**Dipeptide Metabolites from the Marine Derived Bacterium *Streptomyces acrimycini***

Isara L. C. Hernández, Mario L. Macedo, Roberto G. S. Berlinck, Antonio G. Ferreira and Mirna J. L. Godinho

**Introduction**

Marine microorganisms constitute the latest and yet poorly explored source of new biologically active secondary metabolites, mainly because it is assumed that marine microbiology is still a starting research field, and that microbiology methods should be improved in order to enable the isolation and growing of marine microorganisms in artificial media. We have recently started a program aimed to investigate the secondary metabolites of marine-derived microbial strains. Several fungi and bacteria were isolated from sediments, algae and invertebrates, and were grown in different media in order to evaluate their production of secondary metabolites. Crude extracts obtained from the culture media were subjected to different chemical and biological screenings. Based on $^1$H NMR analysis, the crude extract of the actinomycete *Streptomyces acrimycini* was selected for a chemical investigation. We report here the isolation and structure determination of two new dipeptide metabolites isolated from *S. acrimycini*: 8-amino-[1,4]diazonane-2,5-dione (1) and leucyl-4-hydroxyproline (3).

**Keywords**: 8-amino-[1,4]diazonane-2,5-dione, leucyl-4-hydroxyproline, *Streptomyces acrimycini*

**Experimental**

**General experimental procedures**

IR spectra were recorded on a FT-IR Bomem MB102 infrared spectrometer. NMR spectra were run either on a
and restreaked for purity. During two weeks, then single colonies were harvested growing conditions (g/L): soluble starch 10.0, casein 1.0, media. Marine streptomycetes were selected using standard the Universidade de São Paulo. Aliquots of sediments were immediately processed at the Marine Biology Station of São Paulo state, Brazil). Samples of sediments were collected with a Kojak apparatus, at depths between 12 and 15 m in the São Sebastião channel (north coastline of São Paulo). acrimycini was isolated from samples of sediments was grown in 10 L of marine broth 2216 (Streptomyces acrimycini) under the code 02-1309, 1108, 1002. HREIMS m/z 154.07392 (calc. for C7H10N2O2, 154.07423) corresponding to [M-NH3]+. EIMS m/z (rel. intens.): 154 (78) [M-NH3]+, 111 (100), 98 (31), 84 (77), 83 (96), 70 (77), 66 (83). 1H and 13C NMR data: see Table 1.

Leucyl-4-hydroxyproline (3) and N-acetyltyramine (4). Glassy solid. LC-ESIMS m/z (rel. intens.): 245 (2) [M+H]+, 229 [M-CH3]+ (60), 202 (100). Negative-mode HRFABMS m/z 243.13476 (calc. for C11H19N2O4, 243.13448) corresponding to [M-H]+. 1H and 13C NMR data: see Table 1.

N-acetyltyramine (4). Glassy solid. Spectroscopic data in agreement with literature values.7

Results and Discussion

Streptomyces acrimycini was grown in 10 x 1L erlenmeyer flasks, each one containing 500 mL of enriched marine broth (see Experimental), during 10 days at 180 rpm and 27 ºC. Chromatography of the organic crude extract on Sephadex LH-20 (MeOH) yielded several fractions which were further purified by reversed phase chromatography (μBondapak C18, MeOH-H2O 3:7). The dipeptide 8-amino-[1,4]diazonane-2,5-dione (1) was obtained as a glassy solid, [α]D –6.8o (c 0.08, MeOH), IR (film) νmax/cm–1: 3332 (br, νNH), 2925 (νC=H), 1668 (νC=O, amide I), 1456 (νC=O, amide II), 1309, 1108, 1002. HREIMS m/z 154.07392 (calc. for C7H10N2O2, 154.07423) corresponding to [M-NH3]+. EIMS m/z (rel. intens.): 154 (78) [M-NH3]+, 111 (100), 98 (31), 84 (77), 83 (96), 70 (77), 66 (83). 1H and 13C NMR data: see Table 1.

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N-acetyltyramine (4). Glassy solid. Spectroscopic data in agreement with literature values.7

