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

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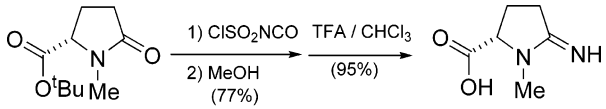
ABSTRACT



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(−)-(5*S*)-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-iminopyrrolidines 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*, *Montrastea cavernosa*, and *Siderastrea siderea* in laboratory and field assays.[5]

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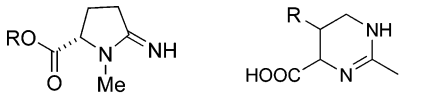
Sponges belonging to the *Cliona* genus are usually burrowing organisms that are able to excavate a variety of calcareous substrates such as rocks, coralline reefs, and oyster shells. Previous chemical investigations on *Cliona* species led to the isolation of different classes of secondary metabolites 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.[9] However, the data were different from those published for pyrostatin B.[9a] 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** and, consequently, to demonstrate that the reported structure of pyrostatin B was incorrect. Furthermore, on searching the literature, we found that the actual NMR data reported for pyrostatin B matched those of ectoine.[10,11]



1 R = H
2 R Me

Ectoine R = H
5-Hydroxyectoine R = OH

Sponge specimens (7 kg) were collected from the Rosario Islands in the Colombian Caribbean and extracted with MeOH and CH_2Cl_2. The methanol and dichloromethane extracts (93 g) were combined, evaporated in vacuo, and then partitioned between CH_2Cl_2 and H_2O. The bioactive aqueous layer was evaporated and further partitioned between water and *n*-BuOH saturated with water, with the activity again remaining in the aqueous layer. After evaporation of the solvent in vacuo, the residue was loaded onto XAD-4 resin, which was washed with distilled water, then with methanol, and finally acetone. The fraction eluted with water, which was found to be the most active, was evaporated under vacuum. Slow addition of methanol to this fraction allowed the sequential precipitation of inorganic media components. Filtration and evaporation of the methanol filtrate to dryness yielded a residue (14 g), which was chromatographed on Sephadex LH-20 (10% MeOH/H_2O) to afford 4.7 g of a 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 $C_6H_{11}N_2O_2$ (calcd 143.0815).

The ^{13}C NMR and DEPT spectra of **1** in D_2O (Table 1) displayed six distinct resonances: two sp^2 quaternary carbons at δ 169.55 ppm (C-2) and δ 177.43 ppm (C-7), suggesting the presence of a C=N carbon and a carboxylic acid, respectively; two methylenes at δ 24.29 ppm (C-4) and δ 30.12 ppm (C-3); a methine at δ 70.36 ppm (C-5); and finally a methyl group at δ 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 $^1H-^1H$ 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.

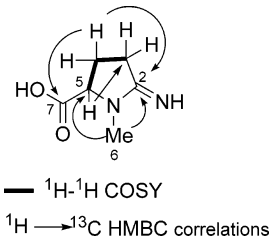


Figure 1. Selected COSY and HMBC correlations observed for **1** in D_2O.

The structure of compound **2** was deduced on the basis of MS and NMR data and the correlation of these data to those of **1**. The high-field shift of the carbonyl carbon to δ 172.35 ppm and the additional resonance at δ 53.40 ppm, corresponding to a methyl group in the ^{13}C NMR spectrum of compound **2**, indicated the formation of the methyl ester derivative of **1**. Furthermore, 2D NMR spectra, including an HMBC spectrum, showed the same set of correlations as compound **1**. This, in conjunction with the MS data that showed a mass 14 amu higher than the respective parent compound, defines **2** as methyl 2-imino-1-methylpyrrolidine-5-carboxylate. This was confirmed by the pseudomolecular

(5) Unpublished results.

