

## Bioactive Cyanopeptides Produced by *Sphaerocavum brasiliense* Strains (Cyanobacteria)

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The halogenated compounds aeruginosin and cyanopeptolin (protease inhibitors) are well documented in several cyanobacteria. In this work, the presence of genetic coding for aeruginosin and cyanopeptolin in *Sphaerocavum* genus and their characterization by mass spectrometry and potential toxic effects were investigated. Three strains of *Sphaerocavum brasiliense* (CCIBt 3094, CCIBt 3096 and CCIBt 3316) isolated from different reservoirs in São Paulo State, and one (CCIBt 3179) from Rio Grande do Norte State were selected for this study. Fragments of *aerA-aerB* and *mcnC-mcnE* gene regions (aeruginosin and cyanopeptolin, respectively) were amplified by polymerase chain reaction (PCR) and sequenced. Compounds were characterized by mass spectrometry. All the studied strains presented aeruginosin and cyanopeptolin synthetase genes. Mass spectrometry showed the presence of several aeruginosin and cyanopeptolin variants. The *Allium* test showed that the ethanolic extract promoted toxic and cytotoxic actions in acute and chronic treatments. These naturally occurring protease inhibitors can play an important role in the physiopathology of human diseases.

**Keywords:** aeruginosin, cyanopeptolin, protease inhibitor, mass spectrometry, *Allium* test, cyanobacteria, *Sphaerocavum* genus

## Introduction

Cyanobacteria are known to produce important bioactive compounds, including those with protease inhibition activity.<sup>1</sup> Numerous protease inhibitor variants produced by cyanobacteria have been described, with such names as aeruginosin, aeruginopeptin, aeruginoside, agardhipeptin, anabaenopeptin, anabaenopeptilide, brunsvicamide, cyanopeptolin, cyanostatin, dehydroradiosumin, hofmannolin, insulapeptolide, microcin, microcistilide, microginin, micropeptin, microviridin, oscillapeptin, nodulapeptin, nostocyclin, nostoginin, nostopeptin, oscillagin, oscillamide, oscillapeptilide, planktocyclin, planktopeptin, spumigin, scyptolin, schizopeptin, somamide, spiroidesin, symplostatin and tasipectin.<sup>2,3</sup> These molecules present significant biological activities, since they act against several different proteases that are

involved in a wide variety of physiological processes in the human body, such as cell cycle progression, food digestion, angiogenesis, blood clotting, blood pressure regulation, antigen presentation, inflammation and apoptosis.<sup>4</sup> Consequently, the characterization of novel protease inhibitors derived from natural product biosynthetic pathways is of great relevance.

Results showing toxicological studies for molecules considered non-toxic are scarce, thus it is imperative that such compounds should be characterized for cytotoxicity, genotoxicity and mutagenicity. The United Nations Environment Programme (UNEP) recommended assays with vascular plants.<sup>5</sup> The *Allium cepa* test is one of the analyses used to determine cytotoxicity and is an excellent model for detecting environmental mutagens.<sup>6</sup>

The genus *Sphaerocavum* is mainly found in eutrophic water bodies, and it was observed in Lago das Garças, Clube da Penha and Billings reservoirs in São Paulo State, Brazil, and also in Rodó Lake, Uruguay.<sup>7</sup>

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The aim of this study was to evaluate the presence of genetic coding for aeruginosin and cyanopeptolin in strains of Brazilian genus *Sphaerocavum* and peptides characterized by mass spectrometry. The *Allium cepa* test system was also used to evaluate cytotoxicity, genotoxicity and mutagenicity.

## Experimental

### Cyanobacterial strains and growth conditions

*Sphaerocavum brasiliense* CCIBt 3094 and CCIBt 3316 were isolated from Billings reservoir, CCIBt 3096 from Garças Lake, all of them from São Paulo State and CCIBt 3179 from a reservoir in Rio Grande do Norte State, Brazil. The strains were cultivated in 300 mL of ASM-1 liquid medium<sup>8</sup> at 25 °C under constant illumination by white fluorescent light (40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 20 days.