Isolation of 1, 3 and 4 from Streptomyces acrimycini

S. acrimycini was grown in 10 L of marine broth 2216 (Difco) with 1% soluble starch, at 25 ºC during 5 days at 200 rpm. Culture media was processed as follows: after filtration on Whatman paper #1, ethyl acetate was added to the culture medium and left overnight under magnetic stirring. The mycelium was extracted with MeOH. The ethyl acetate was separated from the culture medium aqueous phase by liquid-liquid partition. The EtOAc and MeOH extracts were pooled and concentrated to 300 mL of a MeOH-H2O suspension which was partitioned against hexanes. The MeOH-H2O layer was concentrated, dissolved in MeOH and subjected to chromatography on Sephadex LH-20 (MeOH), yielding eight fractions which were analysed by 1H-NMR and by thin-layer chromatography. Based on the chemical and spectroscopic analyses, the fourth fraction was selected for further fractionation by HPLC (μBondapak C18, MeOH-H2O 3:7), yielding 1.2 mg of a mixture of N-acetyltyramine (4) and leucyl-4-hydroxyproline (3), as well as 1.0 mg of pure N-acetyltyramine (4). The fifth fraction arising from the Sephadex LH-20 fractionation was further fractionated by a series of HPLC separations on a μBondapak C18 column (MeOH-H2O 3:7, then H2O-acetonitrile 6:4) and finally purified on an CSC-Inertsil ODS-2 column (H2O-MeOH 9:1), to yield 1.4 mg of 8-amino-[1,4]diazonane-2,5-dione (1).
coupled to CH₂-6 at δ 2.02 (m) and 1.97 (m) (¹³C 22.4), which was in turn coupled to the CH₂-5 methylene group at δ 3.53 (m) (¹³C 45.1). Both ¹H and ¹³C chemical shifts of CH₂-5 agreed with a N-substitution. These data were indicative of an ornithine residue, confirmed by analysis of the gHMBC spectrum. The remaining NMR data indicated the presence of a glycine residue, with a single methylene group at δ 3.99 (d, 17 Hz) and 4.11 (d, 17 Hz) (¹³C 46.2) coupled with two carbonyl carbons at δ 169.9 (C-3) and 164.4 (C-9). We first considered that the structure of this compound corresponded to the diketopiperazine cyclo[Gly-Orn] (2). However, analysis of the NMR data in DMSO-d₆ did not disfavor this hypothesis, since a ¹H coupling in the ¹H-¹H COSY spectrum between CH₂-5 at δ 3.53 with the NH-4 amide exchangeable proton at δ 8.10 was observed. Additionally, both CH₂-5 at δ 3.53 and NH-4 proton at δ 8.10 showed long-range couplings to the carbonyl group at δ 169.9. Therefore, in order to account for the NMR data obtained in DMSO-d₆, the structure should have a lactam group including the ornithine δ-amino group and the glycine acid group, corresponding to 8-amino-[1,4]diazonane-2,5-dione (1). We have been unable to establish the stereochemistry of the ornithine residue in I due to the small amount of compound isolated. To the best of our knowledge, the structure of I is totally unprecedented among dipeptides. A related macrocyclic dipeptide has been recently reported as part of the macrobactins, a group of amphiphilic siderophores recently isolated from Marinobacter sp.⁵,⁶

The second dipeptide isolated from S. acrimumcini was the linear leucyl-4-hydroxyproline (3), isolated in a mixture with N-acetyltyramine (4). Due to the small quantity of the mixture (~1 mg), we have not attempted to separate both compounds in order to avoid any loss of material. Since we have been also able to isolate a pure sample of 4, we could analyse the MS and NMR data of 3 and 4, in order to assign the ¹H (at 500 MHz) and ¹³C (at 125 MHz) signals of both compounds. Analysis of the gHSQC spectrum enabled us to assign all hydrogen bearing carbons of 3. The position of the hydroxyl group in the 4-hydroxyproline moiety was established by analysis of the ¹H-¹H COSY spectrum, which showed sequencial couplings from H-5 (δ 4.38) to H-4a (δ 1.93) and H-4b (δ 2.02), from these two hydrogens to H-3 (δ 4.27), which was in turn coupled with both H-2a (δ 3.21, overlapped by the H₂O signal in DMSO-d₆) and H-2b (δ 3.47). Since the spectra were obtained in DMSO-d₆, we have been able to observe a vicinal coupling between H-3 and the hydroxyl proton at δ 5.08 (d, J 3 Hz). Further support to the 4-hydroxyproline moiety of 3 was obtained by analysis of the gHMBC spectrum, which showed couplings between H-5 and C-4 (δ 3.66), between H-5 and the carbonyl group C-6 at δ 166.6, between H-3 and C-5 (δ 57.1), between H-4b and C-2 (δ 53.7) as well as between H-4b and C-3 (δ 67.0), between H-4a and C-5, and finally between H-2a and C-3. The amide bond between the two amino acid residues was established as the carbonyl group C-6, which chemical shift at δ 166.6 typically resonates at a higher field than the carbonyl group chemical shift of a free carboxylic acid group. Additionally, analysis of the gHMBC spectrum showed long-range couplings between the exchangeable amide N-H proton at δ 7.98 and C-6 (δ 166.6), between the N-H proton and C-5 of 4-hydroxyproline (δ 57.1), as well as between H-8 of leucine (δ 4.04) and C-6 of 4-hydroxyproline. Analysis of the gHMBC, ¹H-¹H COSY and gHMBC spectra defined all hydrogen and carbons of the leucine moiety. ¹H-¹H couplings were observed between

