Supporting Information

Neopetrosiamides, Peptides from the Marine Sponge *Neopetrosia* sp. that Inhibit Amoeboid Invasion by Human Tumor Cells

David E. Williams, Pamela Austin, Ana R. Diaz-Marrero, Rob Van Soest, Teatulohi Matainaho, Calvin Roskelley*, Michel Roberge*, and Raymond J. Andersen*

Departments of Chemistry, Earth & Ocean Sciences, Biochemistry & Molecular Biology, and Cellular & Physiological Sciences, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z1; Institute for Systematics and Ecology, University of Amsterdam, 1090 GT Amsterdam, The Netherlands; Discipline of Pharmacology, School of Medicine and Health Sciences, University of Papua New Guinea, N.C.D., Papua New Guinea

Index:

Page S2: Experimental section
Page S9: Table 1 – Proton NMR data for neopetrosiamide A (1).
Page S11: Table 2 - Carbon NMR data for neopetrosiamide A (1).
Page S12: Table 3 - Proton NMR data for neopetrosiamide B (2).
Page S14: Table 4 - Carbon NMR data for neopetrosiamide B (2).
Page S15: MALDI-TOFMS of neopetrosiamide A (1).
Page S16: 600 MHz Proton NMR of neopetrosiamide A (1).
Page S17: 150 MHz Carbon NMR of neopetrosiamide A (1).
Page S18: 600 MHz COSY spectrum of neopetrosiamide A (1).
Page S19: 600 MHz HMBC spectrum of neopetrosiamide A (1).
Page S20: 600 MHz HSQC spectrum of neopetrosiamide A (1).
Page S21: 600 MHz NOESY spectrum of neopetrosiamide A (1).
Page S22: Expanded 600 MHz NOESY spectrum of neopetrosiamide A (1).
Pages S23 to 25: Summary of Edman Sequencing Analysis
Page S26: 600 MHz proton NMR spectrum of neopetrosiamide B (2).
Page S27: 150 MHz Carbon NMR spectrum of neopetrosiamide B (2).

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Jasco P-1010 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV-600 spectrometer. ^1H chemical shifts are referenced to the residual $\text{MeCN-}d_3$ signal (δ 1.93 ppm) and ^{13}C chemical shifts are referenced to the $\text{MeCN-}d_3$ solvent peak (δ 1.39 ppm). Low and high resolution MALDITOF-MS were recorded on a Bruker Biflex IV mass spectrometer with 2,5-dihydroxybenzoic acid sample matrix. Low resolution ESI-QIT-MS were recorded on a Bruker-Hewlett Packard 1100 Esquire-LC system mass spectrometer. Low resolution FABMS were recorded on a Kratos Concept II HQ mass spectrometer with xenon as the bombarding gas and a 3-nitrobenzyl alcohol sample matrix.

Merck Type 5554 silica gel plates and Whatman MKC18F plates were used for analytical thin layer chromatography. Waters 10 g Sep-Pak's were used for reversed-phase flash chromatography. Reversed-phase HPLC purifications and Marfey's HPLC²⁰ analysis of amino acid derivatives were performed on a Waters 600E System Controller liquid chromatograph attached to a Waters 996 photodiode array detector. All solvents used for HPLC were Fisher HPLC grade.

Animal Material. Specimens of *Neopetrosia* sp. were collected by hand using SCUBA from an overhang at a depth of 10 m, near Milne Bay, Papua New Guinea (10° 33.24 S, 151° 02.34 E) in September 2003. Freshly collected sponge was frozen on site and transported frozen to Vancouver. A voucher sample has been deposited at the Zoological Museum of Amsterdam (ZMA POR. 18339).

Extraction of *Neopetrosia* sp. and Isolation of Neopetrosiamides A (1) and B (2). A sample of sponge (450 g wet wt.; 55 g dried and extracted wt.) was cut into small pieces, immersed in and subsequently extracted repeatedly with MeOH (4 x 500 mL) at room temperature. The combined MeOH extracts were concentrated *in vacuo* and the resultant brown gum was suspended in H₂O (400 mL) and sequentially extracted with EtOAc (3 x 100 mL) and *n*-BuOH (4 x 100 mL). The EtOAc and *n*-butanol extracts were

