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Full Papers

Georgamide, a New Cyclic Depsipeptide with an Alkynoic Acid Residue from an Australian Cyanobacterium

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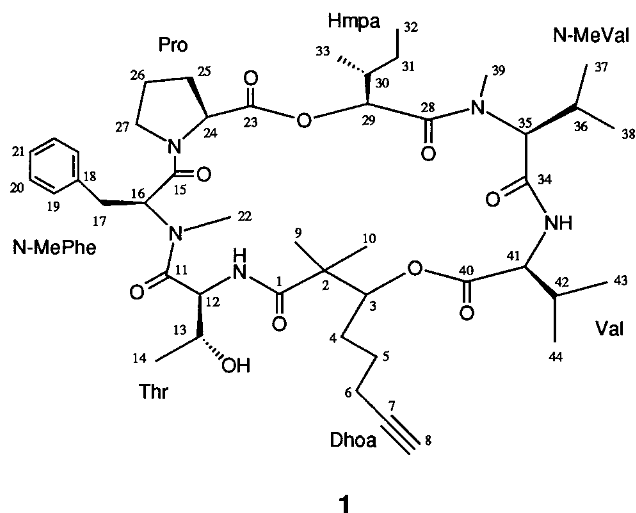
A cyclic depsipeptide, georgamide (**1**), was isolated from an Australian cyanobacterium and characterized by spectroscopic means. The constituent units were five amino acid residues, one each of L-Thr, L-Pro, L-Val, N-Me-L-Val, and N-Me-L-Phe, and two hydroxy carboxylic acids, 2(*S*)-hydroxy-3(*R*)-methylpentanoic acid and the unusual 2,2-dimethyl-3-hydroxy-7-octynoic acid. The stereochemistry was determined by hydrolysis of the peptide followed by derivatization and HPLC comparison with standard samples.

Small peptides, acyclic and cyclic, many of which display remarkable structural and biological activity, have been isolated in impressive numbers from marine cyanobacteria.[1] We recently reported[2] the isolation of two serinol-derived malyngamides displaying weak anti-HIV activity from the organic extract of a cyanobacterium[3] collected at the mouth of the King George River in Northwestern Australia. In the course of this work we found that the HIV inactive extracts contained a novel cyclic depsipeptide as the major secondary metabolite of the organism. Herein we report the isolation and structure determination of this peptide.

Results and Discussion

The algal organic extract (CH_2Cl_2:MeOH) was partitioned between hexane and methanol, and the methanol-soluble portion was then partitioned between methyl *tert*-butyl ether (MTBE)−hexane (9:1) and methanol−water (1:1). The MTBE−hexane extract was subjected to gel filtration on Sephadex LH-20 (hexane−CH_2Cl_2−MeOH, 2:5:1), followed by repeated preparative TLC on silica gel (CH_2Cl_2−EtOAc, 2:1, hexanes−EtOAc, 1:1, and petroleum ether−2-propanol, 10:1) to give georgamide (**1**) (1.85%) as a colorless amorphorous solid.

The molecular formula of **1**, $C_{46}H_{69}O_{10}N_5$, was determined by high-resolution mass spectroscopy. Classical



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amino acid analysis of **1** revealed three common amino acids: one unit each of valine, theronine, and proline. Analysis of the 2D NMR spectra including COSY, TOCSY, HMQC, and HMBC in both $CDCl_3$ and C_6D_6 allowed for the complete spectral assignment of these three amino acids as well as the assignment of structure for *N*-methylvaline (N-MeVal), *N*-methylphenylalanine (N-MePhe), and two non-amino acid moieties (Table 1).