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### Table 1. ¹H and ¹³C NMR assignments of compounds 1 and 3

<table>
<thead>
<tr>
<th>Position</th>
<th>δ ¹³C</th>
<th>δ ¹H (mult, J in Hz)¹</th>
<th>Position</th>
<th>δ ¹³C</th>
<th>δ ¹H (mult, J in Hz)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH-1</td>
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<td></td>
<td>NH-1</td>
<td>n.o.</td>
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<td>CH₂-2</td>
<td>46.2</td>
<td>3.99 (d, 17 Hz) 4.11 (d, 17 Hz)</td>
<td>CH₂-2</td>
<td>53.7</td>
<td>3.21 (d, 12); 3.47 (dd, 4, 12)</td>
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<tr>
<td>C-3</td>
<td>169.9</td>
<td></td>
<td>C-3</td>
<td>67.0</td>
<td>4.27 (bs)</td>
</tr>
<tr>
<td>NH-4</td>
<td>8.10 (s)</td>
<td></td>
<td>NH-4</td>
<td>36.6</td>
<td>1.93 (m); 2.02 (dd, 7, 13)</td>
</tr>
<tr>
<td>CH₂-5</td>
<td>45.1</td>
<td>3.53 (m)</td>
<td>CH₂-5</td>
<td>57.1</td>
<td>4.38 (dd, 7, 10)</td>
</tr>
<tr>
<td>CH₂-6</td>
<td>22.4</td>
<td>2.02 (m); 1.97 (m)</td>
<td>CH₂-6</td>
<td>-</td>
<td>5.08 (d, 3)</td>
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<tr>
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<td>28.2</td>
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<td>166.6</td>
<td></td>
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<tr>
<td>CH₂-8</td>
<td>58.4</td>
<td>4.23 (bt, 9)</td>
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<td>7.98 (bs)</td>
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<td>C-9</td>
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<td>4.04 (dd, 7, 13)</td>
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<td>37.7</td>
<td>1.33 (ddd, 7, 8, 13)</td>
<td>C-10</td>
<td>24.0</td>
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<td>C-11</td>
<td>22.7</td>
<td>0.84 (d, 3.3)</td>
<td>C-11</td>
<td>23.0</td>
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<tr>
<td>C-12</td>
<td>170.7</td>
<td></td>
<td>C-12</td>
<td>23.0</td>
<td>0.86 (d, 3.3)</td>
</tr>
</tbody>
</table>

¹Data obtained at 100 MHz, in DMSO-d₆; ²Data obtained at 400 MHz, in DMSO-d₆; ³Data obtained at 125 MHz in DMSO-d₆; ⁴Data obtained at 500 MHz in DMSO-d₆.
H-8 and H-9α (δ 1.33) and H-9β (δ 1.75). Further hydrogen couplings were observed between both methyl groups CH3-11 at δ 0.84 (d, 3.3 Hz) and CH3-12 at δ 0.86 (d, 3.3 Hz) and H-10 at δ 1.86 (m). 1H-13C Long-range couplings were observed between H-8 and C-9 (δ 37.7), C-10 (δ 24.0), between both H-9α and H-9β and C-8, with the carbonyl group at δ 166.6 (C-6 of 4-hydroxyproline, a J long-range correlation) and with H_C-12, between H-10 and C-8 (δ 52.5) and both methyl groups, as well as between the hydrogens of both methyl groups with C-10 and C-9. The LC-ESIMS of the mixture of 3 and 4 displayed a ion at m/z 229, suggesting the loss of CH4 from the leucyl-4-hydroxyproline molecular ion of small intensity at m/z 245. Negative-mode HRFABMS indicated a parent ion peak [M-H]+ at m/z 243.13476 (calcd. 243.13448, ∆mu +2.8), confirming the molecular formula C11H12N2O4 for 3. Since we obtained only a tiny amount of this mixture, no attempt has been made in order to further purify the dipeptide leucyl-4-hydroxyproline (3), and the stereochemistry of the stereogenic centers were not determined. To the best of our knowledge, this is the first report on the identification of the linear dipeptide leucyl-4-hydroxyproline (3). No chemical investigation of the S. acrimycini secondary metabolites has been previously reported, but only genetic and morphological descriptions.5-11

Although there is abundant literature data about dipeptides isolated from microbial sources,12,16 their true origin remains controversial. Some authors suggest that dipeptides are fermentation artifacts, generated by hydrolysis of proteins present in the growth media.16,17 However, several dipeptides isolated from microorganisms display relevant biological activities.14,15 Recently, it has been demonstrated that several dipeptides present an important role as chemical mediators of bacterial quorum-sensing signalling systems.18 The isolation of unusual metabolites from the previously unstudied marine-derived S. acrimycini reinforce the importance of continuing studies on marine microbiology and on marine microorganisms secondary metabolism.

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References


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