independently combined and evaporated to dryness *in vacuo*. The *n*-BuOH extract exhibited anti-invasive activity down to 12.5 µg/mL against amoeboid invading LS174T human colon cells. The *n*-BuOH extract was chromatographed on a Sephadex LH-20 column eluting with MeOH to give an early eluting bioactive fraction. The bioactive material was further fractionated using reversed-phase Si gel flash chromatography employing a step gradient from 1:1 MeOH/H₂O to MeOH with a final CH₂Cl₂ wash. A 300 mg fraction, eluting with 7:3-4:1 MeOH/H₂O, elicited bioactivity. A portion (88 mg) of this bioactive fraction was further purified via C₁₈ reversed-phase HPLC using a CSC-Inertsil 150A/ODS2, 5 µm 25 x 0.94 cm column, with 3:7 MeCN/(0.05% TFA/H₂O) as an eluent to give a fraction consisting of >95% neopetrosiamides A (**1**) and B (**2**). This was followed by reversed-phase HPLC using the same column but with 3:2 MeOH/(0.05% TFA/H₂O) as an eluent to give one fraction containing pure neopetrosiamide A (**1**) (44.6 mg), and a fraction consisting of a 1:5 mixture of neopetrosiamides A (**1**) and B (**2**). A pure sample of **2** (32.4 mg) was obtained after a final HPLC purification using the same conditions as in the previous step.

Neopetrosiamide A (1) Isolated as a clear glass; $[\alpha]_D^{25}$ -65.2° (c 4.2, MeOH); ¹H NMR, see Table 1; ¹³C NMR, see Table 2; positive ion MALDITOF-MS $[M+H]^+ m/z$ 3071.

Neopetrosiamide B (2) Isolated as a clear glass; $[\alpha]_D^{25}$ -86.2° (c 4.2, MeOH); ¹H NMR, see Table 3; ¹³C NMR, see Table 4; positive ion MALDITOF-MS $[M+H]^+ m/z$ 3071. The NMR data for neopetrosiamide B (**2**) established that the Pro⁴, Pro¹⁴, Pro¹⁷ amide bonds were all *trans*. Thus, the carbon chemical shift of the γ carbons and the carbon chemical shift difference of the β and γ carbons for the three proline amide bonds were as follows: (Pro⁴ δ Cγ 25.0 ppm, Δδ_{Cβ-Cγ} = 4.5 ppm; Pro¹⁴ δ Cγ 25.7 ppm, Δδ_{Cβ-Cγ} = 4.5 ppm; Pro¹⁷ δ Cγ 25.1 ppm, Δδ_{Cβ-Cγ} = 5.3 ppm).

Hydrolysis of Neopetrosiamides A (1) and B (2). Purified metabolites **1** (600 µg, 0.20 µmole) and **2** (500 µg, 0.16 µmole) were hydrolyzed in 0.5 mL 6N HCl (freshly distilled, constant boiling HCl) at 108° with stirring for 16 hours in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction

mixtures were evaporated to dryness and traces of HCl removed from the residual hydrolysates by repeated evaporation from H₂O (3 x 0.4 mL).

Derivatization of Amino Acids with Marfey's Reagent and HPLC Analysis (Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591-596). To a 0.5 mL vial containing 2.0 μ mol of the pure amino acid standard in 40 μ L of H₂O was added 2.8 μ mole of N- α -(2,4-dinitro-5-fluorophenyl)-L-alanine amide (FDAA) in 80.0 μ L acetone followed by 20 μ L of 1N NaHCO₃. The mixture was heated for 1 hour at 40°. After cooling to RT, 10 μ L of 2N HCl was added and the resulting solution was filtered through a 4.5 μ filter and stored in the dark until HPLC analysis.

To the hydrolysate mixtures of neopetrosiamides A (**1**) and B (**2**) in 103 and 86 μ L of H₂O was added 8.5 and 7.1 μ mole of FDAA in 242 and 202 μ L of acetone, respectively, as described above. In each case a 2 μ L aliquot of the resulting mixture of FDAA derivatives was analyzed by reversed-phase HPLC. Either an Alltech Econosil C-18 5 μ , 250 x 4.6 mm column with a linear gradient of (A) 9:1 triethylammonium phosphate (50 mM, pH 3.0 and 1.5)/MeCN and (B) MeCN with 0% B at start to 40% B over 55 min (flow rate 1 mL/min), method i and ii respectively, or for method iii an Alltech Econosil C-18 5 μ , 150 x 2.1 mm column with a linear gradient of (A) 97:3 triethylammonium phosphate (50 mM, pH 1.5)/MeCN and (B) MeCN with 0% B at start to 27% B over 55 min (flow rate 1 mL/min) were used to separate the FDAA derivatives with UV detection at 340 nm. Each peak in the chromatographic trace was identified by comparing its retention time and photodiode array UV spectrum with that of the FDAA derivative of the pure amino acid standard and by coinjection. In all cases a peak at 40.20, 40.04 and 34.49 min, respectively for the three methods used, was observed which was attributed to excess FDAA. Retention times (min) for the three methods are given in parentheses: method (i) D-Arg (26.20), L-Arg (26.69), L-Asn (27.87), L-Ser (28.22), D-Asn (28.85), D-Ser (29.40), L-Thr (29.40), L-*allo*-Thr (29.62), L-Asp (29.96), D-*allo*-Thr (31.08),