### Molecular analyses

Cells of *S. brasiliense* CCIBt 3094, CCIBt 3096, CCIBt 3179 and CCIBt 3316 in the late logarithmic phase were harvested and total genomic DNA extracted according to Fiore *et al.*<sup>9</sup> In order to investigate the presence of microcystin synthetase, one region of each of *mcyA* (MSF/MSR),<sup>10</sup> *mcyB* (FAA/RAA),<sup>11</sup> *mcyD* (mcyDF/mcyDR),<sup>12</sup> *mcyE* (mcyEF2/mcyER4)<sup>12</sup> and *mcyG* (mcyGF/mcyGR)<sup>13</sup> genes was chosen. A microcystin producing strain *Microcystis aeruginosa* SPC777<sup>14</sup> was used as a positive control. Aeruginosin and cyanopeptolin synthetase gene regions were amplified using the primer sets *aerA\_F/aerB\_R* and *mcnC\_F/mcnE\_R*,<sup>15</sup> respectively. All resulting polymerase chain reaction (PCR) products were cloned into the pGEM®-T Easy Vector System (Promega) according to the manufacturer's protocol, followed by DH5 $\alpha$  *E. coli* competent-cell transformation,<sup>16</sup> and recombinant plasmids were purified by the alkaline lysis method.<sup>17</sup> DNA sequencing was performed using T7 and SP6 anchored in the pGEM®-T Easy Vector primers, and the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). The cycle sequencing reaction was performed in a Techne TC-412 thermal cycler (Barloworld Scientific), and the PCR condition was 25 cycles of 95 °C for 20 s, 50 °C for 15 s and 60 °C for 60 s. The sodium acetate/ethanol purified reaction was resuspended in HiDi formamide (Life Technologies), and the samples read in an ABI PRISM 3100 Genetic Analyzer (Life Technologies). The sequenced fragments were assembled into contigs using the Phred/Phrap/Consed software package program (Philip Green, University of Washington), and only bases with a quality > 20 were considered. Products

in 5  $\mu\text{L}$  samples of PCR mixture were visualized after electrophoresis on 1% agarose gels by staining with SYBR Green I (Molecular Probes, Life Technologies) (presented in the Supplementary Information).

### Phylogenetic analyses

The DNA sequences of *aerA-aerB* and *mcnC-mcnE* regions obtained herein were compared with sequences available in the National Center for Biotechnology Information (NCBI) database using the BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST>). Cyanobacterial reference sequences retrieved from GenBank were included in the alignment, which was visually refined and used to generate phylogenetic trees. Phylogenetic trees topology analyses were conducted using neighbor-joining (NJ) and maximum likelihood (ML). NJ was performed using PAUP 4.0,<sup>18</sup> and ML was implemented by the MEGA version 5.0 program package.<sup>19</sup> The best-fitting evolutionary model GTR+G+I was selected using ModelTest under the Akaike information criterion.<sup>20</sup> The robustness of the trees was estimated by bootstrap percentages using 1,000 replications. The accession numbers for *aerA-aerB* and *mcnC-mcnE* nucleotide sequences from *S. brasiliense* CCIBt 3094 were KP769528 and KP769524; for CCIBt 3096, were KP769529 and KP769525; for CCIBt 3179, were KP769530 and KP769526, and for CCIBt 3316 were KP769531 and KP769527, respectively.

### Metabolite extraction

After growth, cyanobacterial cultures were harvested by centrifugation at  $9,000 \times g$  at room temperature. Lyophilized cells (20 mg) were extracted three times in 50 mL of methanol, using glass beads ( $\phi$  3 mm).<sup>21</sup> The suspension was agitated for 1 min and after that, centrifuged at  $9,000 \times g$  at room temperature. The supernatant was collected and evaporated. The dried extract was dissolved in sterile distilled water (10 mL) and applied to a pre-conditioned C<sub>18</sub> solid phase extraction (SPE) cartridge (Applied Separation). The cartridge was washed with water ( $2 \times 10$  mL) and cyanopeptides were eluted with 70% methanol.

