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Isolation and structural characterization of microcystin-LR and three minor oligopeptides simultaneously produced by *Radiocystis feernandoi* (Chroococcales, Cyanobacteriae): A Brazilian toxic cyanobacterium

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Abstract

Several blooms of cyanobacteria naturally occurring in freshwater reservoirs have been associated to numerous fatalities and cases of livestock and human poisoning. Microcystins (Mcs) are the most frequently found cyclic heptapeptide toxins in the cyanobacterial extracts. In previous work, *Radiocystis fernandoi* (strain SPC 714) lyophilized extracts were found to be hepatotoxic to mice with LD_{100} of about 60 mg kg^{-1} and Mc LR was suggested as responsible for that toxicity. Here, we describe the isolation of four oligopeptides from *R. fernandoi* methanol extract by reversed-phase high performance liquid chromatography (RP-HPLC). The major component, which eluted with 65% acetonitrile from acetonitrile/water gradient, was identified as Mc-LR and its structure was confirmed by the presence of molecular related ion species $[M+H]^+$ at *m/z* 996.3, $([M+H\text{-Adda}])^+$ at *m/z* 861.5, $[\text{Arg-Adda-Glu}+H]^+$ at *m/z* 599.8, and $[PhCH_2CH(OMe)]^+$ at m/z 135.1 in the ESI spectra. Two components corresponding to small signals eluted from C18 column, respectively, with 44 and 45% acetonitrile had their structures proposed as isomers of aeruginosin derivatives showing molecular ions at *m/z* 651.7 and a $[CHOI]^+$ immonium at *m/z* 140.1. Finally, the structure of the third minor and most hydrophobic component (68% acetonitrile elution) isolated from *R. fernandoi* extract seemed to correspond to a

Abbreviations: ACN, acetonitrile; Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid; Ahp, 3-amino-6-hydroxy-2-piperidone; Apla, 3-(4-aminophenyl)-lactic acid; Argal, tautomeric argininals (D or L); ASM, 1-modified Artificial Seawater McLachlan medium; CHOI, 2-carboxy-6-hydroxy-octahydroindole; ESI-MS, electrospray ionization mass spectrometry; Hpla, 3-(4-hydroxyphenyl)-lactic acid; Mdha, *N*-methyldehydroalanine; MeOH, methanol; RP-HPLC, reversed phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

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cyclic cyanopeptolin like micropeptin K139, a trypsin inhibitor firstly isolated from *Microcystis aeruginosa*, showing similar ions fragmentation pattern and $[M+H]^+$ at *m/z* 987.6 in its ESI spectra.

Keywords: *Radiocystis fernandoi*; Microcystin LR; Micropeptin; Aeruginosins; Hepatotoxins

1. Introduction

Cyclic peptide hepatotoxins are the most frequently found cyanobacterial toxins in freshwater natural blooms extracts. The first cyanotoxins isolated in the early 1980s from *Microcystis aeruginosa* bloom extracts were referred to as microcystins, cyclic heptapeptides with molecular mass ranging from 800 up to 1100 Da (Chorus and Bartram, 1999).

Microcystins are potent liver toxins, general tumor promoters, inhibitors of protein phosphatases and inhibitors of protein synthesis (Chorus and Bartram, 1999; Solter et al., 2000; Fastner et al., 2001; Ortea et al., 2004). Their potent bioactivities are related mainly to two amino acid residues, an unusual hydrophobic one, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda) at position 5, and *N*-methyldehydroalanine (Mdha) at position 7 (Harada, 2004; Welker et al., 2004a,b). The microcystins general structure is constituted by other D-amino acids, D-alanine, 3-methyl-D-aspartic acid or D-aspartic acid (MeAsp/Asp), D-glutamic acid at positions 1, 3 and 6, respectively, two variable L-amino acids at positions 2 and 4, a β-methylated residue (usually D-methyl aspartic acid) at position 3 and *N*-methylated residues at position 7 (Spoof et al., 2003; Grach-Pogrebinsky et al., 2004; Harada, 2004; Ortea et al., 2004; Diehnelt et al., 2005). On the other hand, Glu at position 6, attached by the γ-carboxyl group to residue in position 7 seems to be less prone to variation, being always present in the known microcystins (Namikoshi et al., 1992; Diehnelt et al., 2005).

