Studies of Australian Ascidians. 5. Virenamides A−C, New Cytotoxic Linear Peptides from the Colonial Didemnid Ascidian *Diplosoma virens*

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Three new linear cytotoxic tripeptides, virenamides A-C have been isolated from extracts of the Didemnid ascidian *Diplosoma virens* collected on the Great Barrier Reef, Australia. Their structures were deduced from 1D and 2D NMR spectral data and confirmed by HPLC analysis of the constituent amino acids after hydrolysis of the peptides and derivatization with 1-fluoro-2,4-dinitrophen-5-yl-L-alanine amide using Marfey's procedure.

Peptides that contain thiazole groups often show high levels of biological activity.[1] The linear peptide, Dolastatin 10, isolated from the sea hare *Dolabella auricularia*, for example, is believed to be the most potent antineoplastic agent presently known.[2] Didemnid ascidians have proven to be a rich source of novel cyclic peptides and some of these also contain thiazole groups.[3] Many of these peptides also show high levels of pharmacological activity.[3] To date no linear peptides have been reported from ascidians from the family Didemnidae; however, in this paper we report the structures of three cytotoxic linear thiazole-containing peptides which have been isolated from the didemnid ascidian *Diplosoma virens* (Hartmayer, 1909).

Results and Discussion

D. virens is a small green colonial ascidian which contains symbiotic prokaryotic algae in its cloacal cavity. It was collected from the rubble zone behind the reef crest at Bramble reef in the central section of the Great Barrier Reef. Extraction of the freeze-dried animals with dichloromethane, followed by methanol, yielded a dark green residue. Rapid silica gel chromatography of this extract afforded a number of peptidic fractions which were combined and repeatedly chromatographed on silica to yield virenamide A (**1**), virenamide B (**2**), and virenamide C (**3**). The non-nitrogenous metabolite, lissoclinolide (**4**), was also obtained. It has previously been reported from another didemnid ascidian, *Lissoclinum patella*, from Fiji, and the spectral data obtained was identical with data reported previously.[4]

The EI mass spectrum of virenamide A (**1**) showed a very weak molecular ion peak at *m*/*z* 538. CI mass spectrometry confirmed the molecular weight as 538, since an intense $MH^{\bullet+}$ peak at *m*/*z* 539 was observed. High resolution mass measurement of the $M^+ - C_5H_9$ peak (*m*/*z* 469.2639) in the EI mass spectrum in combination with ^1H and ^{13}C NMR data (Table 1) implied that the molecule had a molecular formula $C_{31}H_{46}N_4O_2S$. Eleven double bond equivalences were therefore indicated. The IR spectrum showed intense absorptions at 3420, 3337, and 1665 cm^{-1}, which indicated peptide linkages.

The ^{13}C NMR spectrum of virenamide A (Table 1) exhibited signals for all 31 carbons, and DEPT sequence experiments indicated that all but two of the protons were attached to carbons. Signals at 118.3, 142.4, and 170.7 ppm were charactersitic of a C2-substituted thiazole. Two mutually coupled narrow doublets in the ^1H NMR spectrum (7.68 and 7.20 ppm, *J* 3.3 Hz) attached to the carbons with signals at 142.4 and 118.3 ppm, respectively, in the ^{13}C NMR spectrum were characteristic of the C4 and C5 positions of the C2-substituted thiazole. Further downfield two quaternary carbons signals at 171.0 and 174.1 ppm could be assigned to amide carbonyl carbons. A phenyl group and two equivalent trisubstituted double bonds accounted for the remaining carbon signals downfield of 110 ppm. Virenamide A was therefore a linear peptide since all eleven double bond equivalences were accounted for by this data.

A series of homonuclear ^1H NMR decoupling experiments in combination with the results obtained from an XH-correlation experiment established a number of partial connectivities. Thus two valine residues with attached amide protons and a phenylalanine residue lacking an amide proton were suggested. The remaining signals in the ^1H NMR spectrum could be assigned to two equivalent dimethallyl groups.