### Mass spectrometry (MS)

All the dried *S. brasiliense* 70% methanolic extracts were dissolved in a mixture of methanol:formic acid 0.1% (50:50 v/v). The samples were subjected to liquid chromatography coupled with mass spectrometer detector (LC-MS) and the compounds were analyzed using an

Agilent 6410 Triple Quadrupole equipped with Agilent 1200 Series Binary Pump SL pumping system and Agilent 1200 Autosampler (Agilent Technologies). Data acquisition and analysis were performed using Agilent MassHunter Workstation software. The electrospray ionization (ESI) source was employed in positive mode, and set as follows: drying gas was nitrogen heated at 300 °C, gas flow was 10 L min<sup>-1</sup>, capillary voltage was held at 5000 V. The MS/MS parameters were set as follows: dwell time was 200 ms, fragmentor voltage was set at 200 V, the collision gas was nitrogen and collision energy was set according to Table 1. The MS system was operated in full scan and ion precursor mode. The mobile phase A consisted of ultrapure water (Milli-Q) and mobile phase B consisted of methanol, both containing 0.1% (v/v) of formic acid. The flow rate was 0.3 mL min<sup>-1</sup>. Molecular ions were scanned in the range of 100 to 1,800 Da.

#### Allium test

The total cyanobacterial biomass was obtained in 600 mL culture and separated by centrifugation at 9,000 × g for 5 min. Cells were collected and successive extractions with methanol, using glass beads (ϕ 3 mm) to disrupt cells, were performed. The organic extracts were subjected to agitation for 1 min followed by centrifugation at 9,000 × g at 4 °C. The supernatant was then collected and evaporated. For extracellular metabolites, the supernatant was concentrated to 100 mL and subjected to dichloromethane (DCM) extraction using the same volume. The DCM extract was concentrated on a rotary evaporator. The remaining aqueous phase was dried and extracted with ethanol. All the extracts were dried and kept at -20 °C until analysis. Seeds of onion (*Allium cepa*) were pre-soaked for 48 h in petri dishes containing moistened cotton with distilled water and kept at 25 ± 1 °C. An amount of 10 µg of methanol, ethanol and dichloromethane extracts was diluted in Milli-Q water. After root growth of the seeds, they were transferred to petri dishes containing cotton soaked with resuspended extracts. The negative control (Milli-Q water) was also included in the experiment. Root growth and morphological alterations were evaluated for toxicity. For cytotoxicity analysis, the mitotic index was obtained. Five roots were collected after 24 h and 48 h of treatment, fixed in Carnoy solution (3:1, ethanol:glacial acetic acid) and prepared by staining with 2% orcein and crushing the meristematic region. Meristematic cells of five roots (1,000 cells *per* root, 5,000 in total) were analyzed by optical microscopy at 400× magnification and observed for each treatment, taking into account the number of cells in each phase of mitosis. The mitotic index (MI) was obtained by dividing the number of cells in mitosis by the

total number of analyzed cells. The mitotic phase's index was calculated by the sum of cells in each phase divided by the sum of cells in mitosis. The genotoxicity was evaluated by observation of chromosomal damage. The statistical analysis was performed using the BioEstat5.3 program.<sup>22</sup>

#### Trypsin test

The colorimetric Trypsin activity assay kit (Abcam AB 102531) was used for this test and performed according to the protocol described by the manufacturer. Trypsin EC 3.4.21.4 was tested against extracts of *S. brasiliense* CCIBt 3094, CCIBt 3096, CCIBt 3096 and CCIBt 3179 on a 96-well microplate. The extract of *Cylindrospermopsis raciborskii* 339 was used as the negative control. Each assay was performed in duplicate.

