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Structural Studies of Marine Peptides

Annette M. Fernandez,[1] Hai-yin He,[2,3] Leonard A. McDonald,[1,3] Piotr Lassota,[3] Carolyn Discafani,[3] Erik F. Sorensen,[3] Michael C. Edler,[1] Louis R. Barrows,[1] Jon C. Clardy[2*] and Chris M. Ireland[1*]

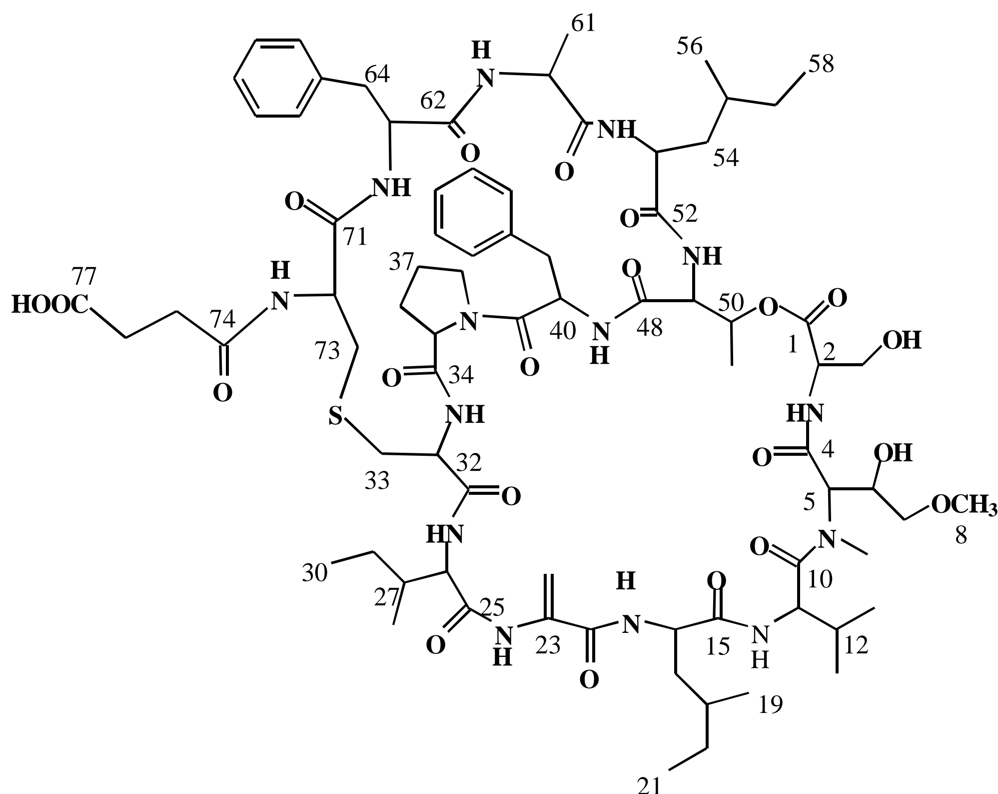
[1]Departments of Medicinal Chemistry and Pharmacology and Toxicology, University of Utah, Salt Lake City, UT 84112, [2]Department of Chemistry, Cornell University, Ithaca, NY 14853-1301 and [3]Wyeth Ayerst Research, Pearl River, New York 10965

Abstract: Previous studies in my lab and others have shown that the didemnid ascidians are a rich source of bioactive peptides, many of which contain cysteine. These cysteine residues in the form of disulfide bridges or five membered heterocyclic rings seem to constrain the peptides to specific conformations. Extracts of the colonial marine ascidian *Didemnum cucculiferum* collected at Beqa lagoon in the Fiji Islands showed potent cytotoxicity in a 25 cell line panel of human solid tumors and activity in a cell based assay for detection of tubulin polymerization inhibitors. Fractionation of the extract yielded a new cyclic peptide vitilevuamide (**1**), which contains the monosulfur bridge amino acid lanthionine. The structure of vitilevuamide was determined using NMR and mass spectrometry. Vitilevuamide inhibits polymerization of tubulin dimers to microtubules at 2 µM. It inhibits binding of vinblastine to tubulin in a non-competitive manner and has litte effect on GTP or dolastatin 10 binding.