The first of the non-amino acid units exhibited signals

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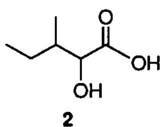
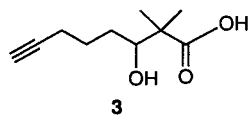
Table 1. NMR Data for **1** in $CDCl_3$

residue	no.	^{13}C NMR	^{1}H NMR (multiplicity, *J*)	COSY	TOCSY	HMBC (H)
Dhoa	1	168.8				3, 12
	2	46.6				3, 9, 10
	3	77.1	5.46 (dd, 8.6 Hz)	4ab	4ab, 5ab	4b, 9, 10
	4a	27.6	1.70 (m)	3, 4b	3, 4b, 5ab, 6a	
	4b		1.47 (m)	3, 4a, 5ab	3, 4a, 5ab	5ab, 6a
	5a	18.1	2.23 (m)	4b, 5b	3, 4ab, 5b, 6a	
	5b		2.16 (m)	4b, 5a	3, 4ab, 5a, 6a	
	6a	24.4	1.89 (m)	5ab, 6b	5ab, 6b	3, 4a, 5ab
	6b		1.47 (m)	6a		
	7	84.0				5ab, 6a, 8
	8	68.8	1.83 (m)			
	9	25.2	1.11 (s)		10	10
	10	16.9	1.30 (s)		9	3, 9
Thr	11	170.8				12, 16, 17a, NH (Thr)
	12	58.7	4.34 (d, 9.5 Hz)	13, NH (Thr)	13, 14, NH (Thr)	14
	13	67.1	4.05 (m)	14, OH	12, 14, OH, NH (Thr)	12, 14, OH
	14	20.1	1.00 (d, 6.9 Hz)	13	12, 13, OH, NH (Thr)	
	OH		4.43 (d, 12.0 Hz)	13	13, 14	
	NH		7.24 (d, 9.4 Hz)	12	12, 13, 14	
N-Me Phe	15	171.7				16, 22
	16	56.6	5.72 (dd, 4.9, 11.8 Hz)	17ab	17ab	17ab, 22
	17a	33.3	2.85 (dd, 4.9, 11.8 Hz)	16, 17b	16, 17b	16, 19
	17b		3.52 (dd, 5.2, 15.3 Hz)	16, 17a	16, 17a	
	18	137.6				17ab, 20
	19	128.1	7.12 (d, 6.4 Hz)	17b, 20		20
	20	128.6	7.15 (dd, 6.9, 7.4 Hz)	19, 21		17ab, 19, 21
	21	126.2	7.07 (t, 6.9 Hz)	20		19
	22	31.7	3.01 (s)			16
Pro	23	167.3				29
	24	57.4	4.48 (t, 8.2 Hz)	25ab	25ab, 26ab, 27ab	27b
	25a	27.5	1.80 (m)	24, 25b	24, 25b, 26ab, 27ab	24, 27b
	25b		1.45 (m)	25a	24, 27b	
	26a	25.8	2.00 (m)	25b, 26b, 27a	24, 25ab, 26b, 27ab	27b
	26b		1.88 (m)	25ab, 26a, 27ab	24, 25ab, 26a, 27ab	
	27a	47.4	3.44 (m)	26a, 27b	24, 25ab, 26ab, 27b	25a
	27b		3.70 (t, 8.3 Hz)	26b, 27a	24, 25ab, 26ab, 27a	
Hmpa	28	169.4				29, 35, 36
	29	77.7	4.59 (d, 5.3 Hz)	30	30, 31ab, 32, 33	33
	30	35.6	1.88 (m)	29, 31b, 33	29, 31ab, 32, 33	29, 32, 33
	31a	24.1	1.51 (m)	31b, 32	29, 30, 31b, 32, 33	29, 32, 33
	31b		1.16 (m)	30, 31a, 32	29, 30, 31a, 32, 33	
	32	11.1	0.83 (t, 7.2 Hz)	31a	29, 30, 31ab, 33	31a
	33	15.2	1.00 (d, 6.9 Hz)	30	29, 30, 31ab, 32	29
N-Me Val	34	172.1				35, 39, 41, NH (Val)
	35	64.8	3.96 (d, 8.9 Hz)	36	36, 38, 39	36, 37, 38
	36	30.7	2.02 (m)	35, 37, 38	35, 37, 38	37, 38
	37	20.1	0.94 (d, 6.4 Hz)	36	35, 36	38
	38	19.8	0.97 (d, 6.4 Hz)	36	35, 36	35, 36, 37
	39	30.6	2.96 (s)			35
Val	40	174.3				9, 10, 41, NH (Val)
	41	53.5	4.64 (d, 5.9 Hz)	NH (Val)	43, 44, NH (Val)	43, 44
	42	31.9	1.88 (m)	41, 43, 44	41, 43, 44, NH (Val)	41, 43, 44
	43	20.4	0.88 (d, 6.4 Hz)	42	41, 42, 44, NH (Val)	41, 42, 44
	44	15.9	0.67 (d, 6.4 Hz)	42	41, 42, 43, NH(Val)	41, 43
	NH		6.85 (d, 6.9 Hz)	41	41, 42, 43, 44	