## Results and Discussion

#### Molecular evaluation of microcystin, aeruginosin and cyanopeptolin sequences

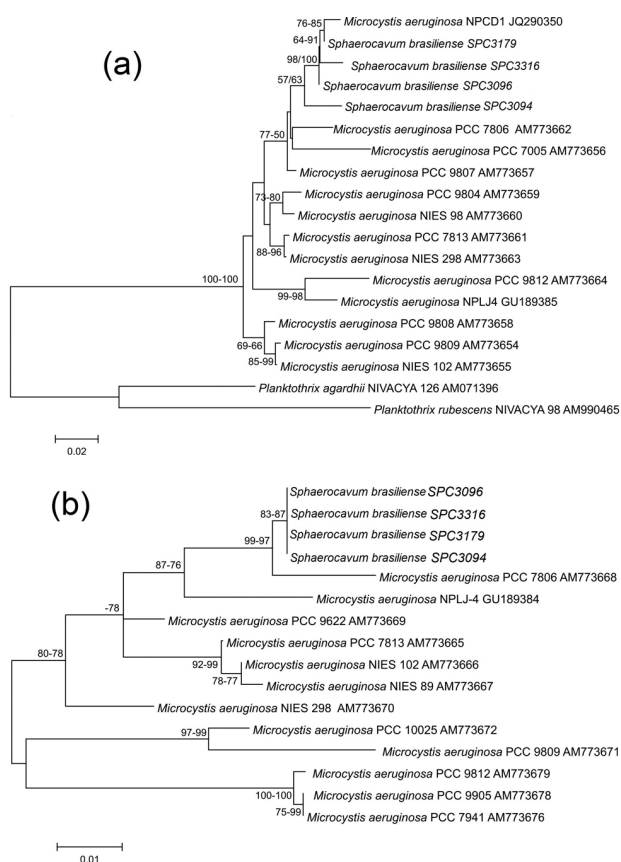
The microcystin synthetase genes (*mcyA*, *mcyB*, *mcyD*, *mycE* and *mcyG*) were chosen as the target sequences to detect the presence of the *mcy* gene cluster in *S. brasiliense* strains. Considering the *mcy* genes amplification for *M. aeruginosa* SPC777 (positive control), the specific primers for the microcystin synthetase genes failed to produce amplicons for all *S. brasiliense* strains.

Previously, we reported the presence of aeruginosin and cyanopeptolin synthetase genes for *S. brasiliense* CCIBt3094, CCIBt3096 and CCIBt3316.<sup>4</sup> In this study all four *Sphaerocavum* strains evaluated presented positive PCR results for aeruginosin and cyanopeptolin synthetase genes. Fragments of 988 bp were obtained from the intergenic region between *aerA* and *aerB* genes, and 586 bp between *mcnC* and *mcnE* genes. The *aerJ* and *mcnD* genes were not found in the obtained sequences.

The phylogenetic tree constructed using the aeruginosin sequences showed that all the *Sphaerocavum* strains grouped in a clade together with a Brazilian *M. aeruginosa* (NPCD-1) strain, a non microcystin producer, while the phylogeny constructed for cyanopeptolin sequences showed that the *Sphaerocavum* strains grouped in a well-defined clade with a *M. aeruginosa* PCC7806, isolated from Braakman reservoir, Netherlands, as the closest lineage (Figure 1).

#### Mass spectrometry

The MS spectrum of the protonated molecule for the methanolic extract confirmed the production of



**Figure 1.** Maximum likelihood topology of aeruginosin in (a) and cyanopeptolin in (b) phylogenetic trees. Number in nodes represents bootstrap of maximum likelihood and neighbor joining, respectively.

several protease inhibitors in the purified extract. These compounds were identified as aeruginosin ( $m/z$  669 and 691), micropeptin ( $m/z$  945 and 959) and cyanopeptolin ( $m/z$  973) for *S. brasiliense* CCIBt 3094. Strains of *S. brasiliense* CCIBt 3096 and CCIBt 3316 showed ions at  $m/z$  653 and 685 corresponding to aeruginosin. Although the molecular results showed that *S. brasiliense* CCIBt 3179 has the genes that encode for aeruginosin and cyanopeptolin, the presence of these compounds, analyzed by mass spectrometry, was not confirmed. Figure 2 shows the fragmentation ions observed for these protease inhibitors, according to different energy collision used. Microcystin molecules were not observed by mass spectrometry analysis.

