J|A|C|S COMMUNICATIONS

Published on Web 01/14/2010

Chemical Synthesis and Biological Activity of the Neopetrosiamides and Their Analogues: Revision of Disulfide Bond Connectivity

Hongqiang Liu,[†] Marc A. Boudreau,[†] Jing Zheng,[†] Randy M. Whittal,[†] Pamela Austin,[‡] Calvin D. Roskelley,[§] Michel Roberge,[‡] Raymond J. Andersen,[||] and John C. Vederas*,[†]

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2, and Departments of Biochemistry and Molecular Biology, Cellular and Physiological Sciences, and Chemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1

Received December 5, 2009; E-mail: john.vederas@ualberta.ca

Marine organisms continue to be investigated as a source of potential therapeutic compounds.[1] Recently, Williams et al.[2] reported the isolation and structures of neopetrosiamides A and B (**1**) from a marine sponge (*Neopetrosia* sp.) collected in Papua New Guinea. They are potent inhibitors of human tumor cell invasion in cancer metastasis at 6 μg/mL. The two peptides are diastereomeric, differing only in the stereochemistry of the methionine sulfoxide at position 24, and contain three intramolecular cross-linking disulfides, as determined by 2D NMR spectroscopy and mass spectrometry. The important bioactivity and complexity of the disulfide connectivity prompted us to undertake the chemical synthesis of the natural peptides and their analogues wherein the methionine sulfoxide was replaced with norleucine or methionine. Such replacement can simplify the synthesis and elucidate the role of this residue in bioactivity.[3] The present synthesis of the proposed structures demonstrated that the disulfide bond connectivity in the neopetrosiamides required reassignment and that the correct connectivity is as shown in structure **2** (Figure 1). This is a rare example of disulfide bond connectivity revision in a disulfide-rich peptide using chemical synthesis.[4] The work provides a cautionary note about the common practice of peptide synthesis using global deprotection of many cysteine residues followed by formation of multiple disulfides through oxidation.

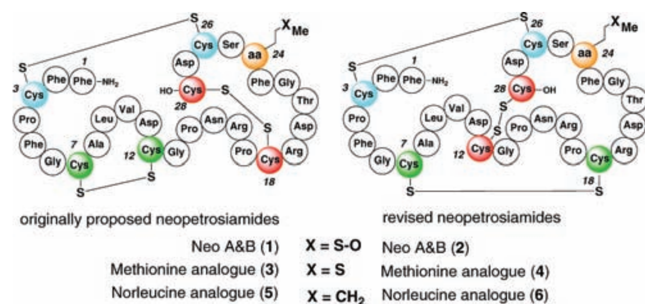
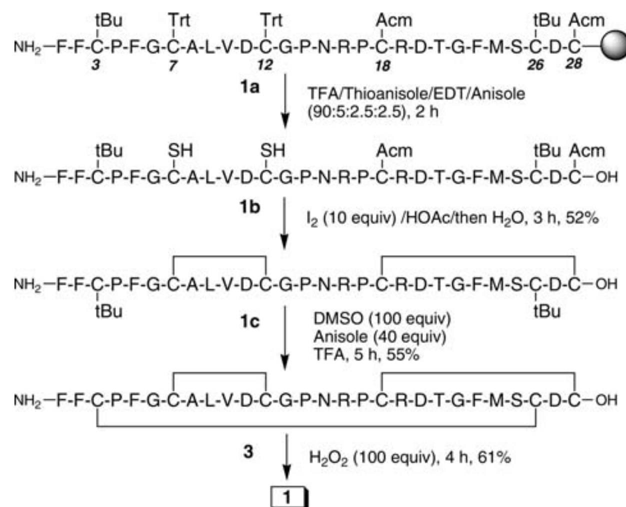


Figure 1. Originally proposed (**1**) and revised (**2**) structures of neopetrosiamides A and B and their analogues.

Chemical synthesis of multiple-disulfide-containing peptides usually involves either directed oxidative folding from a polythiol precursor or, rarely, stepwise introduction of disulfide bonds using orthogonal protection of cysteine residues.[5] Our initial approach involved solid-supported peptide synthesis (SSPS) using Fmoc methodology with acid-labile side-chain protecting groups followed by global deprotection and oxidative folding of a hexathiol precursor with a Cys/Cys_2 redox system. Subsequent oxidation of the methionine at position 24 with hydrogen peroxide[6] gave what appeared to be the desired native disulfide-bond isomer. However, this was accompanied by the production of significant amounts of several other non-native isomers.[7] Therefore, the synthetic strategy was changed to the stepwise formation of disulfide bonds from a linear peptide with orthogonal protecting groups on the cysteines ($Cys^{3,26}$, tBu; $Cys^{7,12}$, Trt; $Cys^{18,28}$, Acm).[8]