Beside microcystins, it has been reported that cyanobacteria can biosynthesize other cyclic, multicyclic and linear oligopeptides, such as nodularins (pentapeptides), anabaenopeptins and cyanopeptolins (micropeptins, microcystilides, aeruginopeptins), micoviridins (multicyclic; 1600–1850 Da), aeruginosins (550–900 Da) and the linear modified peptides microginins (650–950 Da). As well as microcystins, the structures of these peptides generally include unusual amino acids residues, such as 3-(4-hydroxyphenyl)-lactic acid (Hpla) and 2-carboxy-6-hydroxy-octahydroindole (CHOI) in aeruginosins, or 3-amino-6-hydroxy-2-piperidone (Ahp) in cyanopeptolins, β-amino-α-hydroxy-decanoic acid in the linear microginins (Neumann et al., 1997; Fukuta et al., 2004; Harada, 2004; Welker et al., 2004a,b). These peptides have been found to exhibit a wide range of biochemical and pharmacological activities frequently proteases inhibition (Fastner et al., 2001; Bister et al., 2004; Welker et al., 2004a). Furthermore, non-peptide toxins or alkaloidal neurotoxins, the so-called anatoxins, saxitoxins or cylindrospermopsins with molecular mass minor than 450 Da, have been reported (Chorus and Bartram, 1999; Namikoshi et al., 2003; Fastner et al., 2003). These toxins are biosynthesized together to other non-toxic hydroxylated alkaloids.

Microcystins and the other aforementioned bioactive compounds have been isolated worldwide from several genera of cyanobacteria, including *Microcystis*, *Anabaena*, *Planktothrix* (formerly *Oscillatoria*), *Hapalosiphon*, *Aphanocapsa*, *Synechocystis,* and *Nostoc* (Sivonem, 1996; Dawson, 1998; Domingos et al., 1999; Brittain et al., 2000; Martins et al., 2005).

Toxicity of cyanobacterial blooms in Brazilian drinking water reservoirs amounts to 80%, being a great concern for public and livestock healthcare (Azevedo et al., 1994; Carvalho et al., 2004). Recent studies described the detection, isolation and identification of *R. fernandoi* as a new cyanobacterium toxic strain (Azevedo et al., 1994; Domingos et al., 1999; Vieira et al., 2003; Carvalho et al., 2004). Here, we report the isolation and characterization of microcystin-LR as the main compound responsible for the toxicity exhibited by *R. fernandoi* and of other three minor oligopeptides simultaneously produced by this cyanobacterium. Thus, the toxicity of this specie may not be solely attributed to microcystin-LR.

2. Material and methods

2.1. Radiocystis fernandoi culture

The unialgal strain *R. fernandoi* SPC 714 (non-axenic) was isolated from Utinga Reservoir, city of Belém, state of Pará, at 01° 27′ 21″ S and 48° 30′ 15″ W, in 1999 August. This cyanobacterium strain was cultured in 5 L culture bottles with ASM-1 medium, pH 7.4, at 25 °C ± 1 °C, 14:10 h ligth:darkness (20 μmol photon m^{-2} s^{1} light intensity), and moderate aeration rate. The cells were grown until the end of the exponential phase, harvested by centrifugation at 6000*g* (15 min) and lyophilized (Vieira et al., 2003).