An HMBC experiment permitted all of the nonprotonated carbons to be assigned and allowed all of the partial structures to be assembled. Correlations from the allylic methyl singlets at 1.54 and 1.69 ppm to the the olefinic carbons 135.4 and 121.9 ppm confirmed that the molecule contained two coincident dimethallyl groups. Strong correlations from the dimethallyl CH_2 to the α carbon of the phenylalanine indicated that the nitrogen of this amino acid was capped by the two dimethallyl groups. (Evidence for this conclusion was also present in the EI mass spectrum, where fragments were observed at *m*/*z* 188 (27%) and 256 (22%), corresponding to a phenylethylamine residue bearing one and two dimethylallyl groups, respectively.) The most downfield carbon signal 174.1

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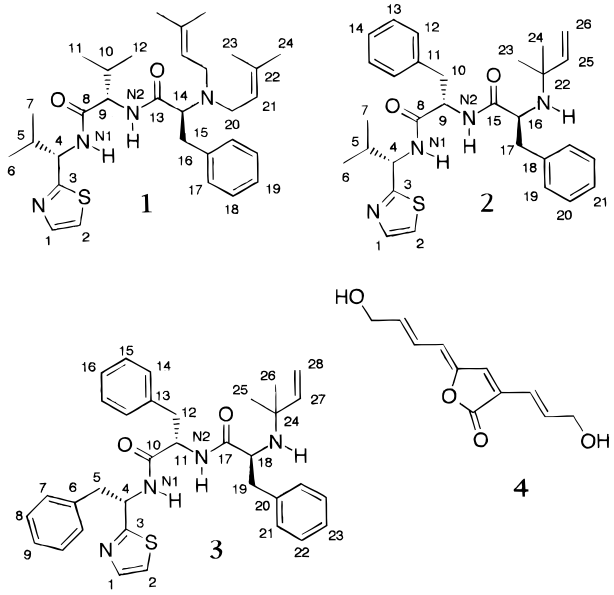
Table 1. ^{1}H and ^{13}C Assignments for Virenamides A−C

