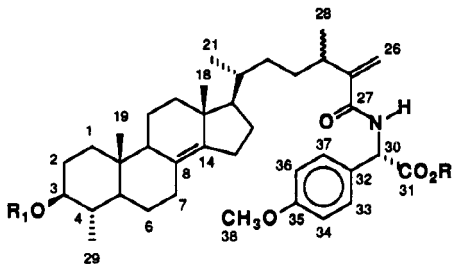
Polymastiamide A, a Novel Steroid/Amino Acid Conjugate Isolated from the Norwegian Marine Sponge *Polymastia boletiformis* (Lamarck, 1815)

Fangming Kong and Raymond J. Andersen*

Departments of Chemistry and Oceanography, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1

Received July 14, 1993

Steroids isolated from marine sponges frequently contain substantially modified side chains.[1] The most commonly encountered modifications involve extensive alkylation and/or dealkylation.[2] Recently, a small group of biologically active steroids containing more complex side chain functional groups have been reported from sponges. Examples include the following: a family of sterol disulfates that contain a side-chain orthoester;[3] the xestobergsterols,[4] which have a C16/C23 bond that generates a new cyclopentane ring involving side-chain and ring D carbon atoms; contignasterol,[5] which contains a cyclic hemiacetal in the side chain; and the kiheisterones,[6] which contain a C21 carboxylic acid and a furan ring in the side chain. As part of an ongoing investigation of the secondary metabolites of cold water marine invertebrates,[7] it was found that extracts of the Norwegian sponge *Polymastia boletiformis* (Lamarck, 1815)[8] exhibited antimicrobial activity. Bioassay guided fractionation of the *P. boletiformis* extracts led to the isolation of the pure antimicrobial metabolite polymastiamide A (1) and a complex mixture of minor analogs. The interesting side chain modification in polymastiamide A (1), which involves linkage to a nonprotein amino acid via an amide bond, is to the best of our knowledge without literature precedent.



1 R = H R_1 = $-SO_3^-$

2 R = CH_3 R_1 = H

Polymastiamide A (1) was isolated as an optically active amorphous solid that gave a parent ion at *m/z* 684.354 80 in the negative ion HRFABMS appropriate for a molecular formula of $C_{38}H_{54}NO_8S$. An intense IR band at 1249 cm^{-1} suggested that the sulfur atom was present as a sulfate functionality. A broad OH/NH stretching band that extended from 3640 to 2400 cm^{-1} with a maximum at 3519 cm^{-1} and carbonyl stretching bands at 1730 and 1656 cm^{-1} were tentatively assigned to carboxylic acid and amide functional groups. Confirmation of the presence of the sulfate and carboxylic acid functionalities came from the reaction of polymastiamide A (1) with HCl/MeOH (12 h, 20 °C) to give the methyl ester **2**. Compound **2** gave a parent ion in the HREIMS at *m/z* 619.4235 appropriate for a molecular formula of $C_{39}H_{57}NO_5$ indicating that the transformation of **1** to **2** involved the loss of one sulfur and three oxygen atoms and the gain of one carbon and three hydrogen atoms. The water-soluble fraction from the methanolysis reaction mixture gave a white precipitate with $BaCl_2$ and the intense band at 1249 cm^{-1} in the IR spectrum of **1**, which was attributed to the sulfate functionality, was absent in the IR spectrum of **2**. Evidence for the conversion of the carboxylic acid in polymastiamide A (1) to a methyl ester in **2** came from the presence of a new three proton resonance at δ 3.72 (s) in the ^1H NMR spectrum of **2** (Table I) and the absence of a carboxylic acid OH stretching band in the IR spectrum.