2.2. Extraction and isolation of microcystin-LR

The lyophilized cells (0.61 g) were submitted to ultrasound (4 × 30 s, 50 W), extracted four times with

methanol (MeOH):H_2O (75:25, v:v) and then centrifuged at 1045*g* for 60 min. The supernatants were combined and evaporated under reduced pressure at 40 °C to remove the organic solvent. The aqueous residue was lyophilized, furnishing 0.13 g of a solid residue, which was reconstituted with 0.5 mL MeOH:H_2O (1:1, v:v) and applied to a Bakerbond® Octadecyl (C_{18}) Prep LC Packing column (9.0×1.9 cm ID, 40 μm particle size). The eluting solvents were 0.1% aqueous trifluoroacetic acid (TFA) solution (30 mL) and MeOH:H_2O (20:80, v:v, 30 mL). The aqueous extract was lyophilized and the methanol extract was vacuum concentrated (Harada et al., 1988; Harada, 1996; Spoof et al., 2003).

2.3. Purification of compounds by RP-HPLC

The aqueous and methanol fractions were pooled and filtered through 0.45 μm Millipore membrane and separated by using a modular *Shimadzu LC-10A* HPLC system comprised of a solvent binary pump (two *LC-10AD* pumps), UV/Vis detector, and *SCL-10Avp system controller* software and workstation. Fractionation was performed by employing a *Shimadzu* C18 analytical (0.46×25.0 cm, 5 μm, 300 Å) or semi-preparative (2.2×25.0 cm, 5 μm, 300 Å) columns, binary gradient of 5–95% solvent B in 30 min, using 0.1% aqueous TFA solution as solvent A and 80% aqueous acetonitrile (ACN) solution (containing 0.09% TFA) as solvent B, flow rate of 1 mL min^{-1} and detection at 238 nm. Four fractions were manually collected and lyophilized

2.4. Analysis of purified products by ESI-TOF MS

ESI-MS analyses were carried out on a Waters® Micromass® Q-Tof micro™ Mass Spectrometer (Micromass Ltd, Manchester, UK) fitted with an electrospray ion source, operated in positive ion mode. The capillary voltage was 3.0–3.5 kV and cone voltages were 30–40 V. MS^2 experiments were performed by Collision Induced Dissociation (CID), carried out by using argon as the collision gas with collision energies in the range from 20 to 50 eV. The instrument ran under control of MassLynx 4.0 data system (Micromass). Mass spectrometer was calibrated by using NaI/CsI in the 100–2000 *m/z* range.

3. Results and discussion

The pre-purification of 610 mg lyophilized cells of *R. fernandoi* furnished 130 mg crude extract which were fractionated by Bakerbond® Octadecyl (C_{18}) Prep LC Packing column to yield an aqueous (10.5 mg) and a methanol (5.8 mg) fractions. Since the total amount of material in these fractions was low, the aqueous and methanol fractions were pooled and fractionated by RP-HPLC. Methanol aqueous solutions with sonication are commonly employed for microcystins extraction. Barco et al. (2005) performed a systematic investigation of solvents employed for microcystin extraction and concluded that acidified methanol is the optimum solvent. Despite the absence of acid in our extraction solvent system, a microcystin could be extracted. In addition to that, the combination of methanol and aqueous extracts probably allowed the first isolation of other cyanotoxins in *R. fernandoi.*

Samples corresponding to the pooled fractions (16.3 mg) were separated by RP-HPLC in four chromatographic products designated by Rf-F1, Rf-F2, Rf-F3 and Rf-F4. Detection was conducted at 238 nm to search for products containing Adda. The predominant chromatogram elution product (Rf-F3; 800 μg of lyophilized mass) eluted from C18 column with retention time of 25.5 min (Fig. 1). Other three components of *R. fernandoi* extract, corresponding to small signals with retention times (RT) of 16.5, 17.0 and 26.5 min in the chromatogram of the Fig. 1, were isolated. The purified and lyophilized fractions Rf-F1 (100 μg), Rf-F2 (<100 μg) and Rf-F4 (100 μg) were individually analyzed by ESI-MS.

