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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 D. 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, and Discipline of Pharmacology, School of Medicine and Health Sciences, University of Papua New Guinea, N.C.D., Papua New Guinea

randersn@interchange.ubc.ca

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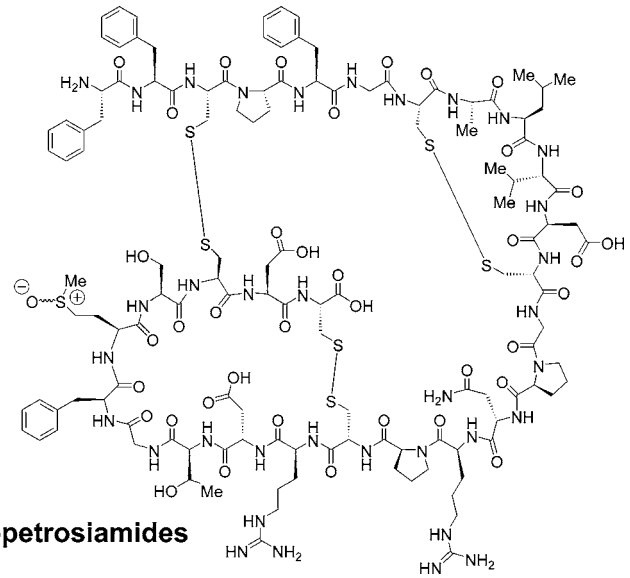
ABSTRACT



Neopetrosiamdes A (1) and B (2), two diastereomeric tricyclic peptides that inhibit amoeboid invasion of human tumor cells, have been isolated from the marine sponge *Neopetrosia* sp. collected in Papua New Guinea. The structures of the neopetrosiamides were elucidated by analysis of MS and NMR data and confirmed by chemical degradation.

Metastasis, the often lethal spread of cancer cells from a primary tumor to remote sites in the body, requires that the cells invade and migrate though the extracellular matrix (ECM).[1−3] There appear to be two distinct mechanisms that tumor cells employ to move through ECM barriers. Mesenchymal migration involves a path-generating process in which the cells use matrix metaloproteinases (MMPs) and serine proteases to degrade the ECM and clear the way for

[†] Chemistry and EOS, University of British Columbia.
[‡] Biochemistry and Molecular Biology, University of British Columbia.
[§] Cellular and Physiological Sciences, University of British Columbia.
[||] University of Amsterdam.
[⊥] University of Papua New Guinea.

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forward motion. Amoeboid migration involves a path-finding process where cells change their shape to move through existing spaces in the ECM rather than enzymatically creating a new path. It has been shown in vitro that if the activity of ECM degrading proteases is inhibited by drugs, tumor cells can switch from mesechymal invasion to amoeboid invasion. Removal of the protease inhibitors in wash-out experiments results in reversion to mesenchymal invasion. This mesenchymal to ameoboid transition (MAT) has been offered as a rationale for the relative lack of efficacy of MMP inhibitors in mouse models of metastasis and in clinical trials. Tumor cells faced with the blockage of one form of migration might simply switch to the other.

The discovery of MAT has shown that simultaneous inhibition of both forms of tumor cell motility will likely be required to effectively control metastasis in vivo.[3] Small molecule MMP inhibitors[4] that effectively block mesenchymal migration in vitro[3] are known. In contrast, there are no documented small molecule inhibitors of amoeboid migration.

Recently, we described the development of a cell-based assay[5] that has proven effective for discovering novel inhibitors of mesenchymal tumor cell invasion and migration.[6] A modification of this assay employing LS174T human colon cancer cells, that are known to migrate exclusively via the amoeboid form, provided a screening method to detect noncytotoxic inhibitors of amoeboid invasion of the ECM. This modified assay has been used to screen a library of marine invertebrate extracts for natural product inhibitors of amoeboid invasion. Crude MeOH extracts of the marine sponge *Neopetrosia* sp. (order Haplosclerida, family Petrosiidae)[7] collected in Papua New Guinea showed promising activity in the assay. Bioassay guided fractionation of the extracts identified the tricyclic peptides neopetrosiamides A (**1**) and B (**2**) as the compounds responsible for inhibition of amoeboid invasion. Details of the isolation and structure elucidation of the neopetrosiamides are presented below.

