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CYCLOXAZOLINE: A CYTOTOXIC CYCLIC HEXAPEPTIDE FROM THE ASCIDIAN *LISSOCLINUM BISTRATUM.*

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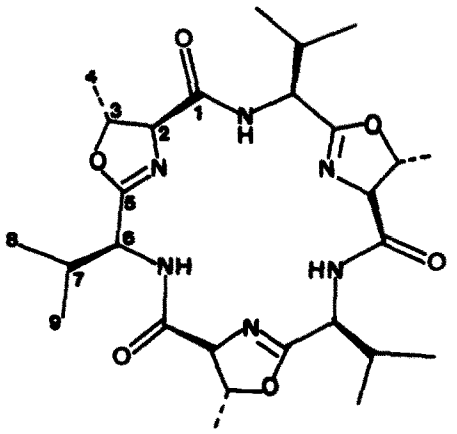
Abstract: *The isolation of a new cyclic hexapeptide is reported from a marine ascidian* ***Lissoclinum bistratum.*** *The structure was determined by NMR, mass spectrometry and X-ray crystallographic techniques. Cytotoxicity against MRC5CV1 and T24 cells expressed as* IC_{50} *was 0.5 μg/mL.*

Didemnid ascidians especially species of *Lissoclinum*, have been a rich source of new classes of biologically active compounds. Compounds from these organisms include the patellamides[1], lissoclinamides[1,2] and patellazoles[3] from *Lissoclinum patella* and the bistratamides and bistratenes from *Lissoclinum bistratum*[4]. Some of these are potent cytotoxins, for example the ulithiacyclamides[5,6] and lissoclinamide 7[7], and others such as the bistratenes[4,8], cause human cells to differentiate. Structure-activity relationships have been discussed for the lissoclinamides[7]. With the exception of the bistratenes all of these compounds contain thiazole or thiazoline amino acids. Crystal structures have been reported for ascidiacyclamide[9] and patellamide D[10]. In this paper we describe the characterization and X-ray crystal structure of another novel cyclic hexapeptide with cytotoxic activity from *Lissoclinum bistratum* containing no thiazole but three oxazoline rings. This compound appears to be identical to westiellamide, which was isolated from the terrestrial blue-green alga *Westiellopsis prolifica*[11].

Cycloxazoline (**1**) was isolated from a methanol/toluene (3:1) extract of frozen *Lissoclinum bistratum* collected from Heron Island Reef on the Great Barrier Reef, Australia. Chromatography of the crude extract on a Whatman Partisil ODS-3 HPLC column yielded the elution profile shown in Figure 1.

Because of its very low absorbance at 254 nm, cycloxazoline was not detected in earlier work[4] using single wavelength detection. Its presence is clearly evident by monitoring at 210 nm (elution time 60 min). The fractions corresponding to cycloxazoline were pooled and evaporated to dryness. (Yield approx. 3 mg per kg wet weight, or 0.2% of dried extract).



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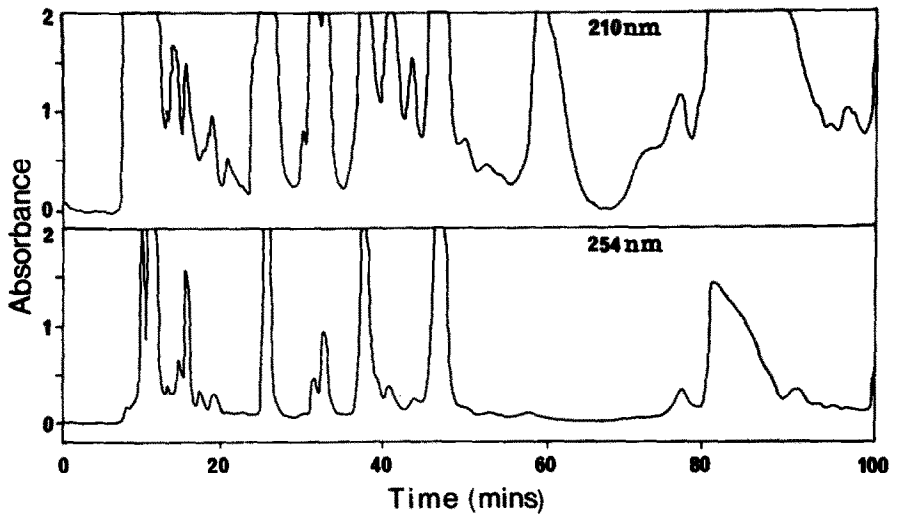

Figure 1. Elution profile of *L. bistratum* extract from the reverse-phase HPLC column.