The $[M+H]^+$ molecular ion observed in the ESI-Q-TOF/MS spectrum of Rf-F1 (RT 16.5 min) was attributed to a compound of 651.3 molecular mass, which differed by only 2 Da from that of non-sulphated aeruginosins 102-A and B, linear tetrapeptides described in the literature as possessing molecular mass of 653 by FABMS (Matsuda et al., 1996). The existence of $[CHOI]^+$ immonium at *m/z* 140.1, as suggested by MS/MS experiments (Fig. 2), indicated that Rf-F1 could be indeed a variant derivative of such aeruginosins (Matsuda et al., 1996). MS/MS data did not give rise to a definitive structure attribution, but a simple dehydrogenation of aeruginosin 102 seems unlikely because fragments correspondent to this product were not found in the MS/MS spectrum (Fig. 2). One possible structural difference between the aeruginosin from *R. fernandoi* and aeruginosins 102-A and B is the presence of 3-(4-aminophenyl)-lactic acid (Apla) instead of 3-(4-hydroxyphenyl)-lactic acid (Hpla) at the first residue. Another piece of evidence in favor to attribution of the previous compound as an aeruginosin is that the spectrum of Rf-F2 (RT 17.0 min) also showed a molecular ion at *m/z* 651.3, probably corresponding to a tautomer of the protonated Rf-F1 molecule. Similar tautomerization was observed in the well-known protease inhibitors leupeptin and antipain, which possess Argal at C-terminus (Matsuda et al., 1996).

The $[M+H]^+$ observed at *m/z* 995.6 in the ESI-Q-TOF/MS spectrum of Rf-F3 (RT of 25.5 min) indicated the presence of Mc LR. Diehnelt et al. (2005) observed a double charged ion $[M+2H]^{2+}$ at *m/z* 498.3 that was not an intense signal in the spectrum. The MS^2 experiment (Fig. 3) by fragmenting the protonated molecule at *m/z* 995.6 showed the presence of the ion $[PhCH_2CH(OMe)]^+$ at *m/z* 135.1, characteristic of Mcs, which is generated by the α-cleavage

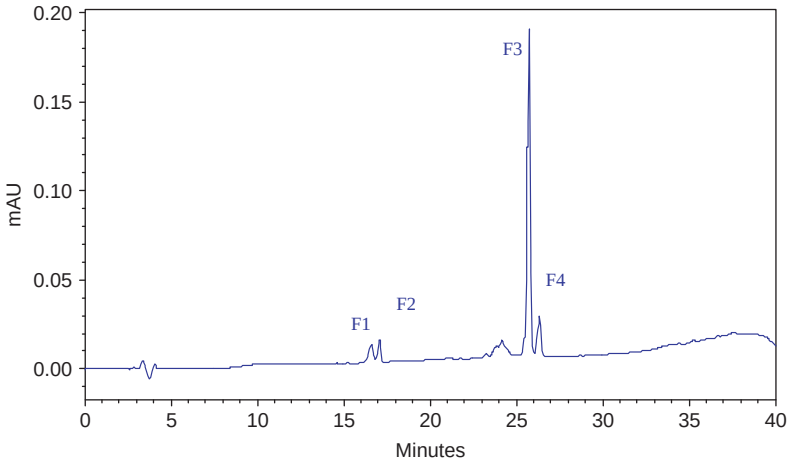

Fig. 1. RP-HPLC profile of *Radiocystis fernandoi* lyophilized cells extract. Experimental conditions: Shimadzu C18 column [(0.46×25.0) cm, 5 μm, 300 Å]; solvent A: 0.1% aqueous TFA solution; solvent B: 80% aqueous acetonitrile (ACN) solution (containing 0.09% TFA); gradient: 5–95% solvent B in 30 min; sample injected volume: 10 μL (ca. 0.5 μg microcystin LR); flow rate: 1 mL min^{-1}; detection: 238 nm. AU, absorbance units.