D-Asp (31,.69), Gly (33.89), D-Thr (33.89), L-Ala (34.86), L-Pro (35.91), D-Pro (38.35), D-Ala (38.91), L-Met (41.01), L-Val (42.47), L-Cys (45.01), D-Met (45.95), L-Leu (48.13), L-Phe (48.13), D-Val (48.13), D-Cys (48.87), D-Phe (52.27), D-Leu (53.38); method (ii) D-Cysteic Acid (24.53), L-Cysteic Acid (25.10), D-Arg (26.81), L-Arg (27.16), L-Asn (29.29), D-Asn (29.71), L-Ser (29.71), D-Ser (30.28), L-Thr (30.88), L-Asp (31.47), D-Asp (32.81), Gly (34.88), D-Thr (34.88), L-Ala (36.61), L-Pro (37.31), D-Pro (39.05), D-Ala (40.04), L-Met (43.12), L-Val (44.12), L-Cys (47.44), D-Met (49.18), L-Leu (49.82), L-Phe (49.82), D-Val (49.82), D-Cys (50.25), D-Phe (53.50), D-Leu (54.47); method (iii) L-Cysteic Acid (26.66), L-Arg (27.33), L-Asn (29.63), L-Ser (30.05), L-Thr (31.06), L-Met(O) (31.64), L-Asp (32.64), Gly (34.49), L-Ala (37.00), L-Pro (38.77), L-Met (46.33), L-Val (47.58), L-Leu (55.55), L-Phe (55.55), L-Cys (56.67).

Hydrolysis of L- and DL-Asparagine. L-Asparagine (400 µg, 3.03 µmole) and DL- asparagine (400 µg, 3.03 µmole) were hydrolyzed in 0.5 mL 6N HCl (freshly distilled, constant boiling HCl) at 108° with stirring for 16 hours in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixtures were evaporated to dryness and traces of HCl removed from the residual hydrolysates by repeated evaporation from H₂O (3 x 0.4 mL). The resulting products were then derivatized with Marfey's Reagent as described above.

Epimerization of L-Cysteic Acid to DL-Cysteic Acid. To 600 µg (3.55 µmole) of L-cysteic acid was added 50 µL of 2N KOH. The reaction mixture was heated to 80°C for 2 hours, cooled and quenched with 50 µL 2N HCl. The resulting mixture was then derivatized with Marfey's Reagent as described above.

Treatment of L-Methionine Sulphoxide with HCl. L-Methionine sulphoxide (300 µg, 1.82 µmole) was heated in 0.5 mL of 6N HCl (freshly distilled, constant boiling HCl) at 108° with stirring for 16 hours in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness and traces of HCl removed from the residual hydrolysate by repeated evaporation from H₂O (3 x 0.4 mL). The resulting products were then derivatized with Marfey's Reagent as described above.

Reaction of L-Methionine Sulphoxide with L-Cystine in the Presence of HCl. L-Methionine sulphoxide (300 μg , 1.82 μmole) and L-cystine (1.0 mg μg , 4.15 μmole) were heated in 0.5 mL of 6N HCl (freshly distilled, constant boiling HCl) at 108° with stirring for 16 hours in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness and traces of HCl removed from the residual hydrolysate by repeated evaporation from H_2O (3 x 0.4 mL). The resulting products were then derivatized with Marfey's Reagent as described above.

Procedure for the Amino Acid Analysis of Neopetrosiamides A (1) and B (2).¹ The amino acid analysis was performed on a Waters Alliance 2690 Separation Module. Dried protein sample was hydrolyzed by a vapour phase or liquid phase reaction, using 6M HCl with 1% phenol at 110°C for 24 hours. The drying, evacuation of air, flushing with pre-purified nitrogen and hydrolysis were done on a Waters PICO-TAG Work Station.