Specimens of *Neopetrosia* sp. (450 g wet wt) were harvested by hand using scuba near Milne Bay, Papua New Guinea. Freshly collected sponge samples were frozen on site and transported to Vancouver over dry ice. Thawed sponge tissue was repeatedly extracted with MeOH (4 × 500 mL) and the combined MeOH extracts were concentrated in vacuo to give a brown gum. The gum was suspended in water (400 mL), and the aqueous suspension was sequentially extracted with EtOAc (3 × 100 mL) and *n*-BuOH (4 × 100 mL). Antiinvasive activity was observed in the *n*-BuOH soluble fraction. Bioassay-guided separation of the *n*-BuOH soluble materials via sequential application of Sephadex LH-20 chromatography (eluent: MeOH), flash reversed-phase column chromatography (eluent: step gradient from 1:1 MeOH/H_2O to MeOH), and repetitive reversed-phase HPLC [first eluent: 3:7 MeCN/(0.05% TFA/H_2O); second eluent: 3:2 MeOH/(0.05% TFA/H_2O)] gave pure samples of neopetrosiamides A (**1**) and B (**2**).

Neopetrosiamide A (**1**) was obtained as an optically active clear glass ($[\alpha]_D = -65.2$ (*c* 4.2, MeOH)). The MALDITOF-MS of **1** gave an $[M + H]^+$ ion at *m*/*z* 3071, indicating a molecular weight of 3070. Peptide **1** gave a sharp well-resolved peak when analyzed by reversed-phase HPLC using a variety of solvent systems and it gave a single clean molecular ion in the MALDITOF-MS. However, in numerous NMR solvents (e.g., MeOH-d_4, DMSO-d_6, MeCN-d_3, etc.) many or all of the resonances in the ^{1}H and ^{13}C NMR spectra of **1** were broadened, resulting in poorly resolved spectra that were not suitable for structural studies. Attempts to simplify the NMR spectra by heating (to 50 °C) or adjusting the pH by addition of TFA or Et_3N did not alleviate the problem. Eventually, it was found that acceptable spectra, with only a single set of well-resolved resonances, could be obtained using 4:1 MeCN-d_3/H_2O as the NMR solvent.

The ^{1}H/^{13}C/DEPT/HSQC NMR data recorded for **1** in 4:1 MeCN-d_3/H_2O identified 129 carbon atoms. Both the ^{1}H and ^{13}C NMR spectra (Supporting Information) of **1** had features that suggested a peptide structure. Detailed analysis of the COSY, HSQC, and HMBC NMR data (Tables 1 and 2, Supporting Information) showed that neopetrosiamide A (**1**) contained one alanine, two arginine, one asparagine, three aspartic acid, three glycine, one leucine, one methionine sulfoxide, four phenylalanine, three proline, one serine, one threonine, and one valine residues, plus six 3-substituted -2-aminopropionic acid residues, for a total of 28 amino acids. The ^{13}C NMR resonances assigned to the substituted methylenes of the 2-aminopropionic acid residues had chemical shifts (δ 37.1−41.2 ppm) appropriate for *S* substitution.

Standard amino acid analysis, with and without performic acid oxidation prior to hydrolysis (to convert cysteine and cystine to cysteic acid), confirmed the presence of the amino acids identified by NMR. In both of these analyses, Asn was converted to Asp, and in the analysis without prior oxidation, methionine sulfoxide was converted to methionine. A control experiment showed that under the hydrolytic conditions used in the amino acid analysis methionine sulfoxide is converted to methionine and cystine is partially converted to cysteic acid if excess cystine is present. Marfey's analysis of the 6 N HCl hydrolysate of neopetrosiamide A (**1**) showed that all of the amino acids had the L configuration. In the Marfey's analysis, Asn was detected as Asp, methionine sulfoxide was detected as methionine, and cysteic acid was detected along with cystine.

The 28 amino acids present in neopetrosiamide A (**1**) contain 129 carbon atoms, which is equivalent to the number identified from the NMR data. A linear peptide constructed

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(7) A voucher sample has been deposited at the Zoological Museum, University of Amsterdam (ZMA POR 18339).

