3	
26	80.6 d	5.17 m	27/27′	
27	37.3 t	3.49t(11.5, 11.3), 3.33 d (11.5)	–	
28	173.6 s	–	27/27′	
29	57.4 d	4.93 dt (10.5, 3, 3)	31, 32	NH2, 30, 31/31′, 32, 33
30	32.5 d	2.68 ddt (7.9, 6.3, 3.4)	31, 32	
31	27.9 t	1.5 m, 1.38 m	–	
32	20.7 q	0.96 d (7)	–	
33	12.9 q	0.96 t (7)	–	
NH1	–	7.86 d (8.2)		
NH2	–	7.58 d (9.1)		
NH3	–	6.82 d (9.1)		

in the peptides.[15–17] Selective irradiation was carried out at the α protons of each residue. For example, irradiating the double doublet at δ 5.35 ppm in the ^{1}H NMR spectrum of **9** excited resonances at δ 6.82 (d, NH), 1.94 (m, H_β), 0.96 (d, Me), 0.75 (d, Me) indicating this to be a valine residue. Further experiments showed lissoclinamide 9 (**9**) contains oxazoline, Pro, Phe, Val, thiazoline and Ile residues as well as a thiazole (Table 1). A gradient-filtered HSQC experiment[18,19] was used to correlate all the protonated carbons with their respective protons. Two and three bond correlations from an HMBC spectrum were used to complete the assignment of the quaternary carbons to their residues, and confirm most of the sequence of the peptide.[20] In lissoclinamide 9 (**9**) the C1–H2 and H3 correlation indicated that C1 was the amide carbonyl in the oxazoline residue, C10–H12 made C10 the Phe amide carbonyl, C17–H19 placed C17 as the thiazole C═O and C25–H26 and H27 indicated that C25 was the thiazoline carbonyl. The remaining quaternaries, C4, C18, C20 and C28 were placed inside amino acid residues by HMBC correlations C4–H2; C18–H19; C20–H21, H22 and NH3; C28–H27/27′. What remained was to determine the sequence of the amino acid residues in the lissoclinamide molecule, and this was evident from the HMBC correlations C1–H29 and NH2; C10–H6 and H9; C17–H11 and NH1 and C25–NH3. Breaks in the assignment of the peptide occurred for the connections of C3–C6; C19–C 20 and C29–C30. However, confirmation of these connections could be derived from mass spectral fragmentation patterns. The FAB-MS indicated that cleavage occurred preferentially on either side of carbonyl C10 in keeping with previously observed fragmentations for cyclic proline containing peptides.[21] After the opening of the macrocycle by cleavage of the C10–C11 or N–C10 bond the next fragmentation was the loss of Pro-CO leaving *m/z* 611 or loss of Phe-CONH leaving *m/z* 561. Loss of the fragment CO-Pro-Oxn by cleavage at C10–C11 and C1–C2 left *m/z* 528, which indicated that the connectivity between the proline and oxazoline (C4–C6) was correct and inferred that the thiazoline–isoleucine linkage (C28–C29) was also correct. A fragment ion at *m/z* 526 is probably formed by the loss of [Pro-Oxn-CO+2H].

Selective 1D TOCSY experiments also proved effective in the assignment of lissoclinamide 10. Analysis of the spectra showed that it contained an oxazoline, a proline, two isoleucines, and two thiazoles. Protonated carbons were assigned by using an HSQC NMR spectrum. The scarcity of sample of lissoclinamide 10 (**10**) meant that few HMBC correlations could be observed. However, the resonances observed allowed a structure to be proposed, when used in combination with ^{13}C chemical shift comparison with other lissoclinamides[4] and lissoclinamide 9 (**9**) and the 1D TOCSY data (Table 2). The key HMBC correlations are C1–H2 and H30; C4–H2; C12–NH1; C26–H28 and NH3

Table 2. ^{1}H and ^{13}C shifts and 2D correlations of lissoclinamide 10 (**10**) at 400 and 100 MHz in $CDCl_3$

