Isolation and Synthesis of \((-\)-(5S)-2-Imino-1-methylpyrrolidine-5-carboxylic Acid from Cliona tenuis: Structure Revision of Pyrostatins

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Received August 23, 2006

ABSTRACT

\((-\)-(5S)-2-Imino-1-methylpyrrolidine-5-carboxylic acid (1), previously reported as the N-acetyl-β-D-glucosaminidase inhibitor pyrostatin B, has been isolated from the organic extracts of the burrowing sponge Cliona tenuis. The structure of 1, including its absolute stereochemistry, was characterized from its spectral data and chemical transformations and confirmed by total synthesis. The synthesis of 1 reveals that the structure of pyrostatin B has been incorrectly assigned. Comparison of NMR spectral data strongly suggests that pyrostatins A and B are identical to 5-hydroxyectoine and ectoine, respectively.

Although substituted pyrrolidines can be found in numerous natural products and pharmaceutically active compounds,\(^1\) 2-iminopyrrolidines are not very common. Two antibacterial compounds bearing a new 2-iminopyrrolidine carboxylic acid structure were isolated recently from Burkholderia plantarii, a bacterial pathogen of rice.\(^2\) Furthermore, recent studies have shown that a series of substituted 2-iminoppyrrolidines are potent and selective inhibitors of human inducible nitric oxide synthases (NOS), and they were postulated as potential therapeutic agents.\(^3\)

Continuing our search for biologically active secondary metabolites and the possible role that these compounds play in the chemical defense of marine sponges,\(^4\) we focused our attention on the Caribbean sponge Cliona tenuis because it aggressively undermines and displaces live coral tissue and because its organic extracts showed a potent lethal activity against corals Madracis mirabilis, Montastrea cavernosa, and Siderastrea siderea in laboratory and field assays.\(^5\)


10.1021/ol062087k CCC: $33.50 © 2006 American Chemical Society

Published on Web 09/19/2006
Sponges belonging to the *Cliona* genus are usually burrowing organisms that are able to excavate a variety of calcareous substrates such as rocks, coralline reefs, and oyster shells. Previous chemical investigations on *Cliona* species led to the isolation of different classes of secondary metabolites such as steroids,\(^6\) linear peptides,\(^7\) and pyrrole alkaloids.\(^8\)

Allelopathic bioassay guided fractionation of the organic extracts of the sponge allowed us to find a very bioactive fraction from which we isolated and characterized compound 1. Analysis of the spectral data indicated that its structure is 2-imino-1-methylpyrrolidine-5-carboxylic acid, which was previously reported as pyrostatin B.\(^3\) However, the data were different from those published for pyrostatin B.\(^9\) Because of this discrepancy, the total synthesis of 2-imino-1-methylpyrrolidine-5-carboxylic acid was addressed. This allowed us to confirm our proposed structure for compound 1. On the basis of these data, we assigned compound 1 as 2-imino-1-methylpyrrolidine-5-carboxylic acid.

Sponge specimens (7 kg) were collected from the Rosario Islands in the Colombian Caribbean and extracted with MeOH and CH₂Cl₂. The methanol and dichloromethane extracts of the sponge allowed us to find a very bioactive fraction. Finally, RP-HPLC purification on a Discovery F5 column [using acetonitrile/water (8:2) and 0.05% formic acid] of 80 mg of that fraction yielded 3.5 mg of compound 1, which was slightly contaminated with some salts. To isolate this compound in a cleaner way, a portion of the same fraction (100 mg) was methylated using methanol and thionyl chloride and then purified by HPLC using acetonitrile/water (1:9) and 0.05% formic acid to afford 3.3 mg of the methyl ester derivative 2.

The prominent (+)-ESIMS pseudomolecular ion [M + H]\(^+\) at \(m/z\) 143 and the pair [M + Na]\(^+\)/[M + K]\(^+\) at \(m/z\) 165 and 181, respectively, for 1 and the corresponding [M − H]\(^−\) at \(m/z\) 141 in the (−)-ESIMS are consistent with a molecular weight of 142 amu. The (−)-HRESIMS of the pseudomolecular [M + H]\(^+\) ion at \(m/z\) 143.0819 established for 1 the molecular formula C₆H₁₁N₂O₂ (calcd 143.0815).

The \(^1\)C NMR and DEPT spectra of 1 in D₂O (Table 1) displayed six distinct resonances: two sp² quaternary carbons at \(\delta\) 169.55 ppm (C-2) and \(\delta\) 177.43 ppm (C-7), suggesting the presence of a C= N carbon and a carboxylic acid, respectively; two methylenes at \(\delta\) 24.29 ppm (C-4) and \(\delta\) 30.12 ppm (C-3); a methine at \(\delta\) 70.36 ppm (C-5); and finally a methyl group at \(\delta\) 31.74 ppm (C-6). The presence of a carboxylic acid was also confirmed from the mass peak in the (−)-ESIMS of 1 at \(m/z\) 97, corresponding to the loss of COOH.