After hydrolysis, the excess HCl was removed from the hydrolysis tube under vacuum and sample was treated with a redrying solution consisting of 2:2:1 MeOH/ H_2O / Et_3N and dried under vacuum. The sample was then derivatized for 20 minutes at room temperature using a derivatizing solution of 7:1:1:1 MeOH/ H_2O / Et_3N /phenylisothiocyanate. The derivatizing solution was removed under vacuum and sample was treated with the redrying solution and dried to remove any traces of phenylisothiocyanate.

The derivatized sample was dissolved in a given amount of sample diluent (pH 7.40) and an aliquot injected into the Waters PICO-TAG column (3.9 mm X 150 mm) running on a Waters PICO-TAG gradient with a column temperature of 38°C. The phenylisothiocyanate-amino acids were detected at 254 nm.

The Waters Alliance 2690 Separation Module system consists of a solvent delivery system, a refrigerated autosampler, a syringe injector and a column heating module. Data acquisition and collection was obtained using a Waters 2487 Dual Wavelength Absorbance Detector. The whole system was controlled using

Waters Millenium chromatography software v. 4.0 running on a Pentium IV PC (Microsoft Windows 2000).

Estimation of Cystine Residues in Neopetrosiamides A (1) and B (2) by Performic Acid Oxidation.¹

Neopetrosins A (1) and B (2) (1-5 µg) were dried in hydrolysis tubes using a centrifugal evaporator, and 10 µL of methanol added. A 10-12 excess of freshly prepared performic acid solution was added with vortex mixing. (The performic acid was prepared by adding 5 vol. of 30% hydrogen peroxide to 95 vol of 88% formic acid in a screw-capped glass tube and allowed to stand at room temperature for 2 hours). The tubes were placed inside a PICO-TAG reaction vessel, evacuated and flushed with pre-purified nitrogen using the PICO-TAG Workstation. The reaction vessel was then sealed and placed in a 5 °C refrigerator for 2.5 hours. The samples were dried by vacuum using a centrifugal evaporator. 50 µL of Milli-Q water was then added with vortex mixing and the samples again dried by vacuum using a centrifugal evaporator. The two samples were then hydrolysed with 6M HCl and analyzed as described above.

Procedure for the Automated Sequence Analysis of Neopetrosiamide A (1).¹ The Procise Protein Sequencing System (supplied by Applied Biosystems, Foster City, CA) was used. The system is composed of four integrated modules that completely automates Edman degradation and phenylthiohydantoin amino acid analysis by sequentially cleaving N-terminal amino acids from protein/peptide chains and analyzes the resulting phenylthiohydantoin amino acid residues. Sample is applied on a solid support either on a glass-fiber disk or bound onto a polyvinylidenedifluoride disk using the ProSorb Sample Preparation Cartridge. The Procise 490 Protein Sequencer used the following reagents and solvents: 5% phenylisothiocyanate in n-heptane; n-methylpiperidine/H₂O/MeOH; 100% TFA; 1:3 TFA/H₂O with 0.1% dithiothreitol; EtOAc; n-butyl chloride; 1:4 MeCN/H₂O. The phenylthiohydantoin amino acid analysis was performed by the on-line Model 140C Microgradient Delivery System which is a dual-syringe, gradient programmable microbore HPLC system using a Spheri-5 PTH 5 µm, 220 X 2.1 mm column from ABI at 55°C, with 3.5% THF/H₂O

with 20 mL of Premix; Buffer Concentrate and 1% (CH₃)₂CO per L and 22:3 MeCN/isopropanol at a flow rate of 325 uL/min. The phenylthiohydantoin amino acid residues were detected at 269 nm using a Perkin-Elmer Series 200 UV/VIS Detector and a Macintosh computer (Apple Power Mac G4 with Mac OS 9.2) equipped with Procise control software and the Model 610A Data Analysis software.

Footnotes

1. Performed at the Advanced Protein Technology Centre, Amino Acid Analysis & Protein Sequencing Facility, Hospital for Sick Children, Rm. 8429 Gerrard Wing, 555 University Avenue, Toronto, Ontario, M5G 1X8, Canada.

Table 1. ^1H NMR Chemical Shifts (ppm) for neopetrosiamide A (**1**) recorded in 4:1 MeCN- d_3 /H $_2$ O at 600 MHz.