INTRODUCTION

Previous studies in my lab and others have shown that the didemnid ascidians are a rich source of bioactive peptides, many of which contain cysteine. These cysteine residues in the form of disulfide bridges or five membered heterocyclic rings seem to constrain the peptides to specific conformations.[1] Extracts of the colonial marine ascidian *Didemnum cucculiferum* (Order: Aplousobranchia, Family: Didemnidae)[2] collected at Beqa lagoon in the Fiji Islands showed potent cytotoxicity in a 25 cell line panel of human solid tumors and activity in a cell based assay for detection of tubulin polymerization inhibitors.[3] Fractionation of the extract yielded a new cyclic peptide vitilevuamide (**1**), which contains the monosulfur bridge amino acid lanthionine. The structure of vitilevuamide was determined using NMR and mass spectrometry. Vitilevuamide inhibits polymerization of tubulin dimers to microtubules at 2 µM. It inhibits binding of vinblastine to tubulin in a non-competitive manner and has little effect on GTP or dolastatin 10

binding. These results suggest that vitilevuamide does not bind at the vinca or peptide sites on tubulin. Vitilevuamide was active *in vivo* against the P388 lymphocytic leukemia with an ILS of 70% at 30μg/kg.



RESULTS

Vitilevuamide was isolated as a white amorphous solid; IR (neat film) V_{max} 3280, 2928, 1734, 1652, 1558, 1538 cm^{-1}; UV (MeOH) λ_{max} 230 nm (ε 2032.38). A molecular formula of $C_{77}H_{114}N_{14}O_{21}S$ was established by HRFABMS (MH^+ 1603.8117, D 3.6 mmu). Hydrolysis of vitilevuamide with 6N HCl at 100° C for 18 hours followed by electrospray mass spectrometry of the crude hydrolysate yielded ions at m/z 90, 106, 116, 118, 120, 132, 146, 166 and 209 consistent with the presence of alanine (ala), serine (ser), proline (pro), valine (val), threonine (thr), isoleucine (ile), homoisoleucine (hil), phenylalanine (phe) and lanthionine (lan), respectively. A detailed analysis of data from the ^{1}H and ^{13}C NMR spectra and a suite of 2D correlation experiments, see Table 1, confirmed the assignments from mass spectrometry including the presence of multiple copies of hil and phe and also indicated the presence of a dehydroalanine (dha), N-methyl methoxinine (nmm) and a succinate unit. Specifically, HMBC correlations from an NH proton singlet at δ 10.11 and a terminal methylene proton at 4.71 (H24a, s) to a quaternary sp^2 carbon at 140.21 defined the dha unit. The nmm proton spin system was defined by scalar coupling between a methine at d 5.68 (H5, d, J = 5.8 Hz) and a methine at 4.31 (H6, m) which was further coupled to diastereotopic methylenes at 3.45 (H7a, m) and 3.61 (H7b, m). HMBC

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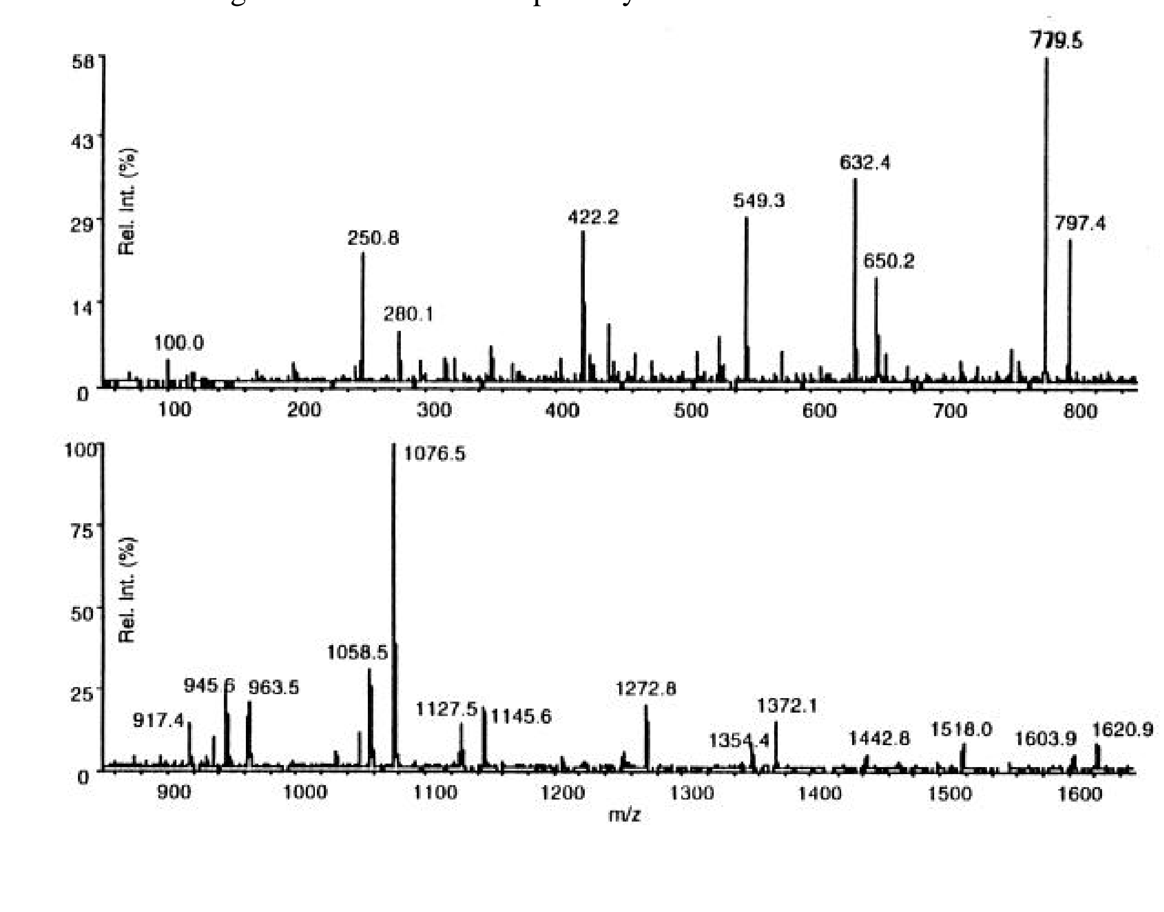
correlations from the O-methyl protons at d 3.09 to C7 and the N-methyl protons at 3.40 to C5 completed the nmm unit.