atom no.	virenamide A (**1**)		virenamide B (**2**)		virenamide C (**3**)	
	δ^{13}C (mult.)	δ^1H (mult, *J* Hz)	δ^{13}C (mult.)	δ^1H (mult, *J* Hz)	δ^{13}C (mult.)	δ^1H (mult, *J* Hz)
1	142.4 (d)	7.68 (d, 3.3)	142.5 (d)	7.69 (d, 3.3)	142.4 (d)	7.69 (d, 3.3)
2	118.3 (d)	7.20 (d, 3.3)	118.1 (d)	7.20 (d, 3.3)	118.8 (d)	7.17 (d, 3.3)
3	170.7 (s)	−	170.6 (s)	−	169.5 (s)	−
4	56.0 (d)	5.20 (dd, 5.7, 8.8)	56.1 (d)	5.16 (dd, 6.8, 8.0)	52.3 (d)	5.53 (ddd, 7.0, 7.0, 7.0)
5	33.2 (d)	2.34 (dqq, 6.2, 6.2, 6.3)	33.1 (d)	2.26 (dqq, 6.8, 6.8, 6.8)	41.6 (d)	3.20 (d, 7.0)
6	17.3 (q)	0.84 (d, 6.2)	19.1 (q)	0.85(d, 6.8)	136.3 (s)	−
7	19.0 (q)	0.86 (d, 6.3)	17.7 (q)	0.86 (d, 6.8)	128.6 (d)	7.25−7.35 (m)
8	171.0 (s)	-	170.6 (s)	−	129.3 (d)	6.95−7.10 (m)
9	58.5 (d)	4.28 (dd, 6.8, 8.9)	53.6 (d)	4.66 (dt, 6.6, 8.0)	127.0 (d)	7.15−7.25 (m)
10	29.9 (d)	2.24 (dqq, 6.8, 6.8, 6.8)	37.9 (t)	3.05 (d 6.6)	170.1 (s)	−
11	17.9 (q)	0.93 (d, 6.8)	137.0 (s)	−	53.8 (d)	4.62 (ddd, 6.9, 7.1, 8.5)
12	19.4 (q)	0.94 (d, 6.8)	128.3 (d)	7.15−7.32 (m)	37.6 (t)	3.00 (dd, 7.1, 13.7)
						3.05 (dd, 6.9, 13.7)
13	174.1 (s)	−	129.4 (d)	7.07 (m)	137.0 (s)	−
14	64.6 (d)	3.75 (dd, 6.3, 6.5)	126.7 (d)	7.15−7.32 (m)	128.3 (d)	7.25-7.35 (m)
15	31.1 (t)	2.88 (dd, 6.3, 14.2)	175.5 (s)	−	129.4 (d)	6.95−7.10 (m)
		3.31 (dd, 6.5, 14.2)				
16	140.0 (s)	-	57.8 (d)	3.23 (bd, 7.0)	126.8 (d)	7.15−7.25 (m)
17	128.2 (d)	7.25 (m)	39.8 (t)	2.38 (dd, 7.0, 12.4)	175.6 (s)	2.97 (bd,12.4)
18	129.1 (d)	7.26 (m)	136.5 (s)	−	57.8 (d)	3.20 (bdd, 9.3, 4.1)
19	125.7 (d)	7.16 (m)	128.5 (d)	7.15−7.32 (m)	39.7 (t)	2.37 (dd, 9.3, 17.3)
						2.94 (dd,4.1, 17.3)
20	48.1 (t)	3.08 (d, 6.5)	129.3 (d)	7.07 (m)	136.5 (s)	−
21	121.9(d)	5.13 (t, 6.5)	126.6 (d)	7.15−7.32 (m)	128.5 (d)	7.25−7.35 (m)
22	135.4 (s)	−	54.6 (s)	−	129.3 (d)	6.95−7.10 (m)
23	18.0 (q)	1.54 (s)	24.9 (q)	0.88 (s)	126.6 (d)	7.15−7.25 (m)
24	25.7 (q)	1.69 (s)	27.8 (q)	0.90 (s)	54.6 (s)	−
25			144.6 (d)	5.14 (dd, 9.6, 18.3)	24.9 (q)	0.81 (s)
26			112.5 (t)	4.82 (d, 18.3)	27.9 (q)	0.87 (s)
				4.83 (d, 9.6)		
27					144.6 (d)	5.11 (dd, 10.5, 16.3)
28					112.7 (t)	4.81 (dd, 16.3, 1.2)
						4.82 (dd, 10.5, 1.2)
N1		7.10 (d, 8.8)		7.09 (d, 8.0)		6.98 (d, 7.0)
N2		7.88 (d, 9.0)		8.12 (d, 8.0)		8.05 (d, 8.5)

ppm was assigned to the phenylalanine carbonyl carbon, since correlations were observed to it from the methylene and α protons of the phenylalanine residue. Correlations were also observed to this carbon from the amide proton at 7.88 ppm and from one of the valine α protons at 4.28 ppm. This allowed one of the valine residues to be connected to the phenylalanine. The remaining valine moiety was masked by a thiazole cap of its carbonyl carbon. Correlations from the second amide proton at 7.10 ppm to the carbonyl carbon 171.0 ppm completed the amino acid sequence and established the connectivity between the two valine residues. Acid hydrolysis followed by derivatization with Marfeys reagent and HPLC analysis[5] indicated that the three amino acids all possessed L absolute stereochemistry. Unexpectedly, no racemization of the amino acid adjacent to the thiazole ring was observed, and some loss of the dimethylallyl groups occurred. Loss of the dimethylallyl substituents was verified by ozonolysis of a sample of virenamide A prior to hydrolysis and derivatization with Marfey's reagent. Under these conditions total loss of the *N*-alkyl substituents was obtained with only peaks which corresponded to derivatized L-valine and L-phenylalanine being observed. Virenamide A was thus shown to have structure **1**.