Residue #	NH	αH	βH	γH	δH	Others ^a
L-Phe¹	8.11 br	4.39	3.23 3.42		7.24 7.24	C ϵH : 7.23, 7.23 C ζH : 7.08
L-Phe²	8.83 d, $J = 3.4$ Hz	4.94	3.00 3.21		7.31 7.31	C ϵH : 7.29, 7.29 C ζH : 7.16
L-Cys³	7.94 d, $J = 9.7$ Hz	4.94	2.51 dd, $J = 11.2, 4.4$ Hz 3.02			
trans L-Pro⁴		3.67	0.95 1.81	1.45-1.50 1.45-1.50	2.04 m 3.36	
L-Phe⁵	8.19	4.64 m	2.57 3.39		7.10 7.10	C ϵH : 7.19, 7.19 C ζH : 7.15
Gly⁶	7.26	3.89 4.03 dd, $J = 17.1, 5.3$ Hz				
L-Cys⁷	8.08	4.32	2.78 3.04			
L-Ala⁸	7.25	4.05	1.37 d, $J = 7.0$ Hz			
L-Leu⁹	6.65 br	4.45	1.43 1.88	1.47	0.72 d, $J = 6.2$ Hz 0.74 d, $J = 6.4$ Hz	
L-Val¹⁰	7.21	3.98 t, $J = 9.1$ Hz	2.22 m	0.91 d, $J = 6.1$ Hz 0.97 d, $J = 6.4$ Hz		
L-Asp¹¹	8.06 d, $J = 9.4$ Hz	4.88 m	2.61 m 2.91			
L-Cys¹²	8.92 d, $J = 4.4$ Hz	4.53 m	3.09-3.14 3.09-3.14			
Gly¹³	7.87 bs	3.72 4.34				
trans L-Pro¹⁴		4.19 m	1.82 2.11	1.92 1.92	3.47 3.55	
L-Asn¹⁵	8.33 d, $J = 7.3$ Hz	4.47	2.72 2.82			NH $_2$: 7.14, 6.48 bs
L-Arg¹⁶	7.05 d, $J = 10.4$ Hz	4.71 m	1.45 1.60	1.42-1.50 1.42-1.50	3.09-3.14 3.09-3.14	NeH: 7.11
trans L-Pro¹⁷		4.39	1.72 m 2.08	1.85 1.85	3.53 3.53	
L-Cys¹⁸	8.23 d, $J = 7.0$ Hz	4.94	2.88 2.93			
L-Arg¹⁹	8.85 d, $J = 9.5$ Hz	4.58 m	1.56 1.56	1.45 1.90	3.05 3.05	NeH: 7.10

L-Asp²⁰	8.48	5.01 ddd, $J = 7.8, 7.8, 7.8$ Hz	2.76 2.94			
L-Thr²¹	8.47	4.27 t, $J = 9.7$ Hz	4.30	0.91 d, $J = 6.1$ Hz		<u>OH</u> : 8.20
Gly²²	7.58 bs	3.68 4.09 dd, $J = 17.8, 5.9$ Hz				
L-Phe²³	8.44 d, $J = 5.1$ Hz	4.35	2.93 3.00		7.21-7.23 7.21-7.23	<u>CεH</u> : 7.28, 7.28 <u>CζH</u> : 7.21
L-MetO²⁴	8.37 d, $J = 6.6$ Hz	3.81	1.96 2.14	1.65 m 2.42		<u>CεH</u> : 2.45 s
L-Ser²⁵	7.23	5.05 m	3.30 m 3.30 m			
L-Cys²⁶	8.49	5.59 t, $J = 10.7$ Hz	1.78 2.30 bd, $J = 13.9$ Hz			
L-Asp²⁷	8.63 d, $J = 8.1$ Hz	4.76 m	2.60 2.72			
L-Cys²⁸	8.17	5.36 t, $J = 9.5$ Hz	2.82 3.20			

^aTwo broad resonances are also observed centered at 6.44 and 6.79 ppm which are likely attributable to the Arg¹⁶ and Arg¹⁹ terminal guanidine NH and NH₂ resonances &/or the three Asp carboxyl OH resonances and the terminal Cys²⁸ carboxyl OH resonance.

Table 2. ^{13}C NMR Chemical Shifts (ppm) for neopetrosiamide A (**1**) recorded in 4:1 MeCN- d_3 /H $_2$ O at 150 MHz.