Atom #	δ ^{13}C ppm	δ ^{1}H ppm (J, Hz)	HMBC C→H	1D-TOCSY H→H
1	172.4	–	2, 30	
2	76.9	4.25 d, (4.8)	5	3, 5
3	81.2	5.46 d (9.5)	5	
4	170.7	–	2	
5	27.8	1.48 d (5.4)		
6	57.5	4.56 t (7.9, 7.8)		7/7′, 8/8′, 9/9′
7	29.8	2.31 m, 2.25 m		
8	26.4	1.90 m		
9	48.5	3.56 m, 2.98 t (10.5, 10.5)		
10	170.9	–	9	
11	53.2	5.01 dq (9.5, 2, 2)	12′	12/12′, NH1
12	41.1	2.88 dd(12.5, 8.2), 2.72 dd (12.5, 9)	NH1	
13	128.1	–		
14	130.8	7.11 m	12/12′	
15	129.5	7.21 m	14	
16	130.3	7.15 m	14	
17	170.7	–		
18	79.4	4.71 m	19	19/19′
19	35.7	3.62 m		
20	175.1	–		
21	55.8	5.17 m	24	22, 23/23′, 24, 25
22	38.5	2.44 m	24	
23	22.9	1.46 m, 1.34 m	24, 25	
24	14.2	0.86 d		
25	12.9	0.95 t		
26	181.4	–	28, NH3	
27	82.3	4.9 m		28/28′
28	38.8	3.48 d (1.9)		
29	173.0	–	30	
30	55.8	5.22 m	33	NH2, 31, 32/32′, 33, 34
31	32.8	2.47 m		
32	23.1	1.42 m	33, 34	
33	15.4	1.14 d (7.6)		
34	13.0	0.95 t (6.3)		
NH1	–	7.08 d (8.2)		
NH2	–	7.85 d (9.3)		
NH3	–	6.90 d (10.3)		

and C29–H30. The final sequence of amino residues was again confirmed from the mass spectral fragmentation patterns. As for lissoclinamide 9, the first bond cleavage again occurred at N–C10 or C10–C11 after which Phe-CONH or Pro-CO were lost leaving *m/z* 577 and 627, respectively. The adjacency of the proline and oxazoline could be inferred from the loss of CO-Pro-Oxn leaving *m/z* 544 and from the loss of [Pro-Oxn-CO+2H] leaving

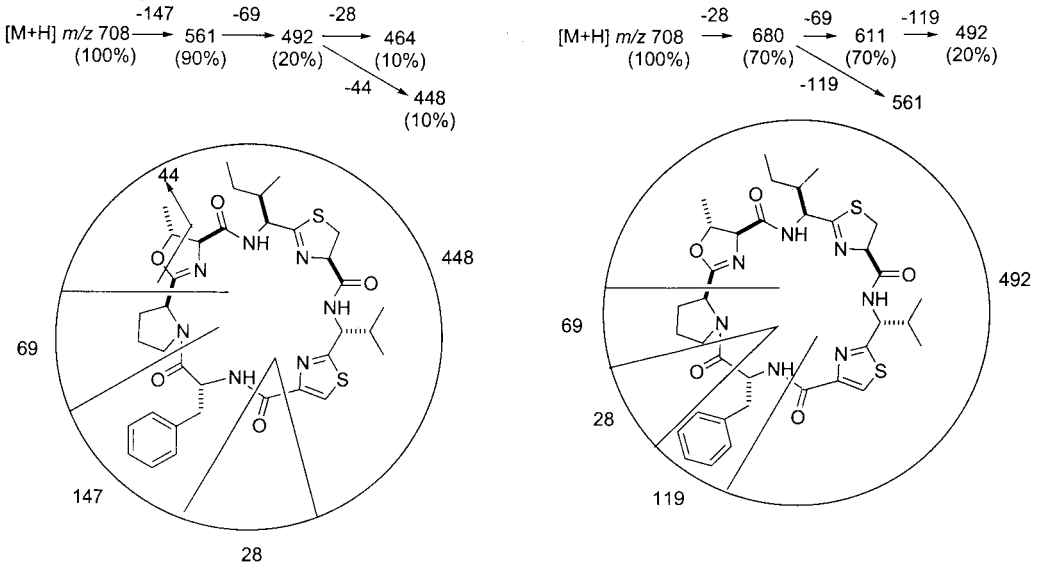

Figure 1. Major fragmentation pathways of lissoclinamide **9**.