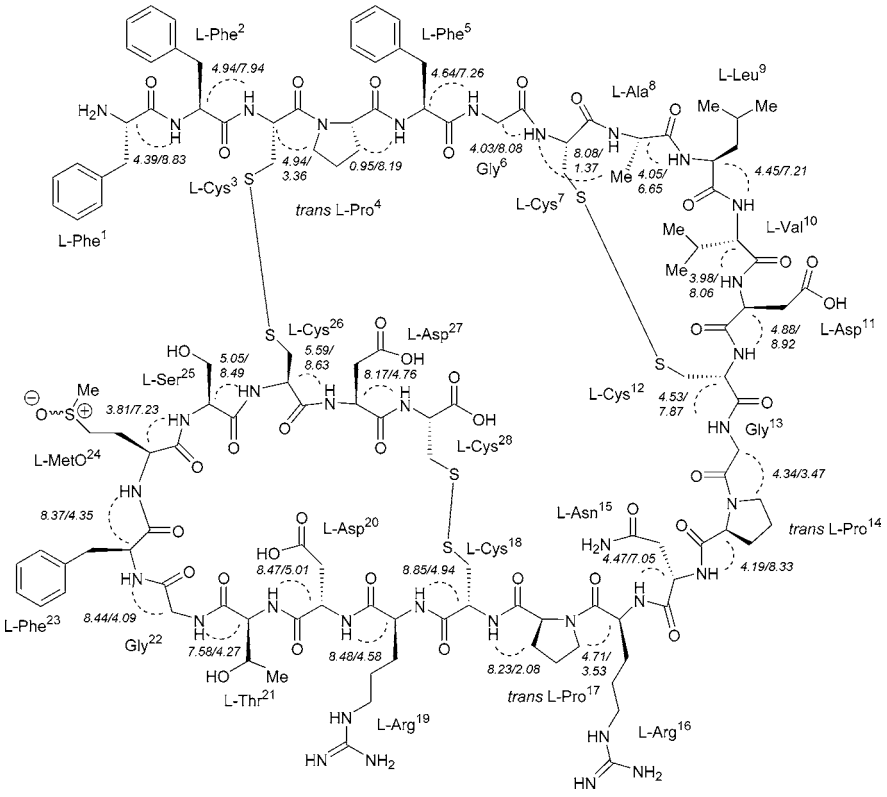

Figure 1. Structure of the diastereomeric neopetrosiamides A and B, which differ only in the configuration at the sulfoxide functionality of the methionine sulfoxide residue (#24), showing the NOESY correlations (dashed arcs, chemical shifts in italics) used to sequence neopetrosiamide A (**1**).

from these 28 amino acids and having three disulfide bridges has a molecular formula of $C_{129}H_{183}N_{35}O_{39}S_7$. The molecular weight of such a linear peptide is 3070.146, in agreement with with the MALDITOF-MS data observed for neopetrosiamide A (**1**). Therefore, it was assumed that neopetrosiamide A (**1**) was a linear peptide with three disulfide bridges.

The sequence of the amino acids in the linear peptide was established by detailed interpretation of NOESY data as shown in Figure 1 and corroborated by HMBC correlations. N−Terminal-initiated Edman degradation sequencing confirmed the NMR-derived amino acid sequence (Supporting Information). NOESY data also established the positions of the disulfide linkages. Correlations observed between one of the β-methylene protons of the Cys^3 residue (δ 2.51) and both the β-methylene protons of Cys^{26} (δ 1.78 and 2.30) and the Cys^{26} NH resonance (δ 8.49) demonstrated the presence of a disulfide bridge between Cys^3 and Cys^{26}. A relatively strong NOESY correlation between the Arg^{19} NH resonance (δ 8.85) and the Asp^{27} NH (δ 8.63) resonance, as well as a weaker correlation between the Arg^{19} NH resonance (δ 8.85) and the Cys^{28} α-methine resonance (δ 5.36), identified a disulfide bridge between Cys^{18} and Cys^{28}. As outlined above, to account for the molecular weight of 3070 measured for neopetrosiamide A in the MALDITOF-MS, there had to be a third disulfide bridge between Cys^7 and Cys^{12}. A weak NOESY correlation observed between the Cys^7 (δ 8.08) and Gly^{13} (δ 7.87) NH resonances supported this assignment.