The amino acid sequence of vitilevuamide was established using a combination of NMR and MS data. Two bond HMBC correlations were observed from the NH of each amino acid to the carbonyl of the *i*-1 amino acid with the exception of the two tertiary amino acids. In the case of the nmm, a correlation was observed from the α proton H5 to the carbonyl of val C10. For the pro, correlations were observed from the β proton at δ 1.94 to the pro carbonyl, C34 and the d proton at 2.35 to the carbonyl of phe, C39. Hydrolysis of vitilevuamide with methanol and ammonia resulted in selective opening of the ester linkage between ser and thr with amidation of ser carboxyl. Tandem MS/MS of the protonated molecular ion at m/z 1621 formed by electrospray ionization yielded the spectrum shown in figure 1. The daughter spectrum showed a series of ions resulting from cleavage at the amide linkages and sequential loss of ammonia, ser, nmm, val, hil, dha, and ile (see figure 2). Subsequent fragmentation occurred after scission of the sulfur bridge of lan accompanied by a hydrogen migration to give dehydroalanine on the C-terminal side and cysteine on the N-terminal side.[4] Fragmentation resumed with loss of 166 mass units corresponding to pro and the newly formed dha, followed by phe, thr, hil, ala, and the dipeptide fragment phe-cys.

Figure 1. Tandem MS of partially linearized vitilevuamide



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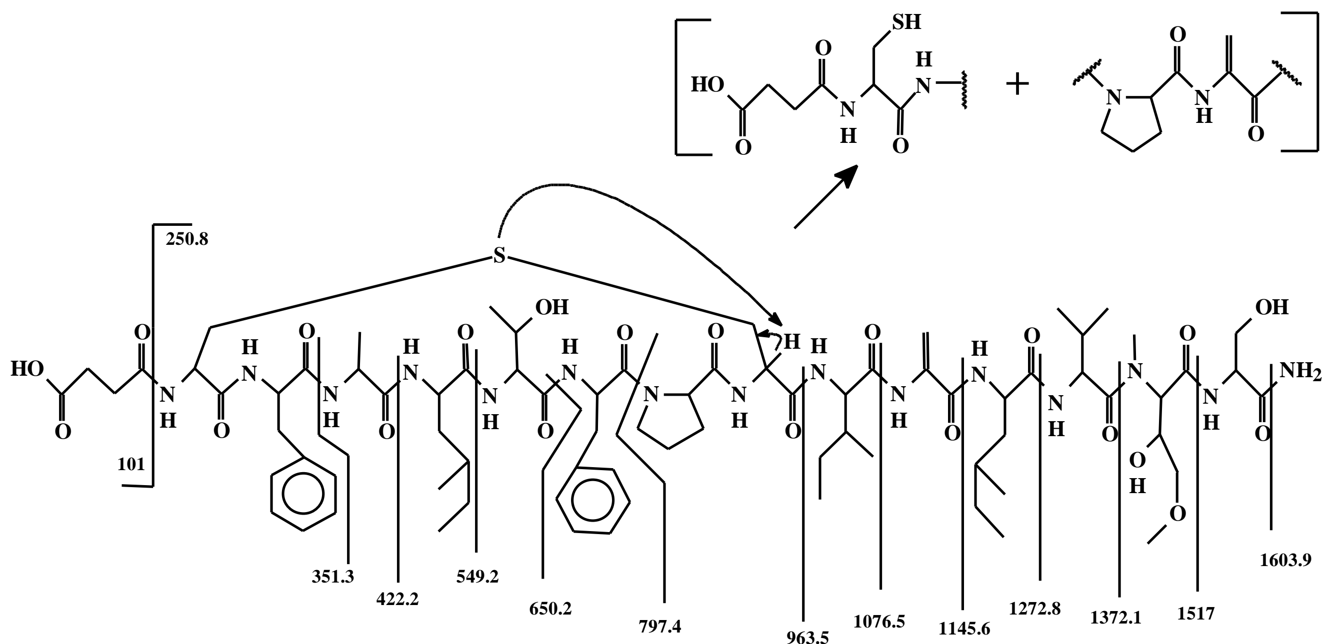

Figure 2. Interpretation of the tandem MS of partially linearized vitilevuamide

Amino acid chirality was established by derivatization of the acid hydrolysate with FDAA (Marfey's Reagent) and analysis of the resulting diastereomers by HPLC.[5] Co-injection with standards indicated the presence of D-ala, D-allothr, D-val, D-phe, L-ser, L-pro, L-ile, and equal amounts of D-allo hil and L-hil. The lan was racemic and the nmm did not survive hydrolysis. The issues of whether the lan racemizes during hydrolysis or occurs in the D,L-meso form in the peptide and the respective positions of the two hil enantiomers is unresolved.

EXPERIMENTAL

General Procedures