in the ^{1}HNMR spectrum reminiscent of those of isoleucine. Resonances at δ 1.88 (H-30), 1.50 (H-31), 0.83 (H-32), and 1.00 (H-33) in $CDCl_3$ were very similar to those reported for isoleucine;[4] however, the chemical shift of the α-carbon in this residue (C-29, 77.7 ppm) was that of an oxymethine, thus indicating that this residue was not isoluecine but 2-hydroxy-3-methylpentanoic acid (Hmpa, **2**).





The structure of the second non-amino acid fragment was deduced as follows. In the HMBC spectrum ($CDCl_3$), geminal dimethyl protons (H-9 and H-10) showed correlations to a quaternary carbon at δ 46.6 (C-2) and an oxymethine carbon at δ 77.1 (C-3). The COSY spectrum allowed for the connection of all protons from H-3 to H-6. H-8 did not show a cross-peak to a carbon in the HMQC spectrum when D3 was set for $J = 135$ Hz, and in the ^{13}C attached proton test (APT) with the same D3 setting, C-8 appeared as a quaternary carbon at δ 68.8. However, in the HMBC spectrum, a quaternary carbon at δ 84.0 (C-7) showed correlation with the H-8 proton at δ 1.83. Moreover, the carbon chemical shifts of C-7 (84.0 ppm) and C-8 (68.8 ppm) matched perfectly with the chemical shifts of a terminal alkyne group.[5] A second ^{13}C APT experiment with D3 set for $J = 235$ Hz verified that C-8 was the methine

carbon of a terminal acetylene. The triple bond between C-7 and C-8 completed the identification of this unit as 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoa, **3**).

Sequencing of the amino acid residues was accomplished with the aid of the HMBC data both in $CDCl_3$ and in C_6D_6. In the HMBC spectrum with $CDCl_3$, a correlation was displayed from the NH proton to the neighboring carbonyl carbon (C-34) between Val/N-MeVal. For residues lacking NH protons, correlations from the α-protons of one amino acid unit to the carbonyl carbons of neighboring residues between N-MeVal/Hmpa (H-35 to C-28), Hmpa/Pro (H-29 to C-23), N-MePhe/Thr (H-16 to C-11), Thr/Dhoa (H-12 to C-1), and Val/N-MeVal (H-41 to C-34) were observed. In the HMBC spectrum with C_6D_6 as solvent, the displayed correlations from the methine protons of one unit to the carbonyl carbons of neighboring residues between Dhoa/Val (H-3 to C-40) and Pro/N-MePhe (H-24 to C-15) completed the identification of the 22-membered ring.

The non-amino acid moiety 2-hydroxy-3-methylpentanoic acid (Hmpa) (isoleucic acid) has been reported on many occasions in the literature.[6,7] On the other hand, 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoa) is rare. It was first reported by Scheuer *et al.* in 1996 as a constituent of the cyclic depsipeptide kulolide-1[8] and later as a constituent of the related kulokainalide-1.[9] Recently Gerwick and co-workers[10] reported its presence in the depsipeptides yanucamide A and B. All of these metabolites are believed to be of cyanobacterial origin.