Table 3. Lissoclinamide 9 ESI-MS fragmentation $[M+H]^+$=*m/z* 708

Fragment ion (MS2)			MS3			MS4		
m/z	Neutral loss	Assignment	*m/z*	Neutral loss	Assignment	*m/z*	Neutral loss	Inference
561 (100%)	147	PheCONH	492	69	Pro	464	28	CO
						448	44	C_2OH_4
680 (35%)	28	CO	611	69	Pro	492	119	PheNH
						567	44	C_2OH_4
611 (30%)	97	ProCO	492	119	PheNH C_2OH_4	–	–	
			567	44	C_2OH_4			

m/z 542. The *m/z* 395 pointed to the loss of a large fragment, Pro-CO-Phe-CONH-Thn.

Ion trap MS^n data

As the MS spectra were complex, additional ion-trap MS^n spectra were obtained up to MS^3 to fully define the sequences and common fragmentation patterns of lissoclinamides 9 (**9**) and 10 (**10**). For lissoclinamide 9 the data are shown in Fig. 1 and Table 3. The predominant cleavage occurred at the Pro-Phe amide bond, after which the loss of Phe occurred, followed by Pro and smaller fragments. An alternative pathway after Pro-Phe amide cleavage was the loss of CO followed by Pro and then Phe. For lissoclinamide 10, the most common cleavage site is also the Pro-Phe amide bond after which a similar fragmentation pathway to lissoclinamide 9 is followed (Fig. 2, Table 4). An alternative fragmentation at the Phe C═O to