Virenamide B (**2**) possessed very similar spectral properties to **1**. Mass spectral data indicated virenamide B had a molecular formula $C_{30}H_{38}N_4O_2S$. Analysis of the ^{1}H and ^{13}C NMR spectra suggested virenamide B was also a linear peptide in which the carbon terminus was capped with a thiazole. The nitrogen terminus of **2**, however, differed from virenamide A since the two dimethallyl groups found in **1** were replaced in **2** by only one dimethallyl group, which in this case was bonded to the nitrogen through the carbon which bore the two methyl groups. (The EI mass spectrum in this case, also exhibited a peak at *m/z* 188 (56%) corresponding to the phenylethylamine residue bearing a dimethylallyl group.) Furthermore, virenamide B contained signals for two phenylalanine residues and only one valine residue. These observations were confirmed by analysis of 2D NMR (DQFCOSY and XH correlation) experiments, and the amino acid sequence was established from analysis



(5) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591.

of an HMBC experiment. Amino acid analysis using the Marfey procedure indicated that all three amino acids possessed L stereochemistry. Virenamide B therefore had structure **2**.

A third peptide, virenamide C (**3**), was isolated as a colorless oil. The structure of **3** differed from **2** only in the replacement of the valine by a phenylalanine residue, as indicated by the close similarities in the ^{1}H and ^{13}C NMR spectra. Lack of signals for the valine residue and their replacement by signals characteristic of another phenylalanine residue in the ^{1}H NMR spectrum of **3** were the only differences observed when the spectra for **2** and **3** were compared. The ^{13}C NMR spectra were also consistent with this change. Amino acid analysis indicated that all three phenylalanine residues possessed L stereochemistry. The molecular formula $C_{34}H_{38}N_4O_2S$ from HREIMS corroborated the structure of virenamide C (**3**).

The virenamides showed modest cytotoxicity toward a panel of cultured cells: virenamide A gave IC_{50}'s of 2.5 μg/mL against P388, and 10 μg/mL against A549, HT29, and CV1 cells. It exhibited topoisomerase II inhibitory activity (IC_{50} of 2.5 μg/mL). Virenamides B and C both gave IC_{50}'s of 5 μg/mL against P388, A549, HT29, and CV1 cells.

Experimental Section

General experimental details have been given in an earlier paper.[6]

Isolation Procedures. *D. virens* (Hartmayer, 1909), a small green colonial ascidan was collected at Bramble Reef, in the central section of the Great Barrier Reef, Australia, in June 1992 using SCUBA (−3 m). A voucher specimen no. G 21263 is housed at the Museum of Tropical North Queensland, Townsville. The ascidians were freeze-dried (38 g) and extracted exhaustively first with CH_2Cl_2 and then CH_3OH. The CH_2Cl_2 extract was concentrated yielding a dark green residue (1.05 g). This residue was rapidly chromatographed on silica gel (Merck silica gel type 60) with a solvent gradient from hexane to CH_3COCH_3 to CH_3OH. The fraction that eluted with hexane/CH_3COCH_3 (1:1) was further chromatographed on silica gel with hexane/CH_3COCH_3 (4:1) to yield lissoclinolide (**4**, 25 mg, 0.07%), virenamide A (**1**, 135 mg, 0.36%), virenamide B (**2**, 75 mg, 0.20%), and virenamide C (**3**, 29 mg, 0.08%), respectively.

Virenamide A (**1**): colorless oil, $[\alpha]_D$ −341° (c 0.14, $CHCl_3$); UV (EtOH) λ_{max} 203 nm (ϵ 7700), 237 nm (ϵ 4300); IR ν_{max} ($CHCl_3$) 3420, 3337, 3018, 2972, 2931, 1665, 1512, 1454, 1390, 1221, 1082, 1047, 928 cm^{-1}; high resolution mass measurement 469.2639, $C_{26}H_{37}N_4O_2S$ ($M^{\bullet+}$ − C_5H_9) requires 469.2637; EIMS, *m*/*z* (relative intensity) 538 (0.1%), 256 (22), 188 (27), 140 (22), 120 (53), 91, (27), 72 (27), 69 (100); CIMS, *m*/*z* (relative intensity) 539 (100), 471 (50), 431 (45).