Scheme 1. Synthesis of the Originally Proposed Structures of Neopetrosiamides A and B



As outlined in Scheme 1, the resin-bound linear peptide with orthogonal protection on the cysteines (**1a**) was treated with trifluoroacetic acid (TFA) to provide **1b**. The first two pairs of disulfide bonds were introduced sequentially in analogy to a literature procedure.[9] The crude peptide purified by HPLC showed a dominant peak with the expected mass of 3169.3 (M + H). To verify the connectivity of the disulfide bonds in **1c**, two other possible disulfide isomers were synthesized using this method by exchanging the protecting groups on the cysteine residues. The HPLC analysis confirmed the desired disulfide connectivity in **1c**. With this bisdisulfide intermediate available, the third disulfide bond was formed using dimethyl sulfoxide (DMSO) as an oxidizing reagent, giving **3** as the major product.[8] After HPLC purification, the tricyclic peptide was treated with hydrogen peroxide to afford the sulfoxide products **1** with the expected mass of 3071.3 (M + H).[6b]

[†] Department of Chemistry, University of Alberta.
[‡] Department of Biochemistry and Molecular Biology, University of British Columbia.
[§] Department of Cellular and Physiological Sciences, University of British Columbia.
[||] Department of Chemistry, University of British Columbia.

10.1021/ja9102925 © 2010 American Chemical Society

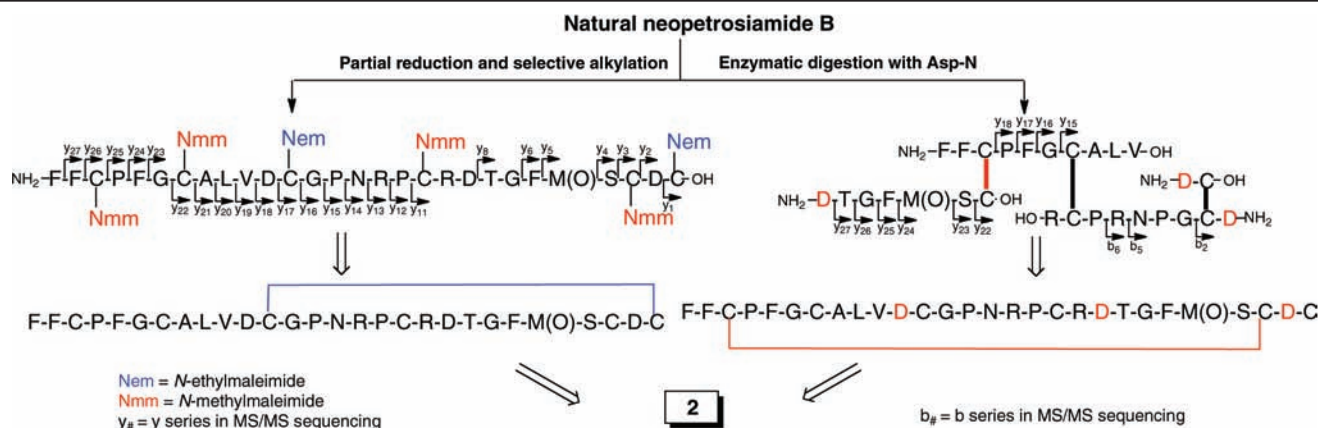


Figure 2. Disulfide mapping using reduction/alkylation of **1** followed by MS/MS analysis revealed the blue disulfide linkage (left), whereas enzymatic cleavage of **1** by an Asp (D)-specific protease and MS/MS analysis demonstrated the red disulfide (right). The results support structure **2**.

To confirm the identity of **1**, extensive HPLC analysis was done using the natural product as a standard. Surprisingly, the compounds showed different HPLC retention times. At this point, the disulfide bond connectivity of **1** was verified by partial reduction of the disulfides, alkylation of the thiol groups with *N*-ethylmaleimide (Nem), and then further reduction and reaction with *N*-methylmaleimide (Nmm).[10] The results suggested that the disulfide pattern in the originally proposed structure was incorrect. NMR studies of nuclear Overhauser effects (NOEs) between Cys residues can be misleading, as the protons in nonconnected residues can still be in close proximity.[11] Disulfide mapping of the natural product was then done using both the partial reduction method and proteolytic cleavage (Figure 2). Partial reduction with selective labeling allowed unambiguous identification of the $Cys^{12}-Cys^{28}$ bond. The remaining four cysteines could not be verified by this method, because only one partially reduced peptide was generated. MS/MS sequencing of the Asp-*N* digestion product verified the $Cys^{3}-Cys^{26}$ bond. The combination of these data suggested that the disulfide pattern of the natural peptide was different from that originally proposed.