Fast atom bombardment mass spectrometry gave a molecular ion peak $(M+H)^+$ of 547, consistent with a molecular weight of 546. Acid hydrolysis followed by chiral gas chromatography yielded equimolar amounts of L-valine and L-threonine. The ^{13}C NMR spectrum was very simple, showing only 9 peaks. Table 1 lists the complete NMR assignments. The data are consistent with a dipeptide of valine and threonine where the threonine moiety has been condensed to form an oxazoline ring. The FAB-MS experiment showing a molecular mass of 546 (3 x 182) indicates that the molecule is a symmetrical trimer of the condensed dipeptide.

Table 1. ^{13}C and ^1H NMR for Cycloxazoline ($CDCl_3$)

Carbon No.	δ (^{13}C)	δ (^1H)	(multiplicity; J (Hz)	^1H - ^1H couplings (COSY 45)
1.	170.54			
2.	73.84	4.22	(dd ; 8.7, 2.1)	H3, H6
3.	82.68	4.80	(dq ; 8.7, 6.1)	H2, H4
4.	21.93	1.59	(d ; 6.1)	H3
5.	168.52			
6.	52.40	4.64	(ddd ; 7.9, 3.1, 2.1)	H2, H7, NH
7.	31.47	2.30	(dqq ; 7.0, 7.0, 3.1)	H6, H8, H9
8.	18.60	0.82	(d, 7.0)	H7
9.	16.90	0.89	(d, 7.0)	H7
	NH	7.77	(d, 7.9)	H6, H2, NH

The long-range coupling between H2 and H6 is sufficiently strong for the splitting to be observed in the one-dimensional ^1H NMR spectrum (J = 2.1 Hz). This is similar to the situation in bistratamide A[4], another cyclic hexapeptide from the same organism.

The vicinal coupling constant ^3J (NHCH) is related to the dihedral angle between NH and α-CH by a Karplus-type relationship[12]. The coupling constant for cycloxazoline is 7.9 Hz, an identical value for the equivalent proton of the octapeptide ascidiacyclamide[2]. This corresponds to a $HN^{\alpha}CH$ dihedral angle of $160° > \Theta \geq 145°$ with the valyl group axial and the NH directed to the centre of the ring. A similar conformation is found for the other cyclic hexapeptides from this organism, bistratamides A and B. The conformation has the three valyl substituent groups on the same surface of the ring system with the other side of the ring unhindered by any group.

The crystal structure of cycloxazoline consists of two independent molecules of cycloxazoline

with no significant differences between their geometries, (Figure 2). Positional parameters are listed in Table 2. The conformation of the cycloxazoline ring is approximately planar. The largest deviations from the plane, defined by the oxazoline rings and the connecting atoms, are 0.77 Å. These deviations are caused by the sp^3 hybridized C*x*6 atoms by the interactions between the N atoms of the oxazoline rings and the adjacent peptide N atom which causes a tilting of the oxazoline rings. Thus, the C*x*6 atoms lie below the mean plane by 0.51 to 0.73 Å and the C*x*2 atoms of the oxazoline rings lie above the plane by 0.55 to 0.77 Å. There are no intramolecular hydrogen bonds stabilizing the conformation as observed in the structure of patellamide D[10]. It is probable that the smaller ring size in the present structure stops the ring folding in a way which would allow intramolecular hydrogen bonding. There are also, no intermolecular hydrogen bonds, the disposition of the peptide N-H groups into the centre of the ring and the peptide O atoms to the outside of the ring preventing their formation. The peptide groups are all planar to within 0.003 Å. The oxazoline rings have envelope conformations in which the atoms C*x*2, C*x*5 and O*x*2 are coplanar to within 0.01 Å and C*x*3 lies 0.1 to 0.2 Å out of this plane. The valyl groups are all axial as deduced for the solution structure from coupling constant data. The dihedral angles, $HN^{\alpha}CH$ (Θ), calculated from the observed dihedral angles C(=O) $N^{\alpha}C^{\beta}C$ and C(=O) $N^{\alpha}CC^{ox}$ range from 160° to 140°, in agreement with the values obtained from coupling constant data.