According to the fragmentation data generated by mass spectrometry (Figures 2a to 2i), aeruginosin ( $m/z$  669, 653 and 685) presented the ions at  $m/z$  140, corresponding to the Choi immonium-(acid 2-carboxy-6-hydroxyoctahydroindole),  $m/z$  221 (Leu-Choi fragment),  $m/z$  266 (Choi-Argininal- $\text{CH}_3\text{N}_2\text{-H}_2\text{O} + \text{H}$ ) and  $m/z$  309 (Choi-Argininal- $\text{NH}_2 + \text{H}$ ), all indicative of aeruginosin. Aeruginosin  $m/z$  669 presented one chlorine ( $m/z$  284) in the portion and aeruginosin ( $m/z$  691) is characterized by

2-carboxy-6-hydroxyoctahydroindole (Choi) moiety and also presented one chlorine. Micropeptin ( $m/z$  945) is well identified by the ion  $m/z$  150 (MTyr-immonium),  $m/z$  209 (Ahp-Leu- $\text{H}_2\text{O} + \text{H}$ ) and  $m/z$  386 (Ahp-Leu-MTyr- $\text{H}_2\text{O} + \text{H}$ ). The other micropeptin ( $m/z$  959) is represented by the ions  $m/z$  181 (Ahp-Leu- $\text{H}_2\text{O-CO} + \text{H}$ ) and  $m/z$  209 (Ahp-Leu- $\text{H}_2\text{O} + \text{H}$ ). Cyanopeptolin ( $m/z$  973) was supported by the ions  $m/z$  70 (Arg fragment) and  $m/z$  150 (MTyr-immonium). Table 1 shows the assignment ions for aeruginosin, cyanopeptolin and micropeptin according to the collision energy used to fragment all the molecules.

### *Allium cepa* test

Different extracts obtained from *Sphaerocavum* strains were evaluated for their potential toxic effects in *Allium cepa*. Morphological alterations in roots such as tumor and hook formations and meristem tip necrosis were not observed after treatments (Figure 3).

On the other hand, the ethanolic extract caused an inhibition of the roots growth in acute and chronic treatments (Figure 4a). This effect was reflected in the mitotic index, where a decrease in the number of cells in mitosis was observed (Figure 4b). In this case, metaphase was the most affected mitotic phase. Thus, both acute and chronic treatments with ethanolic extract promoted a reduction of cells in this phase (Figure 4c).