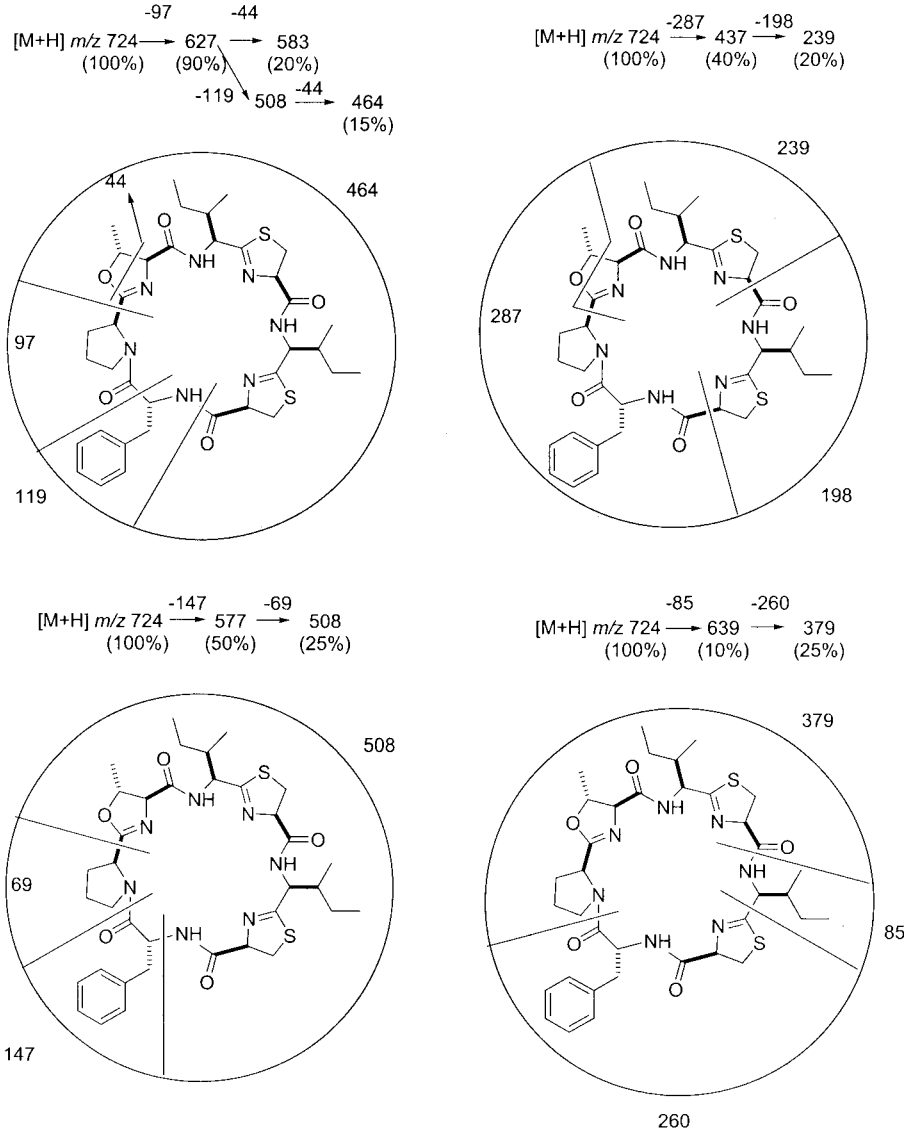

Figure 2. Major fragmentation pathways of lissoclinamide **10**.

Table 4. Lissoclinamide 10 ESI-MS fragmentation $[M+H]^+$=*m/z* 724

Fragment ion (MS2)			MS3		
m/z	Neutral loss	Assignment	*m/z*	Neutral loss	Assignment
696 (60%)	28	CO	627	69	Pro
655 (60%)	69	Pro	627	28	CO
627 (100%)	97	ProCO	583	44	C_2OH_4
			464	119	PheNH
577 (55%)	147	$PheCONH_2$	508	69	Pro
480 (30%)	244	ProCOPheNHCO	437	43	CONH
680 (45%)	44	C_2OH_4	611	69	Pro
639 (70%)	85	IleNH	379	260	ThnCONHPheCO
437 (70%)	287	CONHPheCOPro+CONH	409	28	CO

Table 5. R_f values of amino acids in **9**, **10** and **12** determined by chiral TLC

Compound	L-Phe	D-Phe	L-Val	D-Val	L-Ile	D-Ile	L-Thre	D-Thre	L-Pro	D-Pro	L-Cys
Standards	0.62	0.57	0.63	0.49	0.55	0.47	0.44	0.40	0.73	0.56	0.42
9	–	0.58	–	0.50	0.54	–	–	–	0.72	–	–
10	–	0.59	–	–	0.54	–	–	–	0.72	–	–
12	–	0.58	0.63	–	0.54	–	–	–	–	–	–
9 Ozonised	–	0.58	–	0.50	0.54	–	0.45	–	–	–	0.42
10 Ozonised	–	0.59	–	–	0.54	–	0.45	–	–	–	0.42
12 Ozonised	–	0.59	0.63	–	0.54	–	0.45	–	–	–	–

C_α bond is also observed followed by loss of Pro and then Phe. Initial ring scission near the thiazolines is also observed, and this cleavage leads to the formation of large fragments. In both cases the primary pathway is cleavage at the Pro-Phe amide bond followed by loss of Pro and Phe. This is in agreement with previously published data.[21]

Determination of absolute stereochemistry by hydrolysis and chiral TLC

We determined the absolute stereochemistry of lissoclinamide 9 and 10 by acid hydrolysis followed by chiral TLC.[22] This gave the absolute stereochemistry of the Phe, Val and Ile amino acid residues (Table 5). To determine the absolute stereochemistry of the thiazoline and oxazoline residues, the sample was ozonised before acid hydrolysis to give Cys and Thr, which were subjected to chiral TLC.[23] The proposed stereochemistry of lissoclinamide 9 (**9**) was L-Ile(Thn)-D-Val(Thz)-D-Phe-L-Pro(Oxn) and that of lissoclinamide 10 (**10**) L-Ile(Thn)-L-Ile(Thn)-D-Phe-L-Pro(Oxn). It is known that the C_α next to the thiazole and thiazoline racemises readily during hydrolysis,[24] making the stereochemical assignments for the Ile and Val residues unreliable. For example, recent syntheses of lissoclinamides 4 (**4**) and 5 (**5**)[25] reassign their stereochemistry at R1 and R2 as L-Val and D-Phe instead of the previous D-Val and L-Phe assignments.[2]