A combination of \(^1\)H−\(^1\)H COSY, HSQC, and long-range C−H correlations (HMBC) were used to construct the molecule through quaternary carbons and the nitrogen (Figure 1). On the basis of these data, we assigned compound 1 as 2-imino-1-methylpyrrolidine-5-carboxylic acid.
Purified from the culture broth of Streptomyces isolated from a marine environment. Pyrostatin B and its glutamatic acid, which was protected as the tert-hydroxylated derivative (pyrostatin A) have been reported corresponding to the molecular formula C₁₆H₁₁N₂O₂ (calcd 307.0972).

The structure of 2-imino-1-methylpyrrolidine-5-carboxylic acid (1) was previously assigned to pyrostatin B, a compound purified from the culture broth of Streptomyces sp. SA-3501 isolated from a marine environment. Pyrostatin B and its hydroxylated derivative (pyrostatin A) have been reported and patented as useful therapeutics due to their inhibition of N-acetylglucosaminidase. However, the spectral data obtained for compound 1 did not match those reported for pyrostatin B.

To confirm our proposed structure for compound 1, we decided to carry out the synthesis of 2-imino-1-methylpyrrolidine-5-carboxylic acid. For the synthesis of 1, we employed a modification of the methodology developed by Sattur et al. for the preparation of 2-imino-1-methylazcarbocycles by using chlorosulfonyl isocyanate (Scheme 1). The synthesis started from the commercial (S)-pyroglutamic acid, which was protected as the tert-butyl ester and then N-methylated to give tert-butyl N-methyl (S)-pyroglutamate (3). Treatment of 3 with chlorosulfonyl isocyanate (CSI) and the addition of MeOH afforded tert-butyl (S)-5-(methoxysulfonylimino)-1-methylpyrrolidin-2-carboxylate (4) in 77% yield. The polarity of this intermediate facilitates its purification by silica gel chromatography. In contrast, treatment of 3 with CSI and subsequent hydrolysis with aqueous sodium hydroxide, following the methodology developed by Sattur et al., gave 1 along with the corresponding salts, a situation that made isolation difficult. Finally, removal of the protecting and methoxysulfonyl groups was achieved by treatment of 4 with TFA to give 1 in quantitative yield (Scheme 1).

The spectral data for the synthetic product are practically identical to those of compound 1 (Table 1). The NMR chemical shift data for the C-5 and C-7 positions differ slightly from each other due to the strong pH dependence of the chemical shifts of amino acids in D₂O solution.

<table>
<thead>
<tr>
<th>position/mult</th>
<th>natural compound 1</th>
<th>synthetic compound 1</th>
<th>methylated natural compound 1 (2)</th>
<th>methylated synthetic compound 1 (2)</th>
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<td>67.72</td>
<td>67.49</td>
<td>67.38</td>
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<tr>
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<td>31.74</td>
<td>31.77</td>
<td>31.84</td>
<td>31.74</td>
</tr>
<tr>
<td>7 C</td>
<td>177.43</td>
<td>174.01</td>
<td>172.35</td>
<td>172.27</td>
</tr>
<tr>
<td>OMe</td>
<td>CH₃</td>
<td></td>
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</table>

Under the solvent signal.

Under the solvent signal.

[M + H]⁺ ion at m/z 157.0974 in the (+)-HRESIMS of 2 corresponding to the molecular formula C₁₆H₁₁N₂O₂ (calcd 157.0972).

The structure of 2-imino-1-methylpyrrolidine-5-carboxylic acid (1) was previously assigned to pyrostatin B, a compound purified from the culture broth of Streptomyces sp. SA-3501 isolated from a marine environment. Pyrostatin B and its hydroxylated derivative (pyrostatin A) have been reported and patented as useful therapeutics due to their inhibition of N-acetylglucosaminidase. However, the spectral data obtained for compound 1 did not match those reported for pyrostatin B.

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The spectral data for the synthetic product are practically identical to those of compound 1 (Table 1). The NMR chemical shift data for the C-5 and C-7 positions differ slightly from each other due to the strong pH dependence of the chemical shifts of amino acids in D₂O solution. Furthermore, the synthetic product and compound 1 coeluted when a mixture of both compounds was analyzed by HPLC under the isolation conditions described before. Treatment of synthetic compound 1 with thionyl chloride in MeOH gave the corresponding methyl ester 2 in quantitative yield (Scheme 1). Once again, the NMR spectral data for the synthetic compound are identical to those of 2 (Table 1), showing even better agreement than the comparison between 1 and the corresponding synthetic compound. This is due to the presence of a methyl ester in 2 as opposed to a free carboxylic acid in 1.