The NMR experiments (Table I and supplementary material) which led to the proposed structure for polymastiamide A (1) were initially carried out on the derivative **2** in order to exploit the convenience of collecting data in $CDCl_3$. Subsequently, all of the NMR experiments were repeated on a solution of the natural product **1** in DMSO-d_6. The ^{13}C NMR spectrum (Table I) of methyl ester **2** contained only 37 resolved resonances, which indicated the presence of some element of symmetry in the molecule. Detailed analysis of the COSY, HMQC (heteronuclear multiple quantum coherence),[9] and HMBC (heteronuclear multiple bond connectivity)[10] data for **2** revealed that a *p*-methoxyphenylglycine methyl ester fragment accounted for the symmetry apparent from the ^{13}C NMR spectrum. A pair of scalar coupled ^1H NMR resonances at δ 6.85 (d, J = 8.7 Hz, H34/36) and 7.27 (d, J = 8.7 Hz, H33/37), each integrating for two protons, could be assigned to the para-disubstituted aromatic ring in the amino acid. The ^1H NMR resonance at δ 7.27 (H33/37) showed a HMBC correlation to a deshielded aromatic carbon at δ 159.8, which was also correlated in the HMBC spectrum to a deshielded methyl resonance at δ 3.77 (s). This pair of HMBC correlations established that there was a methoxy substituent on the aromatic ring. A deshielded methine resonance at δ 5.55 (d, J = 7.1 Hz, H30) in the ^1H NMR spectrum showed a HMBC correlation to an aromatic carbon resonance at δ 128.5 (C33/37) which was correlated in the HMQC spectrum to the aromatic proton resonance at δ 7.27 (H33/37). The methine resonance at δ 5.55 (H30) showed an additional HMBC correlation into a carbon resonance at δ 171.7 (C31), which was in turn correlated into the methyl ester proton resonance at δ 3.72. This second set of HMBC correlations established that the methine carbon was the second substituent on the aromatic

(1) Kerr, R. G.; Baker, B. J. *Nat. Prod. Rep.* **1991**, *8*, 465–497.
(2) See: Kerr, R. G.; Kerr, S. L.; Malik, S.; Djerassi, C. *J. Am. Chem. Soc.* **1992**, *114*, 299 and references cited therein.
(3) Koehn, F. E.; Gunasekera, M.; Cross, S. S. *J. Org. Chem.* **1991**, *56*, 1322.
(4) Shoji, N.; Umeyama, A.; Shin, K.; Takeda, K.; Arihara, S.; Kobayashi, J.; Takei, M. *J. Org. Chem.* **1992**, *57*, 2996.
(5) (a) Burgoyne, D. L.; Andersen, R. J.; Allen, T. M. *J. Org. Chem.* **1992**, *57*, 525. (b) Andersen, R. J.; Burgoyne, D. L.; Williams, D. E.; Kong, F.; de Silva, E. D.; Miao, S.; Allen, T. M.; Holmes, C. F. B.; Chen, D.; M. Kent, M. *Gazz. Chim. Ital.* **1993**, *123*, 293.
(6) Carney, J. R.; Yoshida, W. Y.; Scheuer, P. J. *J. Org. Chem.* **1992**, *57*, 6637.
(7) For previous studies see: de Silva, E. D.; Miao, S.; Andersen, R. J.; Shultz, L. W.; Clardy, J. *Tetrahedron Lett.* **1992**, *33*, 2917.
(8) The sponge was identified by Professor Rob van Soest, University of Amsterdam, and a voucher sample has been deposited at the Zoological Museum of Amsterdam (ZMA POR 10170).
(9) Bax, A.; Subramanian, S. J. *J. Magn. Reson.* **1986**, *67*, 565.
(10) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093.