Due to uncertain stereochemical assignments in two amino acid residues in **9**, we were interested in a method which might enable us to confirm the absolute stereochemistry of lissoclinamide 9 (**9**) at C21 and C29. Crystallography will provide the ultimate answer, but due to the difficulty in crystallising small quantities of natural material, thus far only crystallisation of synthetic lissoclinamide 7 (**7**) has been achieved.[11] Synthesis is an alternative, but would be very labour intensive. Previous work has shown lissoclinamide 7 (**7**) with R1 being the unnatural L-Val is rapidly converted to the D-Val stereoisomer in the presence of a base.[11] This indicates that the absolute stereochemistry of the natural product should be the most favourable for the stabilisation of the global conformation of the molecule. Therefore a possible method was to perform NOE restrained molecular dynamics calculations on all four possible stereoisomers of lissoclinamide 9, and determine which has the lowest global energy.

Restrained molecular dynamics of lissoclinamide 9

NOE restrained dynamics studies have been reported for a number of patellamides, including patellamide C (**12**).[9] The solution structure of lissoclinamide 7 (**7**) is available but this was performed using only 6 NOE restraints, all set to 1.8–5.0 Å, and few statistics are given.[11] In our study we derived 28 NOE restraints for lissoclinamide 9 from a T-ROESY experiment, and these were classified as weak (1.8–5.0 Å), medium (1.8–3.5 Å) and strong (1.8–2.5 Å) (Table 6). Of these, 14 were intra residue and 14 inter residue (5 to

Table 6. NOE restraints for lissoclinamide 9 (**9**)

Atom i	Atom j	NOE	Atom i	Atom j	NOE
3	31	w	21	22	m
5	24	w	21	24	m
7′	24	w	21	27	w
9	8′	m	22	24	m
11	9	w	29	2	w
11	9′	w	29	31′	m
11	12	m	29	33	w
11	32	w	30	11	w
12′	9	w	30	29	w
14	2	w	30	32	m
14	11	m	30	33	m
14	12	m	31	2	w
14	12′	m	NH3	23	m
14	29	w	NH2	24	w

Table 7. RMSD and energy data for NOE restrained molecular dynamics calculations on stereoisomers of **9** (all energies in kJ/mol; RMSD's in Å for 10 lowest energy conformations)

Ile	Val	RMSD (BB)	RMSD (Heavy)	E_{tot}	E_{bond}	E_{angle}	E_{noe}	NOE viol <5 kJ/mol	NOE viol >5 kJ/mol	Additional expected strong NOE's
L	D	0.00±0.00	0.76±0.63	57.14	3.61	42.26	4.54	3	0	1
L	L	0.18±0.14	0.73±0.36	82.96	5.17	52.87	16.39	3	1	0
D	D	0.07±0.04	0.58±0.41	103.71	6.13	58.31	20.84	4	1	4
D	L	0.07±0.06	0.61±0.38	48.82	3.00	31.71	8.03	3	0	4

neighbouring residues, and 9 long range). Two ensembles of 100 models were derived by randomising the ϕ and ψ angles of lissoclinamide 9 (**9**) containing all four possible stereoisomers containing D- or L-Ile at C29 and D- or L-Val at C21. Structures were calculated by ab initio simulated annealing and the 30 lowest energy conformations were subjected to a slow cooling refinement protocol. For each of the ensembles the 10 lowest energy structures were used for further analysis. The data for all stereoisomers is summarised in Table 7. It can be seen that the target energy function and distance restraint violation energies vary greatly. The lowest E_{tot} is that of **9**DL (i.e. **9** with D-Ile, L-Val), followed by **9**LD, the stereochemistry determined by hydrolysis, and then **9**LL and finally **9**DD. The last two can be ruled out on the basis of their high E_{tot} (Table 7). The remaining two stereochemistries have low NOE violation energies, and extremely good backbone RMSD's. Using the 10 lowest energy structures for all four stereoisomers we calculated inter-residue H–H distances less than 2.5 Å which appeared in at least 5 out of the 10 structures (Table 7). Such short H–H distances are expected to give rise to strong NOE's. Where such NOE correlations were absent in the T-ROESY spectrum, they are listed in Table 7 under the column headed 'additional expected strong NOE's'. Stereoisomer **9**DL has 4 missing strong NOE's and can therefore be effectively ruled out. The stereoisomer determined by hydrolysis, **9**LD (Fig. 3), has only 1 missing NOE, a slightly higher E_{tot} and only minor NOE violations, and is therefore the most favoured stereoisomer. Using this method the stereochemistry of lissoclinamide 9 is therefore confirmed to be L-Ile(Thn)-D-Val(Thz)-D-Phe-L-Pro(Oxn).