The stereochemistry of the amino acids in georgamide was determined by Marfey's method.[11] Most of the standard amino acid samples used in the analysis for comparison were purchased commercially. *N*-Methyl-L-valine and *N*-methyl-DL-phenylalanine were prepared using the method developed by Quitt *et al.*[12] *N*-Methyl-DL-phenylalanine was also prepared by racemization of *N*-methyl-D-phenylalanine following the method developed by Ichiro Chibata *et al.*[13]

The standard amino acid samples, L- and D-threonine, L- and DL-valine, L- and D-proline, L- and DL-*N*-methylvaline, and DL- and D-*N*-methylphenylalanine, as well as the acid hydrolysate of peptide **1**, were individually derivatized with Marfey's reagent, *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDPAA) and subjected to reversed-phase HPLC analysis with MeCN−aqueous NH_4OAc solution as the solvent system. By comparison of the retention times, it was determined that the amino acids in georgamide were L-threonine, L-valine, L-proline, L-*N*-methylvaline, and L-*N*-methylphenylalanine.

2(*R*)-Hydroxy-3(*R*)-methylpentanoic acid and 2(*S*)-hydroxy-3(*S*)-methylpentanoic acid were commercially available. 2(*S*)-Hydroxy-3(*R*)-methylpentanoic acid and 2(*R*)-hydroxy-3(*S*)-methylpentanoic acid were synthesized according to the procedure developed by Carter *et al.*[14] All four pentanoic acids and the peptide hydrolysate were derivatized individually with *l*-menthol. The derivatives were compared by reversed-phase HPLC analysis with MeCN−water as the solvent system, and the retention times clearly established the stereochemistry of Hmpa in georgamide as 2*S*,3*R*. The stereochemistry of the Dhoa moiety was not established.

The isolation of georgamide, a Dhoa-containing peptide, from a cyanobacterium provides further evidence for the biogenetic source of this unusual moiety. Although the yuanucamides were obtained from a mixed cyanobacterial source, *Lyngbya* and *Schizothrix*,[10] kulolide-1 and kulokainalide-1 were isolated from a mollusc, *Philinopsis speciosa. Philinopsis's* prey is the sea hare, *Stylocheilus longicaudus,* which, in turn, feeds on cyanobacteria; hence the ultimate source of kulolide-1 and kulokainalide-1 was assumed to be cyanobacterial.[8,9]

Experimental Section

General Experimental Conditions. IR spectra were recorded on a Perkin-Elmer FT-IR spectrophotometer (PARAGON 500). Optical rotations were measured on a Rudolph Autopol II polarimeter. ^1H and ^{13}CNMR spectra were obtained on Bruker AC 200 (200 MHz) and Varian Unity 500 (500 MHz) spectrometers. ^1H and ^{13}C NMR chemical shifts are referenced to 7.24 and 77.0 ppm, respectively, for $CDCl_3$ and 7.26 and 128.5 ppm, respectively, for C_6D_6. Fast atom bombardment mass spectrometry (FABMS) data were collected on a JEOL SX102 mass spectrometer operated at an accelerating voltage of 10 kV. Sephadex LH-20 was used for gel filtration, and TLC grade Brinkmann silica gel 60H (7736 EM Sciences) was used for vacuum liquid chromatography (VLC). Preparative TLC was carried out on Sigma-Aldrich precoated silica gel 60F254 plates. Analytical TLC was done on commercial Sigma-Aldrich silica gel on glass. All solvents and chemicals were reagent grade.

The HPLC apparatus used was Beckman Instruments, Inc. (114M solvent delivery module; 340 organizer; 420 controller; 427 integrator; 164 variable wavelength detector). All the solvents were filtered and degassed under vacuum before use. The reversed-phase column was purchased from Alltech/Applied Science, Inc. (Alltech C18 5 μm, length 25 cm; i.d. 4.6 mm).

Plant Material. The organism was collected along the bank and at the head of the King George River, Northwestern Australia, on August 21, 1996. The fine brown filamentous alga grew as a thick mat on a mud substrate. A voucher specimen has been deposited at the Queensland Museum in Brisbane, Australia, specimen number Q66C5927. All attempts to have this alga identified have failed.