at the methoxy group of Adda β-amino acid moiety. The ion fragment at *m/z* 553.4 corresponds to [MdhaAlaLeuMeAspArg+H]$^+$ evidencing the presence of other amino acid residue characteristic of MCs, methyldehydroalanine (Mdha), and also indicating the presence of the residues Leu and Arg at positions 2 and 4, respectively. Additionally, the following molecular ion species have provided full confirmation of Mc LR identity: [Glu-Mdha+H]$^+$ at *m/z* 213.1, [M+2H]$^{2+}$ at *m/z* 498.3, ([M+H-Adda])$^+$ at *m/z* 861.5, [Arg-Adda-Glu+H]$^+$ at *m/z* 599.8, [M+H-Glu]$^+$ at *m/z* 866.6, and [$C_{11}H_{14}O$-Glu-Mdha]$^+$ at *m/z* 375.2 Microcystin LR was thus isolated as the major component

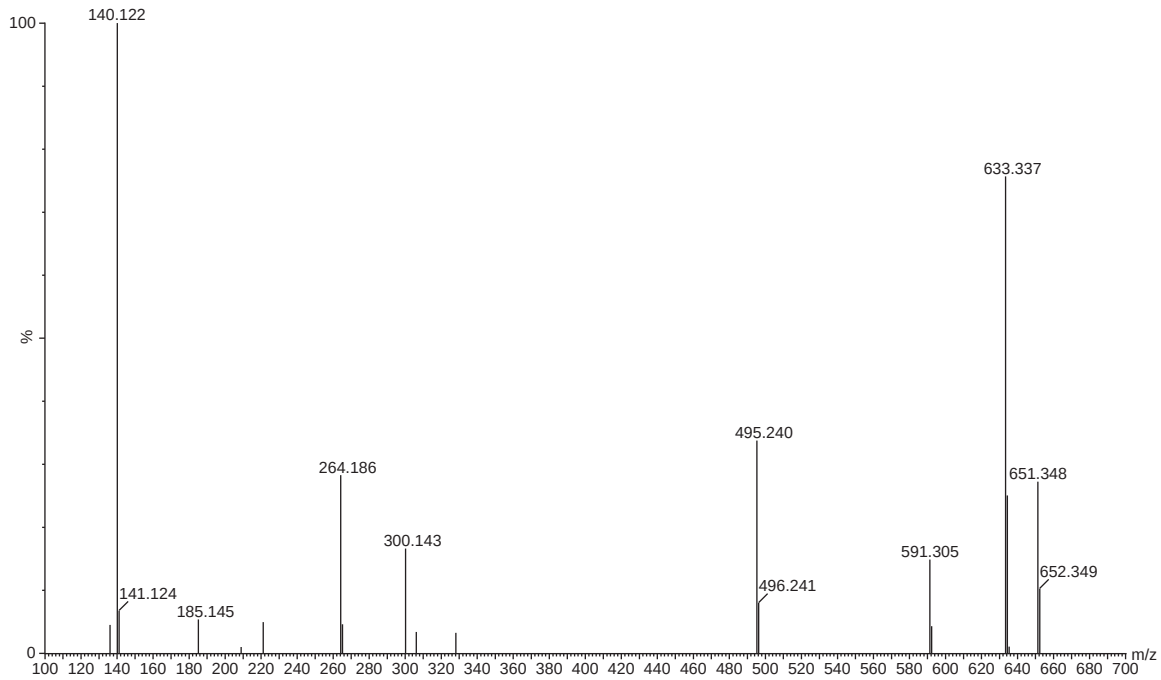

Fig. 2. MS/MS spectrum of *m/z* 651.3 parent ion. Fragmentation was achieved in Waters® Micromass® Q-Tof micro™ Mass Spectrometer by CID using argon as collision gas in the positive mode. Collision voltages were ramped from 20 to 50 eV and data analyzed by MassLynx 4.0 software. The chosen parent ion (*m/z* 651.3) was from *R. fernandoi* fraction 1 (Rf-F1) collected from RP-HPLC (Rf-F1, Fig. 1).