0022-3263/93/1958-6924$04.00/0

Table I. NMR Data for Polymastiamide A (1), Methyl Ester 2, and Model Steroid 3

carbon no.	polymastiamide A (1)[a]		methyl ester 2[b]		3[c]
	δ ^{1}H[d]	δ ^{13}C[e]	δ ^{1}H[d]	δ ^{13}C[e]	δ ^{13}C
1	1.60	35.8	1.68	36.3	36.5
1′	1.03		1.12		
2	2.10	28.1	1.81, dq, J = 13, 4 Hz	31.2	31.5
2′	1.28		1.42		
3	3.53	80.1	3.11, dt, J = 4.4, 10.8 Hz	76.5	71.0
4	1.21	37.1	1.26	39.7	38.2
5	0.89	50.7	0.84	50.7	44.2
6	1.64	24.7	1.69	24.9	28.9
6′	0.87		0.98		
7	2.32	29.2	2.38	29.7	29.6
7′	1.63		1.65		
8		125.8		126.0	126.1
9	1.60	48.7	1.61	49.4	49.2
10		36.8		37.5	36.7
11	1.55	19.48	1.58	20.0	19.9
11′	1.40		1.43		
12	1.85	36.9	1.88, dt, J = 12.2, 4 Hz	37.4	37.2
13		42.1		42.6	42.6
14		141.4		142.1	142.4
15	2.16	25.3	2.16	25.7	25.7
15′	2.08		2.16		
16	1.69	26.5	1.73	27.0	27.0
16′	1.25		1.27		
17	0.97	56.3	1.06	56.6	56.8
18	0.76, s	18.0	0.78, s	18.1	18.2
19	0.63, s	13.6	0.69, s	13.9	12.8
20	1.34	33.9	1.37	34.5	34.4
21	0.83, d, J = 6.5 Hz	18.9	0.86, d, J = 6.6 Hz	19.1	19.0
22	1.32	32.5	1.36	33.1	35.9
22′	0.96		1.01		
23	1.46	31.3	1.52	31.8	23.7
23′	1.13		1.17		
24	2.54	34.9	2.57, tq, J = 6.8, 6.8 Hz	35.8	39.5
25		149.5		150.9	27.9
26	5.61, bs	115.8	5.57, bs	115.6	22.5
26′	5.21, bs		5.22, bs		
27		168.5		168.9	22.7
28	0.98, d, J = 6.8 Hz	19.6	1.05, d, J = 6.8 Hz	19.8	
29	0.87, d, J = 6.2 Hz	15.5	0.97, d, J = 6.3 Hz	15.2	
NH	8.48, d, J = 7.5 Hz		6.65, d, J = 7.1 Hz		
30	5.35, d, J = 7.6 Hz	55.6	5.55, d, J = 7.1 Hz	55.8	
31		172.2		171.7	
COOH	11.0				
32		129.3		128.7	
33	7.31, d, J = 8.7 Hz	129.1	7.27, d, J = 8.7 Hz	128.5	
34	6.88, d, J = 8.7 Hz	113.6	6.85, d, J = 8.7 Hz	114.4	
35		158.8		159.8	
36	6.88, d, J = 8.7 Hz	113.6	6.85, d, J = 8.7 Hz	114.4	
37	7.31, d, J = 8.7 Hz	129.1	7.27, d, J = 8.7 Hz	128.5	
38	3.72, s	55.1	3.77, s	55.3	
39			3.72, s	52.7	

[a] Recorded in DMSO-d_6. [b] Recorded in $CDCl_3$. [c] Recorded in $CDCl_3$ at 20 MHz. [d] 500 MHz. [e] 125 MHz. Assignments are based on HMQC, HMBC, and COSY data.

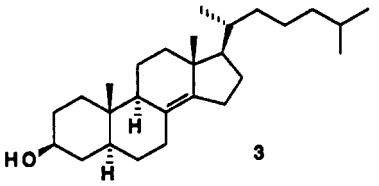
ring and also that it was attached to the methyl ester functionality. The ^{1}H/^{1}H COSY spectrum showed a correlation from the methine resonance (δ 5.55, H30) to a deshielded resonance at δ 6.65 (d, J = 7.1 Hz) that was assigned to an NH proton because it failed to show a correlation to a carbon atom in the HMQC spectrum. Intense fragment ions at m/z 179 (100, $C_{10}H_{11}O_3$) and 194 (90, $C_{10}H_{12}NO_3$) in the EIHRMS of **2** confirmed the presence of the *p*-methoxyphenylglycine methyl ester fragment.

Subtraction of the atoms in the *p*-methoxyphenylglycine methyl ester fragment ($C_{10}H_{12}NO_3$) from the molecular formula of **2** ($C_{39}H_{57}NO_5$) indicated that the remaining portion of the molecule had to account for an elemental composition of $C_{29}H_{45}O_2$ and seven sites of unsaturation. Deshielded resonances at δ 168.9 (s, C27), 126.0 (s, C8), 142.1 (s, C14), 150.9 (s, C25), and 115.6 (t, C26) in the ^{13}C NMR spectrum of **2** were assigned to an amide carbonyl and two olefinic functionalities that had to be present in this second portion of the molecule. The absence of ^{13}C NMR evidence for additional unsaturated functional groups in **2** indicated that this remaining fragment was tetracyclic, and the presence of a series of diagnostic methyl resonances (δ 0.69, s; 0.78, s; 0.86, d, J = 6.6 Hz; 0.97, d, J = 6.3 Hz; 1.05, d, J = 6.8 Hz) in the ^{1}H NMR spectrum suggested that it was steroidal in nature.