^1H and ^{13}C experiments were obtained at 500 and 125 MHz respectively, on a Varian Unity spectrometer. Variable temperature studies were performed at -20, -15, -10, -5, 0, 5, 10, 15, 20, 25, 30 and 35°C using CD_2Cl_2, $CDCl_3$ and C_6D_6. Based on these results, all NMR experiments were run at 22°C in C_6D_6. ^1H chemical shifts were reported in ppm relative to undeuterated benzene resonance at 7.15ppm. ^{13}C chemical shifts are reported in ppm relative to solvent resonance at 128 ppm. UV (in MeOH) and IR spectra were obtained on a HP8452 diode array and a Perkin Elmer 1600 FTIR spectrophotometer, respectively. Low resolution FAB was carried out on a Varian MAT 731 mass spectrometer in 3NBA and CH_2Cl_2 matrices. High resolution FAB was performed on a ZAB-SE spectrometer. Electrospray was carried out on a Fissons Trio 2000 electrospray mass spectrometer. Tandem (MS/MS) was performed on the SIAX API III spectrometer. 5µl of sample was dissolved in 190µl of 50:50:1 H_2O:CH_3OH:CH_3COOH at a flow rate of 2µl/min.

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Extraction and Isolation procedures

Specimens of *Didemnum cuculliferum* were collected by SCUBA in the Fiji Islands. The MeOH extract of the frozen ascidian (350 grams) was repeatedly extracted with 2.5L of MeOH. The crude homogenate (reduced to 50 mL) was separated into increasing polar fractions by extracting first with hexanes (3 x 500 mL) and chloroform (5 x 500 mL) using a modified Kupchan solvent partitioning scheme. The resulting 325.1mg of chloroform extract was subjected to Si gel flash chromatography (column, 2.8 x 46 cm; silica gel 60 Å, stepped gradient elution, $CHCl_3$; 99:1 $CHCl_3$/MeOH; 98:2 $CHCl_3$/MeOH; 97.5:2.5 $CHCl_3$/MeOH) followed by a second silica flash chromatography step (column, 28 x 460 mm; silica gel 60 Å, stepped gradient elution, 3:7 acetone/hexanes; 7:3 acetone/hexanes; acetone). The 70% acetone/hexanes fraction was subjected to RP HPLC (Rainin Microsorb 4.6 x 250mm, 100Å, 5µ silica gel, 90% CH_3CN 10% H_2O, UV detection at 220nm) to yield a clear glassy compound: 10.2mg, 0.0029% of total dry weight. UV (MeOH) λ_{max} 230nm (ε = 2032.38); IR (neat film) ν_{max} 3280, absorbtions centered at 2928, 1734, 1652, 1558,1538 cm^{-1}. Positive FAB MS and electrospray established a molecular weight of 1602.8. The molecular formula of $C_{77}H_{114}N_{14}O_{21}S$ was determined by HR FAB measurement of the protonated molecular ion (1603.8117, Δ 3.6 mmu).