Extraction and Isolation. The organism was ground in a Waring blender with dry ice, mixed with water, and centrifuged to give a water-first extract. The marc was freeze-dried and reextracted with a mixture of CH_2Cl_2−MeOH (1:1 v/v) at 25 °C overnight. The crude organic extract was then concentrated to give a greenish-black sludge.

A sample of the crude extract, 2.68 g, was dissolved in 135 mL of MeOH, and the solution was extracted with hexane (3 × 150 mL). After separation of the layers, the hexane layer was dried over $MgSO_4$ and the hexane was evaporated to give 433 mg of oil. The MeOH layer was diluted with 150 mL of water and extracted with 9:1 methyl *tert*-butyl ether (MTBE)−hexane (2 × 150 mL). The MTBE layer was dried over $MgSO_4$ and concentrated, affording 573 mg of oil. The aqueous MeOH layer was evaporated in vacuo far enough to remove all of the MeOH, and the volume was adjusted to 150 mL with water. This was extracted with EtOAc (3 × 150 mL) to give 52 mg of oil.

The MTBE extract was passed through a Sephadex LH-20 column with hexane−CH_2Cl_2−MeOH (2:5:1) to give 11 fractions. Fraction 3 (162.0 mg) was subjected to repeated preparative TLC in CH_2Cl_2−EtOAc (2:1), then hexanes−EtOAc (1:1), followed by petroleum ether−2-propanol (10:1) to afford a total of 51.0 mg of **1**: colorless amorphorus solid, $[\alpha]_D^{20}$ +8.8° (*c* 0.74, $CHCl_3$); IR ($CHCl_3$) λ_{max} 3415, 2969, 2878, 1751, 1664, 1636, 1498, 1469, 1011, 975 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HRFABMS *m/z* 984.4059 (M + Cs), (calcd for $C_{46}H_{69}O_{10}N_5Cs$, 984.4099).

Derivatization of Amino Acids with Marfey's Reagent.[11] The amino acid standards (0.5 mg) were added to a 1% (w/v) solution of *N*-α-5-fluoro-2,4-dinitrophenylalaninamide (FDPAA, Marfey's reagent) in acetone (100 μL) and 1 M $NaHCO_3$ (40 μL). After heating at 37 °C for 1 h, the reaction mixture was cooled, acidified with 2 N HCl (40 μL), and evaporated to dryness. The resulting product was resuspended in 3 mL of DMSO−H_2O (1:1) solution. The solution was then passed through a short C_{18} column followed by reversed-phase HPLC analysis.

Hydrolysis of Georgamide and Derivatization with Marfey's Reagent.[11] Georgamide (1.1 mg) was suspended in 6 N HCl (1 mL) and incubated at 108 °C for 19 h. It was then concentrated to dryness. The residue was dissolved in H_2O (50 μL), and to the resulting mixture was added a 1% (w/v) solution of FDPAA (Marfey's reagent) in acetone (100 μL) and 1 M $NaHCO_3$ (40 μL). After heating at 37 °C for 1 h, the reaction mixture was cooled, acidified with 2 N HCl (40 μL), and evaporated to dryness. The resulting product was resuspended in 3 mL of DMSO−H_2O (1:1) solution. The solution was then passed through a short C_{18} column followed by reversed-phase HPLC analysis.

Derivatization of the 2-Hydroxy-3-methylpentanoic Acids (Isoleucic Acids) with *l*-Menthol.[15] The 2-hydroxy-3-methylpentanoyl chloride (1.0 mg) was dissolved in CH_2Cl_2 (1 mL). The solution was acidified with 3 drops of trifluroacetic acid and filtered. The filtrate was dried under N_2, giving white needlelike crystals as the residue. The residue was treated with *l*-menthol (1 mg) in toluene and a catalytic amount of *p*-toluenesulfonic acid under reflux for 72 h. The reaction mixture was then washed with saturated $NaHCO_3$ solution and water and dried. The resulting product was resuspended in 1 mL of DMSO−H_2O (1:1) solution. The solution was then passed through a short C_{18} column followed by reversed-phase HPLC analysis.