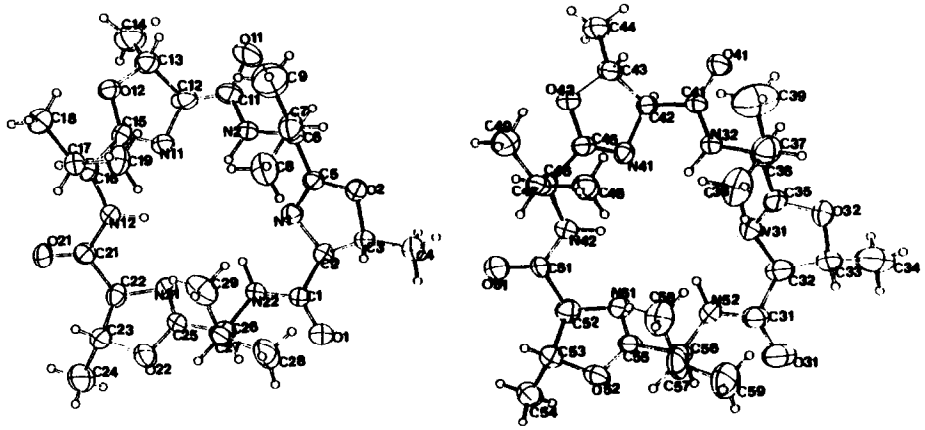

Figure 2. Crystal structure of cycloxazoline: thermal ellipsoid plots (30% probability) of the two independent molecules of cycloxazoline.

Table 2. Positional parameters ($x10^4$) for CYCLOXAZOLINE

	x	y	z		x	y	z
C(1)	1468(8)	-9168(4)	868(3)	C(31)	4915(11)	-11152(5)	3475(3)
O(1)	821(6)	-8901(3)	1162(2)	O(31)	4256(9)	-10948(4)	3793(2)
C(2)	1153(8)	-9020(4)	393(2)	C(32)	4609(11)	-10919(5)	3008(3)
C(3)	-252(7)	-9268(4)	259(3)	C(33)	3122(11)	-11013(7)	2890(3)
C(4)	-1320(8)	-8753(4)	211(3)	C(34)	2352(18)	-10384(8)	2796(4)
O(2)	-6(5)	-9609(3)	-156(2)	O(32)	3148(6)	11410(4)	2491(2)
N(1)	2060(7)	-9350(3)	85(2)	N(31)	5335(7)	-11275(4)	2674(2)
C(5)	1321(9)	-9654(4)	-204(3)	C(35)	4498(10)	-11514(5)	2421(3)
C(6)	1799(9)	-10104(5)	-556(3)	C(36)	4764(7)	-11934(5)	2027(3)
C(7)	1201(11)	-10803(5)	-490(3)	C(37)	3975(12)	-12582(6)	2051(4)
C(8)	1484(11)	-11061(4)	-18(4)	C(38)	4340(13)	-12965(6)	2448(5)
C(9)	1664(16)	-11266(6)	-843(4)	C(39)	4137(17)	-12981(7)	1649(5)
N(2)	3254(7)	-10078(3)	-549(2)	N(32)	6184(7)	-12003(4)	1989(2)
C(11)	4065(12)	-10079(6)	-908(3)	C(41)	6860(8)	-11962(4)	1619(3)
O(11)	3619(9)	-10173(6)	-1282(2)	O(41)	6330(6)	-11941(4)	1253(2)
C(12)	5539(11)	-9973(5)	-822(3)	C(42)	8366(7)	-11947(4)	1663(2)
C(13)	6443(11)	-10454(5)	-1063(3)	C(43)	9069(9)	-12526(5)	1430(3)
C(14)	7468(13)	-10135(6)	-1348(3)	C(44)	9863(10)	-12333(6)	1028(3)
O(12)	7094(6)	-10805(3)	-700(2)	O(42)	9991(6)	-12766(3)	1756(2)
N(11)	5867(7)	-10032(3)	-343(2)	N(41)	8793(6)	-12018(3)	2127(2)
C(15)	6716(8)	-10505(4)	-320(3)	C(45)	9615(9)	-12469(5)	2138(3)
C(16)	7264(8)	-10765(4)	94(3)	C(46)	10285(8)	-12762(4)	2548(3)
C(17)	6941(8)	-11496(4)	181(3)	C(47)	9813(10)	-13489(5)	2623(3)
C(18)	7774(9)	-11958(4)	-111(3)	C(48)	8318(10)	-13543(5)	2612(3)
C(19)	5450(8)	-11635(5)	139(3)	C(49)	10481(11)	-13983(5)	2304(3)
N(12)	6728(7)	-10353(3)	449(2)	N(42)	9916(7)	-12346(4)	2908(2)
C(21)	7316(9)	-10302(4)	846(3)	C(51)	10572(8)	-12306(5)	3275(3)
O(21)	8303(5)	-10577(3)	959(2)	O(51)	11603(7)	-12599(4)	3346(2)
C(22)	6548(8)	-9828(4)	1155(3)	C(52)	9978(9)	-11865(5)	3627(3)
C(23)	6465(9)	-10097(6)	1625(3)	C(53)	9979(9)	-12183(5)	4089(3)
C(24)	7211(10)	-9724(7)	1969(3)	C(54)	10788(10)	-11847(5)	4437(3)
O(22)	5048(6)	-10010(3)	1729(2)	O(52)	8543(6)	-12140(3)	4220(2)
N(21)	5159(7)	-9700(3)	1007(2)	N(51)	8569(7)	-11693(3)	3543(2)
C(25)	4449(8)	-9823(4)	1346(3)	C(55)	7942(8)	-11866(4)	3876(3)
C(26)	2960(8)	-9769(5)	1366(3)	C(56)	6424(9)	-11827(4)	3921(3)
C(27)	2384(9)	-10421(5)	1527(3)	C(57)	5836(9)	-12492(6)	4058(4)
C(28)	916(9)	-10380(6)	1642(4)	C(58)	6037(12)	-13022(5)	3707(4)
C(29)	2698(12)	-10993(5)	1208(3)	C(59)	4393(11)	-12424(8)	4172(4)
N(22)	2443(7)	-9584(3)	936(2)	N(52)	5907(7)	-11579(3)	3507(2)