Analysis of the HMBC, HMQC, and COSY data obtained for **2** provided confirmation of the steroidal substructure and established the location of the functional groups on the steroid skeleton. A deshielded methine resonance at δ 3.11 (dt, J = 4.4 and 10.8 Hz) in the ^{1}H NMR spectrum, which was correlated to a carbon resonance at δ 76.5 in the HMQC spectrum, was assigned to H3. The C3 carbon resonance (δ 76.5) was correlated to a methyl doublet at δ 0.97 (Me29) in the HMBC spectrum. Additional HMBC correlations were observed from the methyl doublet (δ 0.97; Me29) to methine carbon resonances at δ 39.7 (C4) and 50.7 (C5) suggesting that there was a methyl substituent at C4. In the COSY spectrum, the methyl doublet at δ 0.97 (Me29) showed a correlation to the H4 methine resonance at δ 1.26 (HMQC correlation to δ 39.7), and the H4 resonance was further correlated to the H3 methine (δ 3.11) and to a second methine at δ 0.84 which was assigned to H5 by its HMQC correlation to the carbon resonance at δ 50.7. A methyl singlet at δ 0.69 in the ^{1}H NMR spectrum, which was correlated to the C5 carbon resonance at δ 50.7 in the HMBC spectrum, was assigned to Me19. The coupling constants observed for the H3 methine resonance (δ 3.11, dt, J = 4.4 and 10.8 Hz) showed that both H3 and H4 were axial and, therefore, that **2** contained 3β-hydroxyl and 4α-methyl substituents.

The remaining methyl singlet at δ 0.78 in the ^{1}H NMR spectrum of **2** was assigned to Me18. HMBC correlations were observed from the Me18 resonance (δ 0.78) to a methylene carbon at δ 37.4, a methine carbon at 56.6, a quaternary carbon at 42.6, and a fully substituted olefinic carbon at 142.1. The HMBC correlations observed from the Me18 protons required that the olefinic (δ 142.1) and methine (δ 56.6) carbons be situated at C14 and C17, but they did not indicate which type of carbon, either olefinic or aliphatic methine, was at each site. An additional HMBC correlation between a methyl doublet at δ 0.86 (J = 6.6 Hz; C21) and the aliphatic methine carbon at δ 56.6 (C17) was attributed to a Me21/C17 three-bond coupling. The Me21/C17 HMBC correlation located the aliphatic methine and olefinic carbons at C17 and C14, respectively. ^{13}C APT data showed that the three remaining olefinic carbons in **2** included two fully substituted carbons and one olefinic methylene carbon. Therefore, the second olefinic carbon in the steroid nucleus had to be located at

C8. Comparison of the ^{13}C NMR assignments for the steroid nucleus of **2**, obtained from HMQC, HMBC and COSY data, with the literature values for the steroid **3** (Table I),[11] confirmed the proposed constitution for the nucleus and also provided evidence for the relative stereochemistries at C5, C9, C10, C13, C17, and C20 that are shown in **2**.