The NMR data for neopetrosiamide A (**1**) established that the Pro^4, Pro^{14}, and Pro^{17} amide bonds were all trans.[8] 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: (Pro^4 δ Cγ 25.0 ppm, $\Delta\delta_{C\beta-C\gamma} = 4.6$ ppm; Pro^{14} δ Cγ 25.7 ppm, $\Delta\delta_{C\beta-C\gamma} = 4.5$ ppm; Pro^{17} δ Cγ 25.3 ppm, $\Delta\delta_{C\beta-C\gamma} = 5.2$ ppm). NOESY correlations observed between the Cys^3-Hα (δ 4.94)/Pro^4-Hδ_A(δ 2.04), Pro^4-Hδ_B(δ 3.36), between Gly^{13}-Hα_B(δ 4.34)/Pro^{14}-Hδ_A(δ 3.47),Pro^{14}-Hδ_B(δ 3.55), and between Arg^{16}-Hα(δ 4.71)/Pro^{17}-Hδ_A(δ 3.53) &/or Pro^{17}-Hδ_B(δ 3.53) supported the assignment of the trans geometry for the three proline amide linkages. Therefore, the complete structure for neopetrosiamide A is as shown in Figure 1.

Neopetrosiamide B (**2**) was also obtained as an optically active clear glass with the same molecular weight as neopetrosiamide A (**1**). Detailed analysis of the 1D and 2D NMR data obtained for neopetrosiamide B (**2**) (Tables 3 and 4, Supporting Information) indicated that the gross structures of neopetrosiamides A and B were identical. This conclusion was confirmed by the results of amino acid and Marfey

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analyses carried out on **2**. The ^{1}H and ^{13}C NMR spectra for the two peptides were almost superimposable, except for minor chemical shift differences observed in the signals for residues 21−27. The most notable NMR chemical shift differences were found in the methionine sulfoxide residue 24 [neopetrosiamide A (**1**): ^{1}H δ 8.37 (N*H*), 3.81 (α*H*), 1.96/2.14 (β*H*), 1.65/2.42 (γ*H*), 2.45 (SO*Me*); neopetrosiamide B (**2**): 8.53 (N*H*), 3.82 (α*H*), 1.90/2.10 (β*H*), 1.75/2.14 (γ*H*), 2.47 (SO*Me*)]. Therefore, it was apparent that the two peptides differed only by being epimeric at the sulfoxide functionality of the methionine sulfoxide residue. This difference does not show up in the amino acid or Marfey's analyses since, as described above, the methionine sulfoxide is detected as methionine in these degradation experiments.

Marine sponge extracts have been an extremely rich source of complex and frequently bioactive peptides.[9] Novel structural features commonly found in these sponge peptides include D-amino acids, *N*-methylated amino acids, α- or β-methylated amino acids, halogenated amino acids, a wide variety of amino acids with unprecedented extended carbon skeletons and/or amino groups at other than the α carbons, and polyketide derived N-acyl residues. It has been suggested that many of the peptides isolated from sponge extracts are actually biosynthetic products of microorganisms living in association with the source sponge.[10]

The neopetrosiamides share some features with other sponge-derived peptides, but they also represent an interesting structural departure for this group. They are present in the sponge extract at very high concentrations (0.55% dry wt), which is typical of many sponge peptides such as motuporin,[11] the papuamides,[12] and dominicin[13] previously isolated by our group. In addition, they contain only standard L amino acids such as the axinastatins, phakellestatins, hymenamides, and dominicin, although their core peptide is linear instead of cyclic like the others.[9a,13] The feature that really sets the neopetrosiamides apart from other sponge peptides is the presence of the disulfide linkages, which create a tricyclic structure. To the best of our knowledge,[9] the only other sponge peptides with a disulfide bond that generates a cyclic structure are the microcionamides and kendarimide A,[14,15] which are much smaller (7 and 11 versus 28 amino acids), have only one disulfide, and are only monocyclic.

Neopetrosiamides A (**1**) and B (**2**) were active in the amoeboid invasion assay at 6 μg/mL. These new peptides have the potential to be cell biology tools that can be enlisted to find drug targets for inhibiting amoeboid invasion of tumor cells.

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Supporting Information Available: Experimental section, ^{1}H and ^{13}C NMR spectra, and tables of chemical shift values for neopetrosiamides A and B. This material is available free of charge via the Internet at http://pubs.acs.org.

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