Residue #	$\underline{\text{C}}\text{O}$	$\underline{\text{C}}\alpha$	$\underline{\text{C}}\beta$	$\underline{\text{C}}\gamma$	$\underline{\text{C}}\delta$	Others
L-Phe ¹	169.8	54.3	37.7 ^a	134.2	131.9	129.7 ($\underline{\text{C}}\epsilon$), 128.7 ($\underline{\text{C}}\zeta$)
L-Phe ²	172.1 ^b	55.8 ^c	38.0	136.6	130.2	129.6 ($\underline{\text{C}}\epsilon$), 128.4 ($\underline{\text{C}}\zeta$)
L-Cys ³	170.9	50.0	41.2			
<i>trans</i> L-Pro ⁴	173.6	62.6	29.6	25.0	48.1	
L-Phe ⁵	174.3 ^d	53.9	37.6 ^a	139.1	130.4	129.3 ($\underline{\text{C}}\epsilon$), 127.6 ($\underline{\text{C}}\zeta$)
Gly ⁶	173.1 ^e	43.7				
L-Cys ⁷	174.0	55.9 ^c	37.8 ^a			
L-Ala ⁸	175.2	53.1	17.4			
L-Leu ⁹	173.6	52.2 ^f	40.7	26.2	21.8, 23.5	
L-Val ¹⁰	172.8	60.4	32.3	21.0, 21.3		
L-Asp ¹¹	172.9 ^g	49.9	37.0	174.9		
L-Cys ¹²	173.0 ^g	53.9	38.5			
Gly ¹³	169.9	42.7 ^h				
<i>trans</i> L-Pro ¹⁴	174.9	62.5	30.2	25.7	47.7	
L-Asn ¹⁵	172.1 ^b	52.0 ^f	37.3	175.5		
L-Arg ¹⁶	170.8	51.3	30.2	25.2	42.2	158.2 ($\text{C}\zeta$)
<i>trans</i> L-Pro ¹⁷	173.8	60.6	30.5	25.3	48.6	
L-Cys ¹⁸	172.2 ^b	55.3	37.1 ⁱ			
L-Arg ¹⁹	172.9 ^g	53.1	31.3	25.5	41.8	158.0 ($\text{C}\zeta$)
L-Asp ²⁰	173.6	51.3	37.3 ⁱ			
L-Thr ²¹	171.8	59.6	67.4	20.5		
Gly ²²	171.3	42.6 ^h				
L-Phe ²³	173.2 ^e	57.4	36.4	137.7	130.5	129.8 ($\underline{\text{C}}\epsilon$), 128.1 ($\underline{\text{C}}\zeta$)
L-MetO ²⁴	172.5	55.0	23.6	50.9		38.7 ($\underline{\text{C}}\epsilon$)
L-Ser ²⁵	172.3	55.3	63.5			
L-Cys ²⁶	170.4	55.8	38.6			
L-Asp ²⁷	172.0	50.5	40.1	174.4 ^d		
L-Cys ²⁸	174.3 ^d	51.2	37.7 ^a			

^{a-i} Assignments are interchangeable

Table 3. ^1H NMR Chemical Shifts (ppm) for neopetrosiamide B (**2**) recorded in 4:1 MeCN- d_3 /H $_2$ O at 600 MHz.

Residue #	NH	αH	βH	γH	δH	Others ^a
L-Phe¹	8.14 br	4.41	3.22 3.41		7.25 7.25	C ϵH : 7.23, 7.23 C ζH : 7.07
L-Phe²	8.86 d, $J = 2.9$ Hz	4.90	2.98 3.19		7.29 7.29	C ϵH : 7.28, 7.28 C ζH : 7.18
L-Cys³	7.78 d, $J = 9.7$ Hz	4.94	2.50 2.98			
trans L-Pro⁴		3.67	0.94 1.82	1.63 1.63	2.41 m 3.48	
L-Phe⁵	8.22	4.60 m	2.53 3.37		7.09 7.09	C ϵH : 7.20, 7.20 C ζH : 7.15
Gly⁶	7.08	3.91 4.01				
L-Cys⁷	8.17 d, $J = 3.2$ Hz	4.27	2.77 3.02			
L-Ala⁸	8.06 d, $J = 7.5$ Hz	4.03	1.35 d, $J = 7.0$ Hz			
L-Leu⁹	6.58 br	4.47	1.45 1.90	1.47	0.71 d, $J = 6.1$ Hz 0.73 d, $J = 6.1$ Hz	
L-Val¹⁰	7.23	3.98	2.23 m	0.92 d, $J = 6.4$ Hz 0.97 d, $J = 5.9$ Hz		
L-Asp¹¹	8.08 d, $J = 9.4$ Hz	4.89 m	2.61 2.92			
L-Cys¹²	8.96 d, $J = 6.6$ Hz	4.51 m	3.09-3.15 (3.12) 3.09-3.15 (3.12)			
Gly¹³	7.86 br	3.72 4.34				
trans L-Pro¹⁴		4.19	1.81 2.12	1.92 1.92	3.48 3.54	
L-Asn¹⁵	8.34 d, $J = 7.2$ Hz	4.45	2.71 2.83			NH $_2$: 7.17, 6.50 bs
L-Arg¹⁶	7.05	4.70 m	1.45 1.58	1.48-1.50 1.48-1.50	3.09-3.12 3.09-3.12	N ϵH : 7.08
trans L-Pro¹⁷		4.39	1.71 2.09	1.87 1.87	3.53 3.53	
L-Cys¹⁸	8.22 d, $J = 6.8$ Hz	4.90	2.87 2.92			
L-Arg¹⁹	8.92 d, $J = 9.2$ Hz	4.57 m	1.53-1.55 1.53-1.55	1.43 1.43	3.03 3.03	N ϵH : 7.09