Hydrolysis of Georgamide and Derivatization with *l*-Menthol.[15] Georgamide (0.275 mg) was suspended in 6 N HCl (1 mL) and incubated at 104 °C for 20 h. It was then concentrated to dryness and extracted with ether. The ether extract of the acid hydrolysate was dried, and the residue was treated with *l*-menthol (1 mg) in toluene and a catalytic amount of *p*-toluenesulfonic acid under reflux for 72 h. The reaction mixture was then washed with saturated $NaHCO_3$ solution and water and dried. The resulting product was resuspended in 1 mL of DMSO−H_2O (1:1) solution. The solution was then passed through a short C_{18} column followed by reversed-phase HPLC analysis.

Stereochemistry of Georgamide by HPLC Analysis. The standard derivatized amino acid samples as well as the derivatized acid hydrolysate of georgamide were individually subjected to HPLC analysis. In the case of L- and D-threonine, L- and DL-valine, and L- and D-proline, the FDPAA derivative of the sample was injected into the HPLC with MeCN−aqueous NH_4OAc (100 mM) (20:80) as the eluent and an eluent speed of 1 mL/min, detector λ_{max} at 340 nm. Unreacted FDPAA appeared as a broad peak at 26.67 min. The retention times, in minutes, of the amino acid derivatives were as follows: L-thr 4.66, D-thr 7.29, L-val 9.62, D,L-val 9.73 (L) and 25.06 D), L-pro 6.33, D-pro 8.99.

In the case of L- and D-*N*-methylvaline and D- and DL-phenylalanine, the FDPAA derivative of the sample was injected into the HPLC with MeCN−aqueous NH_4OAc (50 mM) (18:82) as the eluent and an eluent speed of 1 mL/min, detector λ_{max} at 340 nm. One broad peak at 31.92 min was attributed to the unreacted FDPAA. The retention times, in minutes, of the amino acid derivatives were as follows: *N*-methyl-L-val 17.76, *N*-methyl-DL-val 17.76 (L) and 37.34 (D), *N*-methyl-D,L-phe 29.10 (L) and 40.16 (D), *N*-methyl-D-phe 38.70.

Analysis of the FDPAA derivatized peptide hydrolysate with MeCN−aqueous NH_4OAc (100 mM) (20:80) as the eluent showed three sharp strong peaks with t_R of 4.64, 9.47, and 6.27 min. Co-injecting with standard amino acid derivatives confirmed that the stereochemistries of threonine, valine, and proline in georgamide were all L. It should be noted, however, that standard samples of the allothreonines were not available for comparison. Although it is highly unlikely that the retention times of the allothreonine FDPAA derivatives would be identical to those of the normal series, this possibility cannot be ruled out.

Analysis of the FDPAA derivatized peptide hydrolysate with MeCN−aqueous NH_4OAc (50 mM) (18:82) as the eluent showed two peaks with t_R of 17.82 and 28.76 min. Co-injecting with the standards confirmed that the stereochemistries of the *N*-methylvaline and *N*-methylphenylalanine in georgamide are L.

When analyzing the *l*-menthol derivatized isoleucic acids on HPLC with MeCN−H_2O (20:80) as the eluent, an eluent speed at 1 mL/min, and detector λ_{max} at 260 nm, the retention time of the standards were as follows: 2*R*,3*R* 25.28 min, 2*S*,3*R* 68.24 min, 2*R*,3*S* 22.30 min, and 2*S*,3*R* 8.18 min.

Analysis of the *l*-menthol derivatized peptide hydrolysate with MeCN−H_2O (20:80) as the eleuent showed one peak at 8.04 min. Co-injecting the derivatized peptide hydrolysate with the standards confirmed the stereochemistry of the isoleucic acid in georgamide as 2(*R*),3(*S*).

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Supporting Information Available: NMR spectral data for **1** in C_6D_6. This material is available free of charge via the Internet at http://pubs.acs.org.

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