3

Further analysis of the HMBC, HMQC, and COSY data for **2** revealed the nature of the link between the steroidal and *p*-methoxyphenylglycine fragments. A pair of broad one proton singlets at δ 5.22 (H26′) and 5.57 (H26) in the ^1H NMR spectrum, which were both correlated to the same ^{13}C NMR resonance at δ 115.6 (C26) in the HMQC spectrum, were assigned to an olefinic methylene functionality. HMBC correlations were observed from both of the olefinic methylene proton resonances (δ 5.22 (H26′) and 5.57 (H26)) to a fully substituted olefinic carbon resonance at δ 150.9 (C25) and to a carbonyl resonance at δ 168.9 (C27), revealing an $\alpha\beta$-unsaturated carbonyl substructure. An additional HMBC correlation between the NH proton resonance at δ 6.65 and the carbonyl resonance at δ 168.9 (C27) showed that the *p*-methoxyphenylglycine fragment was linked to the $\alpha\beta$-unsaturated carbonyl via an amide bond. The acrylamide substructure defined above could be most readily accommodated by the C25, C26, and C27 carbons of a standard steroidal side chain skeleton. A methyl doublet at δ 1.05 (J = 6.8 Hz, Me28) in the ^1H NMR spectrum of **2** was correlated in the HMBC spectrum to the α-olefinic carbon at δ 150.9 (C25) indicating the existence of a methyl substituent at C24. The COSY spectrum showed coupling between the methyl doublet (δ 1.05, Me28) and a methine at δ 2.57 (H24) that was correlated in the HMQC spectrum to a carbon at δ 35.8 (C24). HMBC correlations from the olefinic methylene protons at δ 5.22 (H26′) and 5.57 (H26) to the methine carbon at δ 35.8 (C24) confirmed the presence of the methyl substituent at C24. COSY, HMQC, and HMBC data (see supplementary material) confirmed the indicated side chain connectivity between C24 and C20 in **2**.

Reaction of **2** with aqueous HBr followed by derivatization with Marfey's reagent[12] and HPLC analysis showed that *p*-hydroxyphenylglycine had been formed in the hydrolysis reaction. Comparison of the HPLC retention times of the Marfey's reagent derivative of the *p*-hydroxyphenylglycine liberated from **2** by hydrolysis with authentic standards revealed that the amino acid residue in **2** had the L configuration.

The initial conversion of polymastiamide A (**1**) to the derivative **2** involved hydrolysis of a sulfate functionality and formation of a methyl ester. With the structure of **2** established, it was apparent that the sulfate group had to be attached at the C3 hydroxyl group and that the *p*-methoxyphenylglycine residue contained a free carboxylic acid in polymastiamide A (**1**). The NMR and MS data for polymastiamide A (**1**) (Table I) were completely consistent with these conclusions.

Polymastiamide A (**1**) is the first example of a new type of marine natural product that is formed by combination of steroid and α-amino acid components.[13] Its discovery in the extracts of *Polymastia boletiformis* adds to the rich diversity of steroid modifications that are known from marine sponges.[1-6] Polymastiamide A (**1**) exhibited in vitro antimicrobial activity against various human and plant pathogens (MIC's in a 1/4-in. disk assay: *Staphylococcus aureus* (100 μg/disk); *Candida albicans* (75 μg/disk) and *Pythium ultimum* (25 μg/disk)) but it was found to be inactive against HIV-1.

Experimental Section

Isolation. Specimens of *Polymastia boletiformis* were collected by hand using SCUBA at depths of 20–25 m on vertical rock faces off Korsnes Peninsula on Fanafjiord south of Bergen, Norway, in July 1992. Freshly collected sponge was frozen on site and transported to Vancouver over dry ice. A portion of the frozen sponge (152 g) was cut into small pieces, immersed in MeOH, and soaked at room temperature for 2 days. The methanol extract was concentrated in vacuo to give a red suspension that was partitioned between an aqueous solution (300 mL) of 1:1 H_2O/MeOH and hexanes (200 mL × 2). The aqueous layer was concentrated and lyophilized to give a brown solid (12.2 g, mostly salt). The solid was suspended in MeOH, and the suspension was filtered. The filtrate was concentrated in vacuo and chromatographed on Sephadex LH-20 in MeOH to give a fraction containing steroid-like compounds (378 mg) which was desalted by reversed-phase silica column chromatography (eluent: first water, then 1:1 H_2O/MeOH) to afford 193 mg of a steroid mixture. Subsequent chromatography on Sephadex LH-20 (eluent: 40:10:4 EtOAc/MeOH/H_2O) yielded almost pure polymastiamide A. Final purification was achieved by repeated Sephadex LH-20 chromatography (eluent: 40:10:4 EtOAc/MeOH/H_2O) to give pure polymastiamide A (**1**) (34 mg).