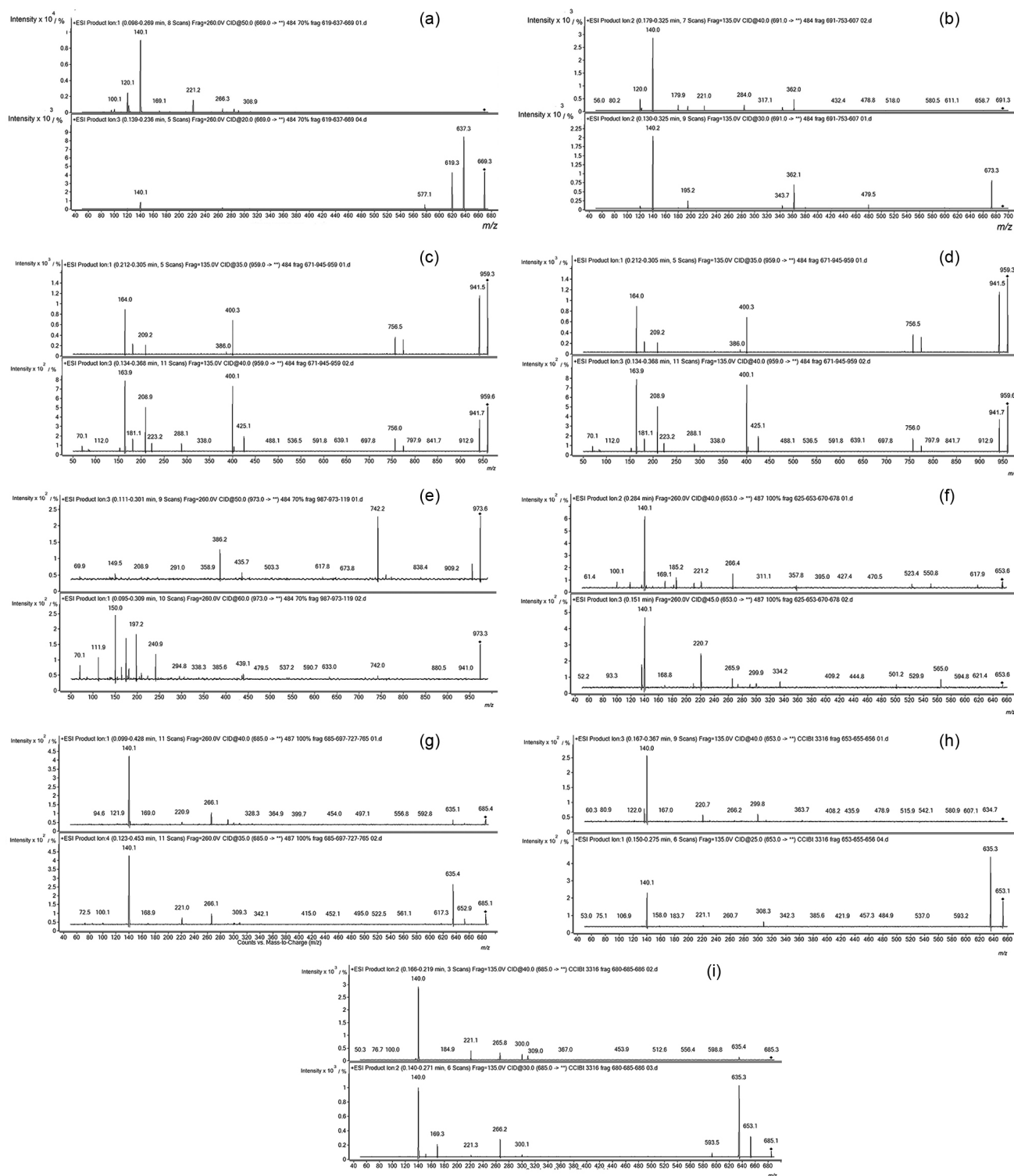
An interesting finding was that the roots treated with dichloromethane extract after 48 h did not present cells in mitosis demonstrating a cytotoxic effect (Figure 5), even though the roots continued growing, probably by cell elongation. Chromosomal damage was not observed for all treatments.

### Trypsin test

Trypsin (EC 3.4.21.4) was tested against the extracts listed in Table 2. All the extracts were positive for the presence of protease inhibitor, except for *S. brasiliense* CCIBt 3179 and *C. raciborskii* 339 extracts. Extracts from *S. brasiliense* CCIBt 3094, CCIBt 3096, and CCIBt 3316 tested for trypsin inhibition showed inhibitory activity from 56 to 77%.

*Sphaerocavum* is a tropical and subtropical genus proposed by Azevedo and Sant'Anna<sup>7</sup> from samples collected from eutrophic reservoirs in São Paulo State, and also from Uruguai. Since the genus was proposed, *S. brasiliense* was reported for eutrophic lakes in different parts of the world.<sup>24,25</sup> *Sphaerocavum brasiliense* CCIBt 3094, isolated from Billings reservoir, in São Paulo State, is the type species and belongs to the family



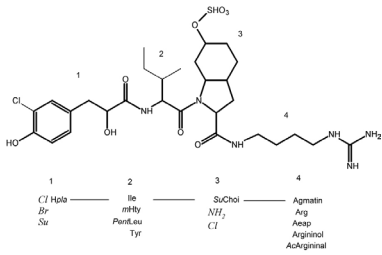


**Figure 2.** ESI-MS spectra of *Sphaerocavum* strains 70% methanolic extracts in the positive mode (precursor mode). *S. brasiliense* CCIBt 3094: in (a)  $m/z$  669 fragmentation (CE = 50 and 20 V); in (b)  $m/z$  691 fragmentation (CE = 40 and 30 V); in (c)  $m/z$  945 fragmentation (CE = 35 and 30 V); in (d)  $m/z$  959 fragmentation (CE = 35 and 40 V); in (e)  $m/z$  973 fragmentation (CE = 50 and 60 V). *S. brasiliense* CCIBt 3096: in (f)  $m/z$  653 fragmentation (CE = 40 and 45 V); in (g)  $m/z$  685 fragmentation (CE = 40 and 35 V). *S. brasiliense* CCIBt 3316: in (h)  $m/z$  653 fragmentation (CE = 40 and 25 V); in (i)  $m/z$  685 fragmentation (CE = 40 and 30 V). CE describes the collision energy.