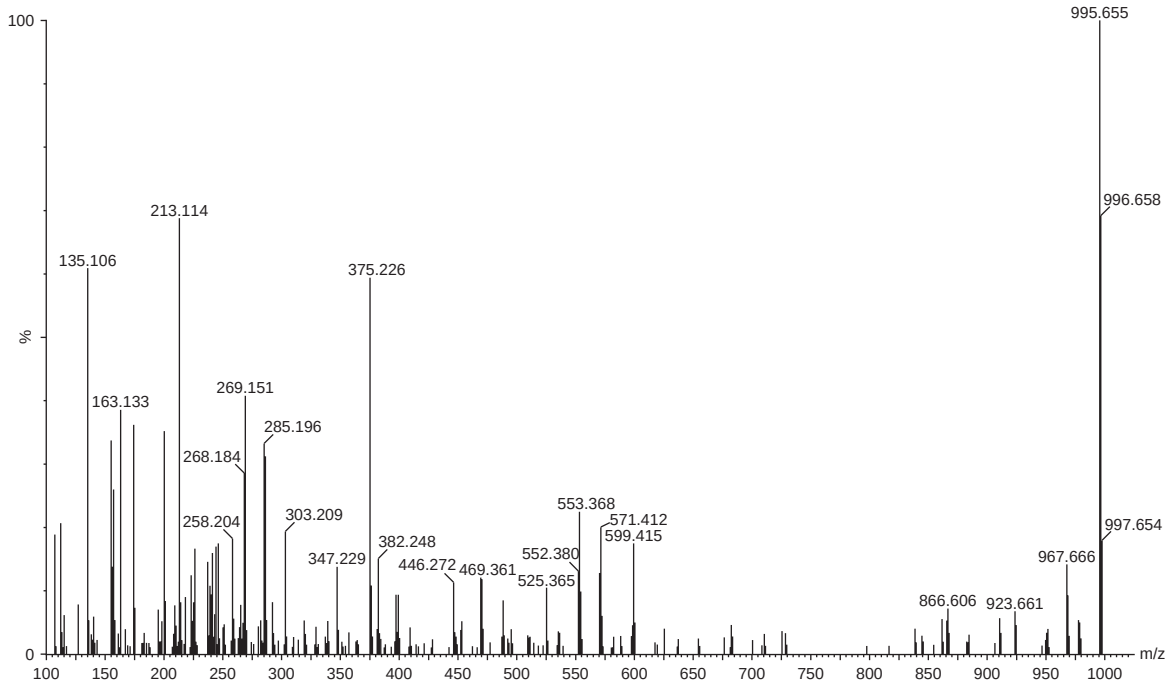

Fig. 3. MS/MS spectrum of *m/z* 995.6 parent ion. Fragmentation was achieved in a Waters® Micromass® Q-Tof micro™ Mass Spectrometer by CID using argon as collision gas in the positive mode. Collision voltages were ramped from 20 to 50 eV and data analyzed by MassLynx 4.0 software. The chosen parent ion (*m/z* 995.6) was from *R. fernandoi* fraction 1 (Rf-F3) collected from RP-HPLC (Rf-F3, Fig. 1).