Polymastiamide A (1): off-white amorphous solid; $[\alpha]^{21}_D$ +67.4° (MeOH, c = 1.1); UV λ_{max} 224 nm (ϵ 8840), 272 nm (ϵ 1520), and 278 nm (ϵ 1270); IR (KBr) 3519, 2945, 1730, 1656, 1613, 1514, 1465, 1380, 1249, 980, 827, and 630 cm^{-1}; ^1H NMR see Table I; ^{13}C NMR see Table I; negative ion HRFABMS m/z 684.35480 ($C_{38}H_{54}NO_8S$ ΔM −3.24 ppm).

Methanolysis of Polymastiamide A (1). Polymastiamide A (**1**) (12 mg) was dissolved in 3 mL of MeOH containing five drops of concentrated HCl, and the reaction mixture was stirred overnight at room temperature. The reaction mixture was partitioned between EtOAc and H_2O. Evaporation of EtOAc layer gave the methyl ester **2** that was further purified on normal phase HPLC (eluent: 3:1 hexane/EtOAc) (yield 4.5 mg).

Methyl Ester 2: amorphous white solid; $[\alpha]^{21}_D$ +66.6° (MeOH, c = 0.35); UV λ_{max} 226 nm (ϵ 10 620), 272 nm (ϵ 1590), and 278 nm (ϵ 1420); IR (KBr) 3316, 2933, 2872, 1741, 1651, 1615, and 1507 cm^{-1}; ^1H NMR see Table I; ^{13}C NMR see Table I; LREIMS m/z 619, 601, 586, 194, and 179; HREIMS M$^+$ m/z 619.4235 ($C_{39}H_{57}NO_5$ ΔM −0.2 mmu).

Determination of the Absolute Configuration of the *p*-Methoxyphenylglycine Residue. Methyl ester **2** (3 mg) was dissolved in 4 mL of 5 N HBr and refluxed overnight with stirring. The cooled reaction mixture was evaporated to dryness, and traces of HBr were removed from the residue by repeated evaporation from 10-mL aliquots of H_2O. The residual hydrolysate was treated with excess Marfey's reagent,[12] 5-fluoro-2,4-dinitrophenyl-L-alanineamide (FDAA), for 1 h at 40 °C. The FDAA derivative was analyzed by reversed-phase HPLC. A linear gradient of (A) 9:1 triethylammonium phosphate (50 mM, pH 3.0)/MeCN and

(11) Kho, E.; Imagawa, D. K.; Rohmer, M.; Kashman, Y.; Djerassi, C. *J. Org. Chem.* **1981**, *46*, 1836.
(12) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591.

(13) While this manuscript was in review, a report appeared describing a starfish steroid in which a 24-carboxylic acid functionality is linked via an amide bond to D-cysteinolic acid. See: De Riccardis, F.; Minale, L.; Riccio, R.; Iorizzi, M.; Debitus, C.; Duhet, D.; Monniot, C. *Tetrahedron Lett.* **1993**, *34*, 4381.

(B) MeCN with 0% at the start to 40% (B) over 40 min (flow rate 0.7 mL/min) was used to separate the FDAA derivative which was detected by UV absorbance at 340 nm. The peaks in the chromatograph were identified by comparing their retention times and photodiode array UV spectra with those of pure amino acid derivative standards and by coinjection. The hydrolysate showed peaks at 54.2 min (major) and 57.1 min (minor). The amino acid standards gave the following retention times in minutes: 57.9 for D-*p*-hydroxyphenylglycine; 55.1 for L-*p*-hydroxyphenylglycine injected as a racemic mixture, 57.7 for D-*p*-hydroxyphenylglycine injected as a pure enantiomer. In all cases a peak at 42 min was observed, which was attributed to the excess FDAA. Coinjection of the hydrolysate sample with the authentic amino acid derivatives confirmed that the *p*-methoxyphenylglycine residue in polymastiamide A had the L configuration.

Acknowledgment. The authors thank Mike Le Blanc and Sandra Millen for collecting *P. boletiformis* and Professor R. van Soest for identifying the sponge. We also wish to thank Professor Kenneth Rinehart and Dr. Sue Cross for conducting the HIV-1 bioassay. Financial support was provided by a grant to R.J.A. from the Natural Sciences and Engineering Research Council of Canada and by a UBC Killam Postgraduate fellowship awarded to F.K.

Supplementary Material Available: ^{1}H, ^{13}C, COSY, HMQC, and HMBC spectra for polymastiamide A (1) and the methyl ester **2** (10 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.