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Table 1. ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) Spectral Data of Natural and Synthetic **1** and Their Methyl Ester Derivatives (**2**) in D_2O

position/mult		natural compound **1**		synthetic compound **1**		methylated natural compound **1** (**2**)		methylated synthetic compound **1** (**2**)	
		δ_C	δ_H mult (J Hz)	δ_C	δ_H mult (J Hz)	δ_C	δ_H, mult (J)	δ_C	δ_H, mult (J)
2	C	169.55		170.11		170.30		170.27	
3	CH_2	30.12	2.95 m	29.68	2.95 m	29.64	3.05 m	29.53	3.03 m
4	CH_2	24.29	2.12 m	23.59	2.23 m	23.41	2.63 m	23.32	2.62 m
			2.51 m		2.55 m		2.35 m		2.35 m
5	CH	70.36	4.37 dd	67.72	4.60 dd	67.49	4.7[a]	67.38	4.69 dd
			(9.5, 4.2)		(9.8, 3.6)				(9.8, 3.2)
6	CH_3	31.74	3.04 s	31.77	3.05 s	31.84	3.15 s	31.74	3.15 s
7	C	177.43		174.01		172.35		172.27	
OMe	CH_3					53.40	3.88 s	53.29	3.88 s

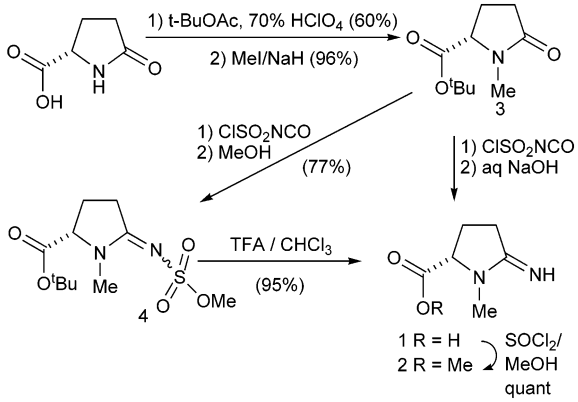
[a] Under the solvent signal.

$[M + H]^+$ ion at *m*/*z* 157.0974 in the (+)-HRESIMS of **2** corresponding to the molecular formula $C_6H_{11}N_2O_2$ (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.[9] Pyrostatin B and its hydroxylated derivative (pyrostatin A) have been reported and patented as useful therapeutics due to their inhibition of *N*-acetylglucosaminidase.[12] 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-methylazacarbocycles by using chlorosulfonyl isocyanate (Scheme 1).[13]

Scheme 1



The synthesis started from the commercial (*S*)-pyroglutamatic acid, which was protected as the *tert*-butyl ester and then N-methylated to give *tert*-butyl *N*-methyl (*S*)-pyroglutamate (**3**).[14] 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 D_2O solution.[15] 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** {$[\alpha]_D$ of $-10.1°$ (*c* 0.1, H_2O)} to that of the synthetic methyl ester derivative obtained from (*S*)-pyroglutamatic acid {$[\alpha]_D$ of $-7.5°$

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(c 0.3, H_2O)}. On the basis of these data, the absolute structure of **1** was thus established as (−)-(5*S*)-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 strorage, 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.

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

pyrostatin B[9a]			ectoine		
C	δ_C (mult)	δ_H mult (J Hz)	**C**	δ_C (mult)[10]	δ_H mult (J Hz)[11]
2	161.2 (C)		**2**	161.5 (C)	
3	38.0 (CH_2)	3.30 ddd (14.0, 8.6, 5.0) 3.46 ddd (14.0, 5.6, 5.6)	**6**	38.0 (CH_2)	3.28 ddd (13.5, 8.4, 4.8) 3.44 ddd (13.5, 5.4, 5.4)
4	22.1 (CH_2)	2.07−2.19 m	**5**	23.6 (CH_2)	2.11 m
5	53.9 (CH)	4.07 dd (5.6, 5.6)	**4**	53.6 (CH)	4.06 dd (5.4, 5.4)
6	18.9 (CH_3)	2.24 s	**2′**	19.1 (CH_3)	2.22 s
7	177.4 (C)		**4′**	177.0 (C)	

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*)-pyroglutamatic 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.

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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.

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