To confirm the newly proposed structure, the chemical synthesis of **2** was undertaken using the same synthetic method. The linear peptide was synthesized via reshuffling of the protecting groups on the cysteine residues ($Cys^{3,26}$, tBu; $Cys^{7,18}$, Trt; $Cys^{12,28}$, Acm). The stepwise synthesis afforded the desired peptide with the correct disulfide pattern, which was confirmed by enzymatic digestion and MS/MS sequencing. The synthetic peptide **2** and the natural product displayed the same HPLC behavior and disulfide fingerprint pattern.[12]

At this point, the two norleucine analogues **5** and **6** were also prepared using the established methodology. Biological testing of all of the synthetic peptides was done with a cell-based assay.[2] The synthetic peptide **2** displayed the expected inhibition of human tumor cell invasion comparable to that of the natural peptide isolated from *Neopetrosia* sp. Neither **1** nor its analogues **3** and **5** showed activity, indicating a crucial role for the correct disulfide pattern. Interestingly, the synthetic precursor **4** with the unoxidized methionine was fully active, and the norleucine analogue **6** displayed only slightly reduced activity. This indicates that the sulfoxide is not essential for bioactivity.

In summary, an efficient synthesis of neopetrosiamides A and B and their analogues has been accomplished using a stepwise disulfide bond formation method. The structure of the natural peptide has been revised and confirmed by chemical synthesis. This work illustrates the importance of using controlled disulfide formation rather than oxidative folding to ensure the attainment of the correct disulfide patterns.

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada, the Alberta Heritage Foundation for Medical Research, the Canadian Cancer Society, and the Canada Research Chair in Bioorganic and Medicinal Chemistry for financial support.

Supporting Information Available: Experimental procedures, HPLC traces, and compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Blunt, J. W.; Copp, B. R.; Hu, W. P.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. *Nat. Prod. Rep.* **2009**, *26*, 170. (b) Molinski, T. F.; Dalisay, D. S.; Lievens, S. L.; Saludes, J. P. *Nat. Rev. Drug Discovery* **2009**, *8*, 69. (c) Li, J. W. H.; Vederas, J. C. *Science* **2009**, *325*, 161.
(2) Williams, D. E.; Austin, P.; Diaz-Marrero, A. R.; Van Soest, R.; Matainaho, T.; Roskelley, C. D.; Roberge, M.; Andersen, R. J. *Org. Lett.* **2005**, *7*, 4173.
(3) Derksen, D. J.; Boudreau, M. A.; Vederas, J. C. *ChemBioChem* **2008**, *9*, 1898.
(4) Selected examples of syntheses to correct peptide sequence and stereochemistry: (a) Cotton, J.; Crest, M.; Bouet, F.; Alessandri, N.; Gola, M.; Forest, E.; Karlsson, E.; Castaneda, O.; Harvey, A. L.; Vita, C.; Menez, A. *Eur. J. Biochem.* **1997**, *244*, 192. (b) Nishio, H.; Inui, T.; Nishiuchi, Y.; De Medeiros, C. L. C.; Rowan, E. G.; Harvey, A. L.; Katoh, E.; Yamazaki, T.; Kimura, T.; Sakakibara, S. *J. Pept. Res.* **1998**, *51*, 355.
(5) (a) Armishaw, C. J.; Alewood, P. F. *Curr. Protein Pept. Sci.* **2005**, *6*, 221. (b) Witt, D. *Synthesis* **2008**, 2491.
(6) (a) Escher, S. E.; Sticht, H.; Forssmann, W. G.; Rosch, P.; Adermann, K. *J. Pept. Res.* **1999**, *54*, 505. (b) Duenas, E. T.; Keck, R.; De Vos, A.; Jones, A. J. S.; Cleland, J. L. *Pharm. Res.* **2001**, *18*, 1455.
(7) See the details and HPLC traces in the Supporting Information.
(8) Schulz, A.; Kluver, E.; Schulz-Maronde, S.; Adermann, K. *Biopolymers* **2005**, *80*, 34.
(9) Soll, R.; Beck-Sickinger, A. G. *J. Pept. Sci.* **2000**, *6*, 387.
(10) Goransson, U.; Craik, D. J. *J. Biol. Chem.* **2003**, *278*, 48188.
(11) Rosengren, K. J.; Daly, N. L.; Plan, M. R.; Waine, C.; Craik, D. J. *J. Biol. Chem.* **2003**, *278*, 8606.
(12) Bulaj, G.; Zhang, M. M.; Green, B. R.; Fiedler, B.; Layer, R. T.; Wei, S.; Nielsen, J. S.; Low, S. J.; Klein, B. D.; Wagstaff, J. D.; Chicoine, L.; Harty, T. P.; Terlau, H.; Yoshikami, D.; Olivera, B. M. *Biochemistry* **2006**, *45*, 7404.

JA9102925