Partial Linearization of Vitilevuamide Using Ammonia

1.5 mL of saturated NH_3 in HPLC grade MeOH was added to 1.2mg of vitilevuamide in 5 mL MeOH. The solution in a capped vial was placed at 0^oC for 18 hours. The resulting mixture was then chromatographed by RP HPLC (Waters NOVAPAK C_{18}; 4.6 x 100mm column, isocratic elution at 90% MeOH/H_2O to afford the partially linear peptide. Low resolution ESMS established a molecular weight of 1620.8. $C_{77}H_{118}N_{15}O_{21}S$ requires a molecular weight of 1620.8269. This partially linearized molecule was then subjected to tandem mass spectroscopy for sequential analysis.

Hydrolysis and Derivatization

Hydrolysis of the peptide was carried out in 5 mL of 6N HCl under a nitrogen atmosphere in a sealed bomb at 104 ^oC for 18 hours. After traces of HCl were removed by repeated evaporation in vacuo, the residual hydrolysate was suspended in 500µl of water and derivatized with (1-fluoro-2,4-dinitrophen-5-yl) L- alanineamide (FDAA) using Marfey's procedure. HPLC analysis (Waters NOVAPAK C_{18}; 4.6 x 100mm column, linear gradient elution, triethylammonium phosphate (50mM, pH 3.0) / acetonitrile, 90:10-60:40 in 45 mins; 1.0mL/min; UV detection at 340nm) of the FDAA derivatized amino acid standards established the stereochemistry of the constituent amino acids with the exception of homoisoleucine, lanthionine and N-methyl methoxinine.

The conditions for the stereochemistry determination of isoleucine incuded gradient cyano HPLC (Rainin Microsorb (4.6 x 250mm) at MeOH/1% Acetic acid, 35:65-42:58 for 30 mins at 1mL/min) with UV detection at 340nm. For homoisoleucine, gradient cyano HPLC (Rainin Microsorb (4.6x250mm) at MeOH/1% Acetic acid, 25:75-90:10 at 1mL/min) with UV detection at 340nm was used.

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Table 1 NMR[a] assignments for vitilevuamide in C_6D_6

Atom	δ ^{13}C	(mult.)	δ ^{1}H	(mult., *J* (Hz))	HMBC correlations
N1			8.66	(d, 8.79)	C14
1	170.09	(s)			
2	54.77	(d)	5.06	(ddd, 2.47,2.58,5.05)	C13, C14
3	63.60	(t)	3.91	(dd, 2.47,11.48)	C13
			4.33	(m)	
4	169.32	(s)			
5	57.03	(d)	5.68	(d,5.86)	C5,C14,C51
6	68.05	(t)	4.31	(m)	
7	70.17	(t)	3.61	(m)	
			3.45	(m)	
8	58.65	(q)	3.09	(s)	
9	32.49	(q)	3.40	(s)	C5
OH			3.77	(d, 4.44)	
N3			8.12	(d, 6.90)	C4, C35, C55
10	173.63	(s)			
11	56.63	(d)	4.42	(m)	
12	30.58	(d)	2.26	(bm)	
13	19.12	(q)	1.03	(bm)	
14	18.95	(q)	0.93	(bm)	C35, C55, C66
N4			8.09	(d, 10.66)	C1, C45
15	175.07	(s)			
16	54.09	(d)	5.27	(ddd, 4.46,10.45)	
17	42.77	(t)	2.29	(bm)	C1, C54, C67
			1.80	(bm)	
18	30.29	(d)	2.17	(m)	
19	18.72	(q)	1.10	(d, 6.78)	
20	30.74	(t)	1.48	(bm)	
			1.28	(bm)	
21	11.55	(q)	0.93	(bm)	
N5			10.11	(s)	C8, C15
22	168.26	(s)			
23	140.21	(s)			
24	111.11	(t)	4.71	(s)	C8, C15, C16
			4.83	(s)	