Cytotoxicity of cycloxazoline was determined for MRC5CV1 fibroblasts and T24 bladder carcinoma cells. The IC_{50} value was 0.5μg/mL for both cell types, at least a hundred fold more toxic than the other hexapeptides from *Lissoclinum bistratum*, bistratamides A and B, and one of the most toxic compounds isolated from these organisms.

Using the same methodology as described above, Prinsep *et al.* assigned the same structure to a compound, westiellamide, isolated from a terrestrial cyanophyte. They reported an IC_{50} value of 2μg/mL against KB cells.

Cycloxazoline was originally named trisoxazoline in a patent application[17], however this name had already been used in an earlier unrelated patent[18].The fact that the same compound occurs in a terrestrial cyanophyte and a marine symbiotic alga provides evidence that the cyclic peptides isolated from the *Lissoclinum* species of ascidians originate from the *Prochloron* symbiont.

EXPERIMENTAL SECTION

Extraction of Compounds.

L. bistratum was collected at Heron Island Reef on the Great Barrier Reef, and extracted as previously described[4]. The extract was chromatographed on a Whatman Partisil ODS-3 Magnum 9 preparative HPLC column equilibrated in 77% methanol. The absorbance of eluting compounds was monitored using a Waters 990 photodiode array detector.

NMR Spectroscopy.

Proton and ^{13}C NMR spectra were obtained with a C-5 dual probe in a JEOL GX400 spectrometer. Deuterated chloroform (Merck) was used as the solvent and chemical shifts are reported relative to tetramethylsilane (TMS).

Mass Spectroscopy.

A Kratos MS 25 RFA instrument was used with an Iontech saddle-field FAB source and argon gas. The sample was dissolved in methanol at a concentration of 1 mg/mL and diluted five times with glycerol for FAB analysis.

Acid Hydrolysis and Chiral Gas Chromatography.

Cycloxazoline (0.3 μmol) was hydrolysed in 6N HCl (1 mL) *in vacuo* overnight at 110°C. After evaporation to dryness the amino acids were converted to their N-pentafluoropropionyl isopropyl esters[13] and applied to a Chirasil-Val GC column for separation of D and L isomers[14].

X-ray Crystallography.

Crystal data: a=10.017(2), b=20.247(3), c=30.344(5) A, V=6154 A^3, $C_{27}H_{42}N_6O_6$, orthorhombic space group $P2_12_12_1$, Z=8, MoKα radiation (λ=0.71069 A), 4173 reflections collected, 2595 with I>2.5 σ(I) used in the refinement. Data collected on an Enraf-Nonius CAD4 diffractometer; structure solved by direct methods using SHELXS-86[15] and refined by blocked-matrix least squares using SHELX-76[16] to a final R of 0.052, R_w0.056. The largest peak in a final difference map was 0.2 eA^{-3} in height.

Cytotoxicity Assays.

The incorporation of [Methyl-^{3}H] thymidine into DNA was measured after treatment of two cell lines MRC5CV1 (SV40-transformed fibroblasts) and T24 (transitional cell carcinoma of the bladder) with cycloxazoline. Details of the method have been described previously[4].

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Supplementary Material Available.

Lists of bond lengths, bond angles, thermal parameters, torsion angles, hydrogen atom positional and thermal parameters and structure factors are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Linsfield Road, Cambridge CB2 1EW. Any request should be accompanied by the full literature citation for this communication.

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