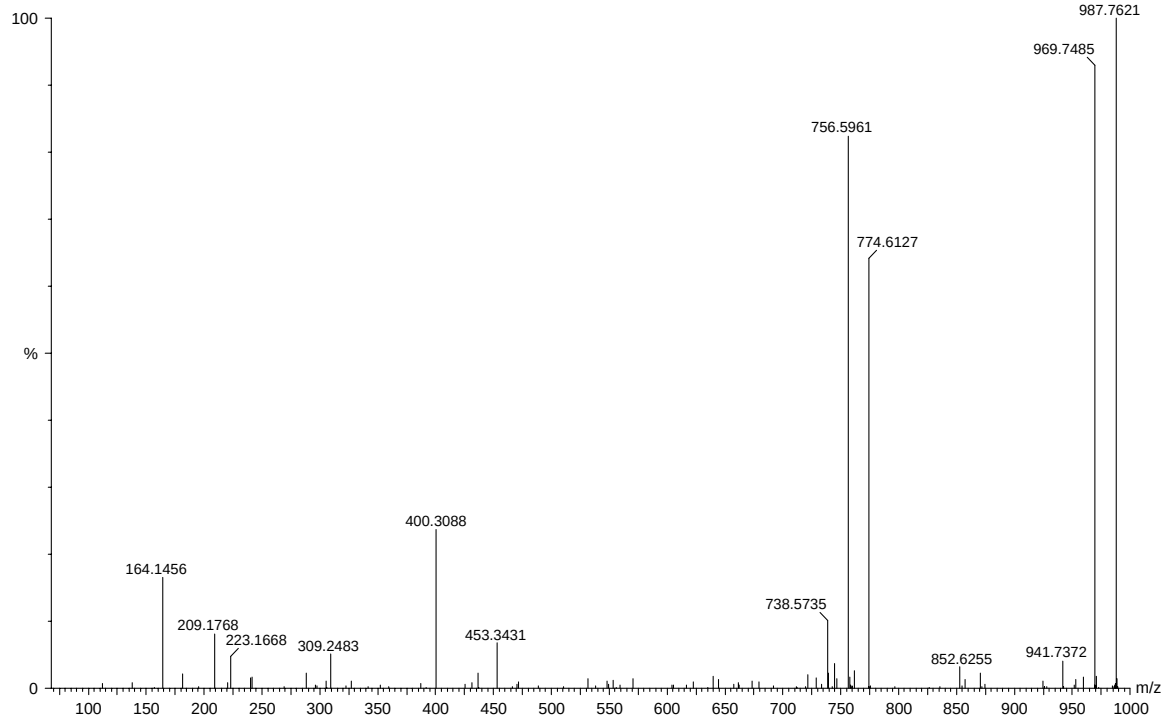

Fig. 4. MS/MS spectrum of *m/z* 987.7 parent ion. Fragmentation was achieved in a Waters® Micromass® Q-Tof micro™ Mass Spectrometer by CID using argon as collision gas in the positive mode. Collision voltages were ramped from 20 to 50 eV and data analyzed by MassLynx 4.0 software. The chosen parent ion (*m/z* 987.7) was from *R. fernandoi* fraction 1 (Rf-F4) collected from RP-HPLC (Rf-F4, Fig. 1).