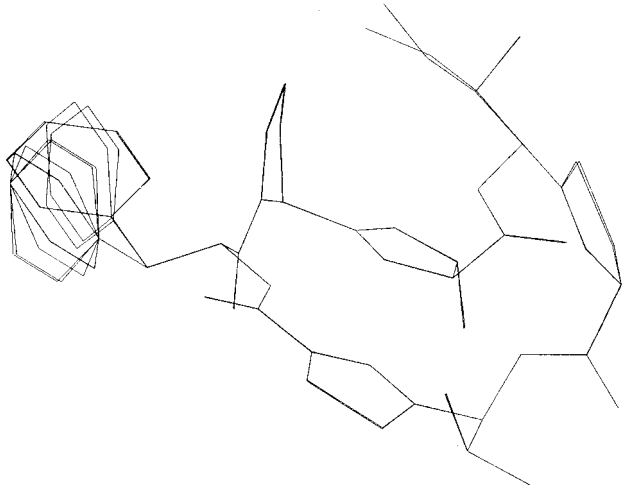

Figure 3. Backbones of the ten lowest energy structures of **9** with L-Ile and D-Val (stereoisomer determined by hydrolysis).

The use of NOE restrained molecular dynamics therefore offers an independent and more rapid way of confirming the absolute stereochemistry of the lissoclinamides than total synthesis. This method can be used on small quantities of naturally derived substance, and is more reliable than hydrolytic methods which may racemise stereocentres.

Experimental

General experimental

HPLC was performed using an ODS 10 μm spherical particle/100 Å pore size column and UV detection at 254 nm. All NMR spectra were recorded with a 400 MHz instrument at 26°C in $CDCl_3$ using residual $CHCl_3$ as internal reference at 7.27 ppm. Selective 1D TOCSY's were acquired by creating a shaped pulse at the desired resonance frequency and incorporating this into the 1D TOCSY pulse sequence.[16,17] An array of spectra was acquired using different spin-lock periods (10–180 ms in 10 ms increments), with four transients per increment. ROE's were obtained with a T-ROESY pulse sequence with a 400 ms mixing time.[26,27] NOE's were classified into weak (1.8–5.0 Å), medium (1.8–3.5 Å) and strong (1.8–2.5 Å). The envelope for Me groups was opened up by 0.5 Å. ROE restrained molecular dynamics studies were carried out using Axel Brünger's X-PLOR.[28] All mass spectrometry was carried out at Department of Biomolecular Mass Spectrometry, Universiteit Utrecht, Utrecht, the Netherlands. HRMS were obtained on a quadrupole-time of flight instrument with electrospray ionisation. In addition FAB-MS was used to observe fragmentations followed by MS^n work using an ion trap mass spectrometer in electrospray ionisation mode. All samples were dissolved in HPLC grade methanol.

Collection and identification

The sample of *L. patella* (Order Enterogona, Family Didemnidae, coll no. 96323) was collected in November 1996 at a depth of 15 m from a sloping reef, Halmahera, Indonesia (1°39.839′ N; 127°29.444′ E). The sample was identified by Miranda Sanders of the University of California, Santa Cruz. A voucher specimen is preserved at the Marine Natural Products Laboratory, Department of Chemistry, University of Aberdeen (voucher number 96323).