Virenamide B (**2**): colorless oil, $[\alpha]_D$ −775° (c 0.10, $CHCl_3$); UV (EtOH) λ_{max} 209 nm (ϵ 13600), 240 nm (ϵ 4500); IR ($CHCl_3$) ν_{max} 3420, 3337, 3018, 2975, 1657, 1517, 1478, 1454, 1424, 191, 1211, 1047 cm^{-1}; high resolution mass measurement 427.2168, $C_{23}H_{31}N_4O_2S$ ($M^{\bullet+}$ − C_7H_7) requires 427.2168; EIMS, *m*/*z* (relative intensity) 518 (0.1%), 427 (4), 188 (56), 120 (100), 91, (17), 69 (47); CIMS, *m*/*z* (relative intensity) 519 (100), 188 (13), 120 (14).

Virenamide C (**3**): colorless oil, $[\alpha]_D$ −634° (c 0.05, $CHCl_3$); UV (EtOH) λ_{max} 202 nm (ϵ 10900), 240 nm (ϵ 4800); IR ($CHCl_3$) ν_{max} 3415, 3329, 2964, 2928, 2856, 1665, 1604, 1498, 1454, 1442, 1380, 1366, 1172, 1101, 1001 cm^{-1}; high resolution mass measurement 566.2717, $C_{34}H_{38}N_4O_2S$ requires 566.2715; EIMS, *m*/*z* (relative intensity) 566 (0.3%), 475 (4), 188 (66), 120 (100); CIMS, *m*/*z* (relative intensity) 567 (100), 188 (41), 120 (50).

Stereochemistry of Virenamides. In a typical hydrolysis a peptide (1.0 mg) was heated in 6 N HCl (10 mL) in a sealed glass tube at 105 °C for 40 h. The resulting hydrolysate was freeze-dried, dissolved in distilled water (200 μL), and derivatized with 1-fluoro-2,4-dinitrophen-5-yl-L-alanine amide (FDAA) (1.25 mg) in acetone (500 μL) and 1 N sodium bicarbonate (100 μL) at 50 °C for 2 h. Upon completion of reaction the solution was acidified with 2 N HCl (500 μL) and stored in the dark until it was analyzed. HPLC analysis (C18 Activon goldpak column; linear gradient elution, triethylammonium phosphate (50 mM, pH 3.0)/acetonitrile, 80:20−50:50 in 40 min, and then held at 50:50 for 60 min; 1.0 mL/min; UV detection at 340 nm) of the FDAA derivatized hydrolysates established the stereochemistry of the constituent amino acids. Each peak in the chromatographic trace was identified by comparing its retention time with that of the FDAA derivative of the pure amino acid standard and by coinjection. The acid hydrolysate of virenamide A (**1**) showed a large peak at 42.14 and a small peak at 47.07 min; in addition peaks were observed at 66.2 and 67.5 min (presumably prenylated phenylalanine derivatives). When the virenamide A was ozonolyzed prior to hydrolysis and derivatization, only peaks at 42.75 and 48.34 min were observed. Virenamide B (**2**) also showed peaks with the same retention times, while virenamide C (**3**) only showed a peak at 47.98 min. The amino acid standards gave the following retention times in minutes: 41.62 for L- and 47.90 for D-valine; and 46.74 L- and 52.81 for D-phenylalanine. Coinjection with the authentic derivatives was necessary because of the similarity of the retention times for L-phenylalanine and D-valine, together with variations in elution times of up to 1 min for sucessive injections. In all cases, the peak which eluted around 47−48 min was confirmed to be due to L-phenylalanine.

Ozonolysis of Virenamide A. Virenamide A (1.0 mg) was dissolved in dry CH_2Cl_2 (5 mL) and a stream of ozone in oxygen was bubbled through the solution until excess ozone was detected (starch/iodine paper). The solvent was removed under vacuum, and the sample was hydrolyzed and derivatized as before.

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