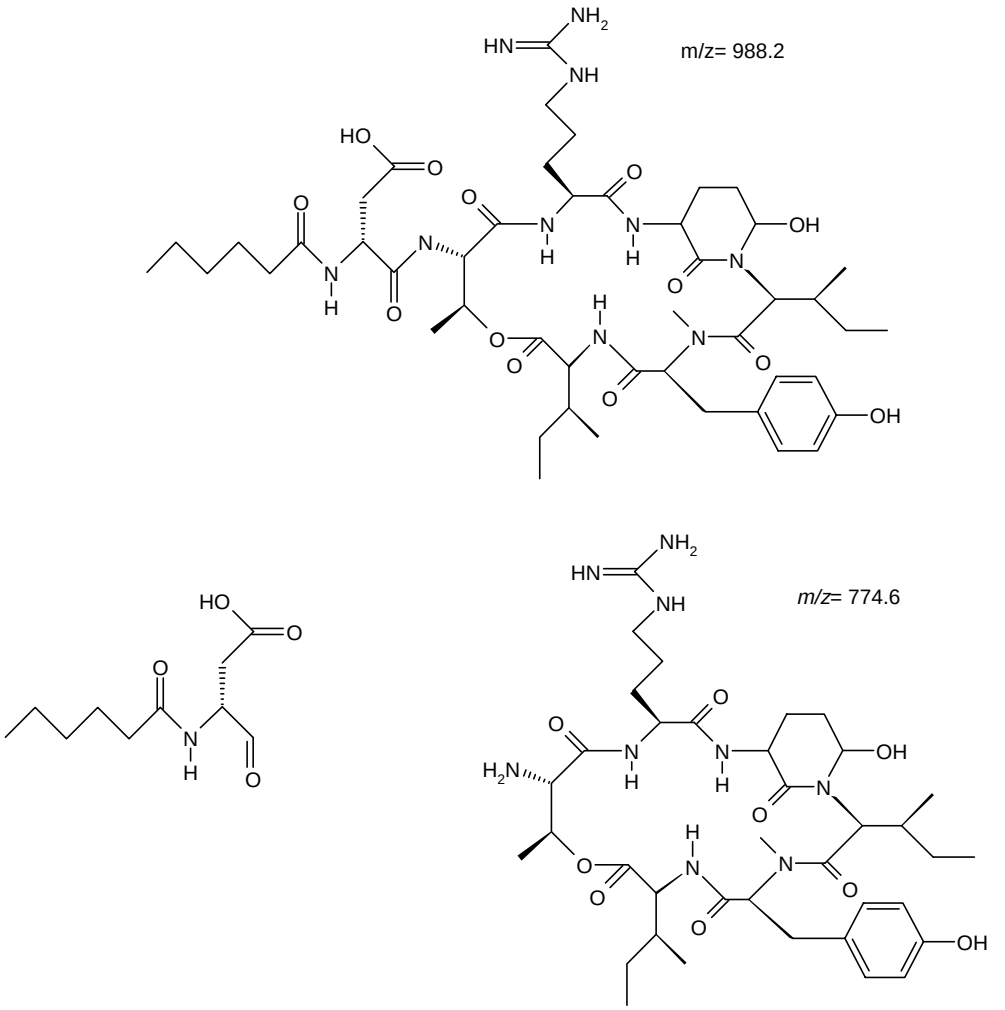

Fig. 5. Structure proposed for Rf-F4 which corresponded to known micropeptin K139 (theoretical $[M+H]^+ = 988.2$). The cleavage of a hexanoyl-glutamyl residue in a micropeptin K139 could originate the observed fragment peak at *m/z* 774.6 (theoretical *m/z*: 774.9) corresponding to $[M+H\text{-}213.1]^+$.

of the pooled fractions (corresponding to 0.0013% of the dried cells).

Finally, the spectrum of Rf-F4 (RT 26.5 min) presented in Fig. 4 shows a major product with $[M+H]^+$ at *m/z* 987.7. The structure that could present such ions fragmentation pattern corresponds to Micropeptin K139, a trypsin inhibitor firstly isolated from *M. aeruginosa*, which possesses molecular mass of 987.2 (theoretical *m/z* 988.2), and molecular formula as $C_{47}H_{74}N_{10}O_{13}$ (Fig. 5) (Harada, 2004; Welker et al., 2004a). The peak observed at *m/z* 774.6 (theoretical *m/z*: 774.9) corresponding to $[M+H\text{-}213.1]^+$ could have originated by the cleavage of a hexanoyl group and the exocyclic glutamic acid residue in Micropeptin K139 (Fig. 5).

From our results we concluded that the production of microcystin LR by cyanobacterium *R. fernandoi* is predominant, confirming its toxicity previously described. Three other oligopeptides were purified and identified as two new tautomers compounds of the aeruginosins family and as a micropeptin, which could probably exert protease inhibition activities in the similar way to those known related compounds. Thus, *R. fernandoi* produces at least three different classes of toxins.

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