Extraction and isolation

The sample was preserved by immersing in a 1:1 EtOH:seawater mixture. After 24 h the mixture was decanted and discarded, after which the organism was transported back

to Aberdeen at ambient temperature. The organism was extracted with MeOH for 24 h (3×) and CH_2Cl_2 (3×) and the concentrated extracts combined. The crude oil was partitioned between water and CH_2Cl_2, after which the CH_2Cl_2 layer was stripped of solvent and the resulting oil partitioned between *n*-hexane and 10% aqueous MeOH. The MeOH layer was then phase adjusted to 50% aqueous MeOH and extracted with CH_2Cl_2. The CH_2Cl_2 fraction was then subjected to Sephadex LH20 size exclusion chromatography (1:1 MeOH:CH_2Cl_2), and separated into three fractions. The last fraction was subjected to ODS-HPLC (20% aq. MeOH) to give **9** (9 mg), **10** (4 mg) and **12** (50 mg).

Determination of absolute stereochemistry by chiral TLC

Compounds **9**, **10** and **12** were subjected to acid hydrolysis in 6N HCl at 110°C for 72 h. To determine the absolute stereochemistry of the chiral centres in the oxazoline and thiazoline moieties, a portion of each was subjected to ozonolysis prior to acid digestion to give threonine and cysteine, respectively.[23] The acid digest was subjected to chiral TLC using chiral plates (ODS impregnated with a proline derivative and Cu^{2+}),[22] and visualised with ninhydrin spray reagent. Two different solvent systems were utilised. For proline the solvent system used was MeOH:H_2O:MeCN 1:1:4. For the other amino acids, the solvent used system was MeOH:H_2O:MeCN 5:5:3. The R_f values of the digests are given in Table 5.

Lissoclinamide 9 (9). pale yellow oil; IR ($CHCl_3$ soln.) 3424 (NH str.) 3387, 3319, 2924 (CH str.), 2853,1739, 1653 (C=O str.), 1541, 1457, 1257, 1030, 960, 772 (Phe CH def.), 668.; UV (*c* 0.2 mg/ml $CHCl_3$) λ_{max} 308 nm, ϵ=3370; Circular dichroism spectrum (0.02 mg/mL in MeOH) 209 nm ΔA=0.30 mdeg, 254 nm ΔA=0.28 mdeg; HRESIMS *m/z* 708.2982 $[M+H]^+$ calcd for $C_{35}H_{46}N_7O_5S_2$ 708.3001 (Δ 2.7 ppm); MS fragmentation data—see Table 3; NMR data—see Table 1.

Lissoclinamide 10 (10). pale yellow oil; IR ($CHCl_3$ soln.) 3443 (NH str.), 3387, 3319, 2974, 2929 (CH str.), 2853, 1636, 1603 (C=O str.), 1458, 1251, 1029, 966, 774 (Phe CH def.), 658; UV (*c* 0.2 mg/ml $CHCl_3$) λ_{max} 308 nm, ϵ=2900; Circular dichroism spectrum (0.02 mg/mL in MeOH) 209 nm ΔA=−0.28 mdeg, 250 nm ΔA=0.22 mdeg; HRESIMS *m/z* 724.3353 $[M+H]^+$ calcd for $C_{36}H_{50}N_7O_5S_2$ 724.3341 (Δ 1.7 ppm); MS fragmentation data—see Table 4; NMR data—see Table 2

ROE restrained molecular dynamics calculations

Twenty-eight NOE restraints were derived from the T-ROESY spectrum and classified as weak, medium or strong. ROE's were quantified by contour counting. Dihedral restraints were included for the three H_N–N–C_α angles. Restrained molecular dynamics calculations were carried out using XPLOR 3.851[28] using a force field with repulsive non-bonded terms. Two ab initio simulated annealing calculations (YASAP 3.0: 120 ps total time simulated annealing from 2000 to 100 K, 200 steps minimisation) were used to calculate structures with for all four stereoisomers with D- or L-Ile, D- or L-Val and D-Phe starting from a starting conformation with randomised ϕ and ψ angles. From each ensemble 30 structures were refined using a simulated annealing with slow cooling protocol (600 ps, cooling from 1500 to 100 K, 4000 steps of minimisation). The lowest 10 energy structures from each ensemble were selected to represent these structures. The overlay and display of structures was achieved using Molmol.[29]

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