L-Asp²⁰	8.51	4.99 m	2.76 2.92 d, $J = 5.7$ Hz			
L-Thr²¹	8.45 d, $J = 9.4$ Hz	4.28 t	4.30	0.88 d, $J = 5.9$ Hz		
Gly²²	7.61 bs	3.71 4.09				
L-Phe²³	8.52	4.31	2.95 3.02		7.23 7.23	CεH: 7.33, 7.33 CζH: 7.26
L-MetO²⁴	8.53	3.82	1.90 2.10	1.75 m 2.14		CεH ₃ : 2.47 s
L-Ser²⁵	7.23	5.02 m	3.20 m 3.27 m			
L-Cys²⁶	8.40 d, $J = 9.7$ Hz	5.54 t, $J = 10.9$ Hz	1.67 2.23 m			
L-Asp²⁷	8.63 d, $J = 8.1$ Hz	4.77 m	2.55 2.69			
L-Cys²⁸	8.17	5.36 t, $J = 9.5$ Hz	2.82 3.20			

^aTwo broad resonances are also observed centred at 6.43 and 6.77 ppm which are likely attributable to the Arg¹⁶ and Arg¹⁹ terminal guanidine NH and NH₂ resonances &/or the three Asp carboxyl OH resonances and the terminal Cys²⁸ carboxyl OH resonance.

Table 4. ^{13}C NMR Chemical Shifts (ppm) for neopetrosiamide B (**2**) recorded in 4:1 MeCN- d_3 /H $_2$ O at 150 MHz.

Residue #	$\underline{\text{C}}\text{O}$	$\underline{\text{C}}\alpha$	$\underline{\text{C}}\beta$	$\underline{\text{C}}\gamma$	$\underline{\text{C}}\delta$	Others
L-Phe ¹	169.6	54.1	37.6 ^a	134.1	131.9	129.5 ($\underline{\text{C}}\epsilon$), 128.7 ($\underline{\text{C}}\zeta$)
L-Phe ²	172.3 ^b	56.2	37.9 ^c	136.7	130.5	129.6 ($\underline{\text{C}}\epsilon$), 128.4 ($\underline{\text{C}}\zeta$)
L-Cys ³	171.9	49.7 ^d	40.8			
<i>trans</i> L-Pro ⁴	173.3	62.6	29.5	25.0	48.3	
L-Phe ⁵	174.3 ^e	53.8	37.8 ^c	139.0	130.4	129.2 ($\underline{\text{C}}\epsilon$), 127.5 ($\underline{\text{C}}\zeta$)
Gly ⁶	173.3 ^f	43.7				
L-Cys ⁷	173.9	55.9	37.6 ^a			
L-Ala ⁸	175.3	53.2	17.2			
L-Leu ⁹	173.7	52.2 ^g	40.6	26.0	21.7, 23.4	
L-Val ¹⁰	172.6	60.3	32.3	21.3, 21.4		
L-Asp ¹¹	173.3 ^f	49.7 ^d	36.9 ^h	175.0		
L-Cys ¹²	172.9 ⁱ	53.8	38.3			
Gly ¹³	169.9	42.6				
<i>trans</i> L-Pro ¹⁴	175.0	62.4	30.2	25.7	47.7	
L-Asn ¹⁵	172.1 ^b	51.8 ^g	37.2	175.6		
L-Arg ¹⁶	170.6	51.3	30.1	25.2	42.1	158.1 ($\text{C}\zeta$)
<i>trans</i> L-Pro ¹⁷	173.2 ⁱ	60.5	30.4	25.1	48.5	
L-Cys ¹⁸	172.1 ^b	55.3	37.1 ^h			
L-Arg ¹⁹	173.2 ⁱ	53.0	31.2	25.5	41.7	157.9 ($\text{C}\zeta$)
L-Asp ²⁰	173.6	51.2	37.2 ^h	174.3 ^e		
L-Thr ²¹	171.6	59.3	67.1	20.4		
Gly ²²	171.2	42.6				
L-Phe ²³	173.6 ⁱ	57.9	36.4	137.4	130.3	130.2 ($\underline{\text{C}}\epsilon$), 128.1 ($\underline{\text{C}}\zeta$)
L-MetO ²⁴	172.6	55.1	24.0	50.9		38.4 ($\underline{\text{C}}\epsilon$)
L-Ser ²⁵	172.3 ^b	55.2	63.2			
L-Cys ²⁶	170.1	55.8	38.5			
L-Asp ²⁷	172.0	50.6	40.2	174.3 ^e		
L-Cys ²⁸	174.3 ^e	51.0	37.6 ^a			