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N6			8.00	(d, 5.23)	C7, C31, C48
25	172.49	(s)			
26	60.92	(d)	4.19	(bm)	C10, C48
27	36.94	(d)	1.89	(bm)	
27	11.55	(q)	0.93	(bm)	
28	15.01	(q)	1.21	(d, 6.76)	
29	25.10	(t)	1.78	(bm)	
			1.31	(bm)	
30	10.90	(q)	0.84	(bm)	
31	172.84	(s)			
32	50.94	(d)	4.79	(dd, 7.17,9.59)	C7, C47
33	31.95	(t)	3.16	(bs)	C7
			2.01	(dd, 15.34,17.38)	
34	174.99	(s)			
35	61.42	(d)	5.16	(dd, 7.6,15.1)	C2, C61
36	29.95	(t)	1.94	(bm)	C2, C44
			1.79	(bm)	
37	25.11	(t)	1.61	(bm)	C30
			1.44	(bm)	
38	47.01	(t)	3.20	(bm)	
			2.35	(bm)	
N9			10.49	(d, 4.95)	C11
39	169.96	(s)			
40	55.40	(d)	4.31	(m)	C11, C49
41	37.13	(t)	3.21	(bm)	C13, C17, C19, C37
			3.05	(bs)	C13, C17, C19, C37
42	136.28	(s)			
43	130.12	(d)	7.41	(d, 7.38)	C23
44	128.89	(d)	7.28	(m)	C17, C18
45	127.57	(d)	7.08	(d, 7.37)	C19
46	128.89	(d)	7.28	(m)	C17, C18
47	130.12	(d)	7.41	(d, 7.38)	C23
N10			8.72	(d, 6.26)	C3, C11
48	170.14	(s)			
49	57.03	(d)	4.95	(dd, 1.12,6.32)	C3, C11, C27
50	73.25	(d)	5.51	(ddd, 1.23,5.86,6.49)	C13
51	19.94	(q)	1.14	(d, 6.69)	
N11			9.22	(d, 9.87)	C3, C10

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52	174.94	(s)			
53	49.81	(d)	5.62	(ddd, 3.35,10.05,10.89)	
54	42.29	(t)	1.97	(m)	C3
			1.87	(bm)	
55	31.83	(d)	1.78	(m)	
56	13.39	(q)	1.15	(d, 6.56)	
57	27.78	(t)	1.93	(bm)	
			1.40	(bm)	
58	11.22	(q)	1.03	(bm)	
N12			8.40	(d,10.43)	C6
59	171.49	(s)			
60	48.60	(d)	5.40	(q, 6.7,10.47)	
61	30.15	(d)	1.35	(bm)	C10, C43
N13			6.43	(d, 7.78)	C6, C9, C38, C50
62	173.28	(s)			
67	128.39	(d)	7.28	(m)	
68	127.00	(d)	7.10	(d, 7.58)	
69	128.39	(d)	7.28	(m)	
70	129.82	(d)	7.50	(d, 7.38)	
N14			6.54	(d, 8.22)	C6
71	172.54	(s)			
72	55.40	(d)	4.44	(bm)	
73	39.48	(t)	3.33	(m)	C2, C36, C52
			2.59	(dd, 11.66,14.12)	
74	173.28	(s)			
75	30.41	(t)	3.38	(bm)	
			1.86		
76	28.61	(t)	2.29	(bm)	
			1.78	(bm)	
77	176.82	(s)			

[a] Proton and carbon data were acquired at 500 and 125 MHz, respectively.
[b] From a DEPT experiment.
[c] The HMBC experiment was optimized to observe $^{n}J_{CH}$ couplings of 8 Hz.

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REFERENCES

1. C.M. Ireland, D.M. Roll, T.F. Molinski, T.C. McKee, T.M. Zabriskie, J.C. Swersey, *Mem. Cal. Acad. Sci.* 13, 41-57 (1988); b) B.S. Davidson. *Chem. Rev.* 93, 1771-1791 (1993).
2. Voucher specimens were identified by Dr. Françoise Monniot, Museum d'Histoire Naturelle Paris, France.
3. J.M. Kokoshka, C.M. Ireland, L.R. Barrows. *J. Nat. Prod.* 59, 1179 (1996).
4. A.G. Craig. *Biological Mass Spec.* 20, 195 (1991).
5. P. Marfey. *Carlsberg Res. Commun*. 49, 591-96 (1984).

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