The absolute stereochemistry of the asymmetric center at C-5 of the iminopyrrolidine ring was deduced by comparison of the optical rotation of compound 2 {[α]D of −10.1° (c 0.1, H₂O)} to that of the synthetic methyl ester derivative obtained from (S)-pyroglutamic acid {[α]D of −7.5°}.


(c 0.3, H2O). On the basis of these data, the absolute structure of 1 was thus established as (−)-(5S)-2-imino-1-methylpyrrolidine-5-carboxylic acid.

Careful analysis of the NMR data reported for pyrostatin B showed that some chemical shifts are incompatible with the proposed structure. For example, the carbon chemical shift of the N-methyl group at δ 18.9 ppm is unexpected because this value is usually higher. Taking into account the data reported for pyrostatin B, a literature search for other possible structures showed that its reported NMR data match those of 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid, named as ectoine or THP(B). This metabolite was first isolated from extremely halophilic and phototrophic species of the bacterial genus Ectothiorhodospira.16 (S)-Ectoine is now commercially available and is sold for its osmoprotectant activities against a wide variety of organisms, for its protective effects in Escherichia coli during drying and storage, and as a stabilizer for enzymes.17 The perfect agreement of the NMR chemical shifts reported for pyrostatin B with those of ectoine is evident from the data in Table 2.

Furthermore, the NMR data reported for pyrostatin A also matched those of 5-hydroxyectoine [THP(A)], which is produced by Streptomyces parvulus.10,11 This finding suggests a new unreported biological activity for these compounds as N-acetyl-β-D-glucosaminidase (NAG) inhibitors. The role of N-acetyl-β-D-glucosaminidase in the degradation of extracellular matrix components suggested the usefulness of hexosaminidase inhibitors as potential antiinvasive antitumor agents.18 Furthermore, it was found that the activity of this enzyme is increased markedly in patients with diabetes, leukemia, or cancer and their inhibitors can help to elucidate the mechanism of these diseases.9,19

In summary, we have isolated and characterized 2-imino-1-methylpyrrolidine-5-carboxylic acid from a very active allelopathic fraction obtained from the sponge Cliona tenuis. The structure of this compound was previously reported for the natural N-acetylglucosaminidase inhibitor named pyrostatin B. The synthesis of this compound from (S)-pyroglutamic acid indicated that the published structure does not correspond to that of the natural product. Comparison of NMR spectral data strongly suggests that pyrostatins A and B are identical with 5-hydroxyectoine and ectoine, respectively. An investigation into the allelopathic activity of 1 and 2 against corals is underway.

### Acknowledgment
This work was financially supported by Grants from Xunta de Galicia (PGIDIT05RMA10302PR), Ministerio de Educación y Ciencia (CQT2005-00793), and COLCIENCIAS from Colombia (grant 1101-09-13544). L.C. thanks Program Al/án, the European Union Program of High Level Scholarships for Latin America for a scholarship (E04D033392CO), and Universidad Nacional de Colombia for the leave of absence to perform doctoral studies.

### Supporting Information Available
Experimental details and spectral data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

### Table 2. Reported 13C NMR and 1H NMR Spectral Data for Pyrostatin B and Ectoine [THP(B)]

<table>
<thead>
<tr>
<th></th>
<th>δ_C (mult)</th>
<th>δ_H (mult) (J Hz)</th>
<th>δ_C (mult)</th>
<th>δ_H (mult) (J Hz)</th>
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</thead>
<tbody>
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<td>161.2 (C)</td>
<td>3.30 ddd (14.0, 8.6, 5.0)</td>
<td>2</td>
<td>161.5 (C)</td>
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<tr>
<td>3</td>
<td>38.0 (CH2)</td>
<td>3.46 ddd (14.0, 5.6, 5.6)</td>
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<td>38.0 (CH2)</td>
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<tr>
<td>4</td>
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<td>2.07–2.19 m</td>
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<td>23.6 (CH2)</td>
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<td>53.9 (CH)</td>
<td>4.07 dd</td>
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<td>53.6 (CH)</td>
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<tr>
<td>6</td>
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<td>2.24 s</td>
<td>2'</td>
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<td>177.4 (C)</td>
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Org. Lett., Vol. 8, No. 21, 2006

(17) Sigma (E 2271) and Fluka (81619) catalogues.