^{a-i} Assignments are interchangeable

MALDITOF-MS of neopetrosiamide A (**1**).

600MHz ^1H NMR Spectrum of Neopetrosiamide A (1) in 4:1 MeCN- $\text{d}_3/\text{H}_2\text{O}$

150MHz ^{13}C NMR Spectrum of Neopetrosiamide A (1) in 4:1 MeCN- d_3 /H $_2\text{O}$

600MHz COSY60 NMR Spectrum of Neopetrosiamide A (1) in MeCN-d₃/H₂O

600MHz HMBC NMR Spectrum of Neopetrosiamide A (1) in MeCN-d₃/H₂O

600MHz HSQC NMR Spectrum of Neopetrosiamide A (1) in MeCN-d₃/H₂O

600MHz NOESY NMR Spectrum of Neopetrosiamide A (1) in MeCN-d₃/H₂O

Expanded 600MHz NOESY NMR Spectrum of Neopetrosiamide A in 4:1 MeCN-d₃/H₂O

SEQUENCING ANALYSIS SUMMARY

Dr. David Williams
University of Toronto
Biochemistry

Customer Code
Williams

Sample Name
PNG03-408:BuOH-A-B-1-3

Sample Code
P022205CA

Analysis Date
February 22, 2005

Residue: Amino Acid: Comments:

Residue 1 F - Phe

Residue 2 F - Phe

Residue 3 Cys-SH possibly

Residue 4 P - Pro

Residue 5 F - Phe

Residue 6 G - Gly

Residue 7 Cys-SH possibly

Residue 8 A - Ala

Residue 9 L - Leu

Residue 10 V - Val

SEQUENCING ANALYSIS SUMMARY

Dr. David Williams
University of Toronto
Biochemistry

Customer Code

Williams

Sample Name

PNG03-408;BuOH-A-B-1-3

Sample Code

P022205CA

Analysis Date

February 22, 2005

Residue: Amino Acid: Comments:

Residue 11 D - Asp

Residue 12 Cys-SH possibly

Residue 13 G - Gly

Residue 14 P - Pro

Residue 15 N - Asn

Residue 16 R - Arg

Residue 17 P - Pro

Residue 18 Cys-SH possibly

Residue 19 R - Arg

Residue 20 D - Asp

SEQUENCING ANALYSIS SUMMARY

Dr. David Williams
University of Toronto
Biochemistry

Customer Code

Williams

Sample Name

PNG03-408:BuOH-A-B-1-3

Sample Code

P022205CA

Analysis Date

February 22, 2005

Residue: **Amino Acid:** **Comments:**

Residue 21 T - Thr

Residue 22 G - Gly

Residue 23 F - Phe

Residue 24 M - Met

Residue 25 S - Ser

Residue 26 Cys-SH possibly

Residue 27 D - Asp

Residue 28 Cys-SH possibly

Residue 29

Residue 30

600 MHz ^1H NMR Spectrum of Neopetrosiamide B (2) in 4:1 MeCN- $\text{d}_3/\text{H}_2\text{O}$

150 MHz ^{13}C NMR Spectrum of Neopetrosiamide B (2) in 4:1 MeCN- $\text{d}_3/\text{H}_2\text{O}$