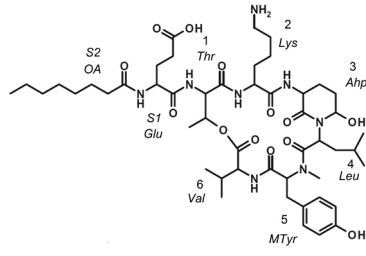
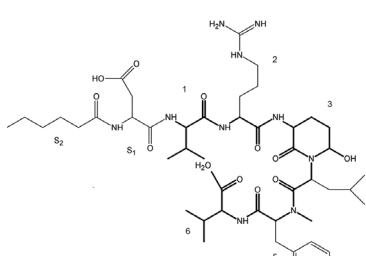
Merismopediaceae, subfamily Merismopedioideae due to the two plains of cell division and slightly flattened, but not flat colonies.

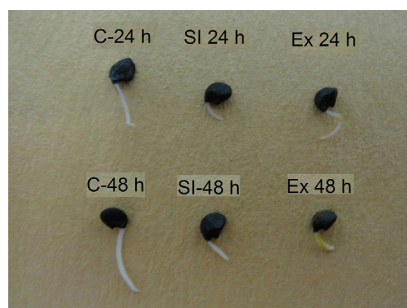
The genetic coding for the protease inhibitors aeruginosin and cyanopeptolin synthetase and their production were confirmed for the evaluated *Sphaerocavum* strains. These

**Table 1.** Assignment of cyanopeptide fragmentations

Cyanobacteria	Collision energy / V	Fragmentation ions / $m/z$	Fragment assignment	Chemical structure	Reference
<i>S. brasiliense</i> CCIBt 3094	20	669	–		Aeruginosin structure and ions assignments adapted from Welker and von Döhren <sup>2</sup>
		637	M-CH <sub>3</sub> OH		
		619	M-CH <sub>3</sub> OH-H <sub>2</sub> O + H		
		577	M-C <sub>2</sub> H <sub>5</sub> O		
	50	309	Choi-Argininal-NH <sub>2</sub> + H		
		291	Choi-Argininal-NH <sub>2</sub> -H <sub>2</sub> O + H		
		266	Choi-Argininal-CH <sub>3</sub> N <sub>2</sub> -H <sub>2</sub> O + H <sup>a</sup>		
		284	Cl-Hpla-Leu-CO + H		
		221	(Leu-Choi) fragment		
		140	Choi-immonium ion		
	30	100	Argal-fragment		
		691	–		
		673	M-H <sub>2</sub> O + H		
		344	CH <sub>3</sub> OH-H <sub>2</sub> O + H		
		284	Cl-Hpla-Leu-CO + H		
	40	221	(Leu-Choi) fragment		
		140	Choi-immonium ion		
<i>S. brasiliense</i> CCIBt 3096	40	653	–		
		266	Choi-Argininal-CH <sub>3</sub> N <sub>2</sub> -H <sub>2</sub> O + H <sup>a</sup>		
		221	(Leu-Choi) fragment		
		169	Choi + H		
		140	Choi-immonium ion		
		100	Argal-fragment		
	35	685	–		
		309	Choi-Argininal-NH <sub>2</sub> + H		
		291	Choi-Argininal-NH <sub>2</sub> -H <sub>2</sub> O + H		
		266	Choi-Argininal-CH <sub>3</sub> N <sub>2</sub> -H <sub>2</sub> O + H <sup>a</sup>		
		221	(Leu-Choi) fragment		
	25	140	Choi-immonium ion		
		100	Argal-fragment		
		653	–		
		635	M-H <sub>2</sub> O + H		
<i>S. brasiliense</i> CCIBt 3316	40	266	Choi-Argininal-CH <sub>3</sub> N <sub>2</sub> -H <sub>2</sub> O + H <sup>a</sup>		
		221	(Leu-Choi) fragment		
		140	Choi-immonium ion		
		685	–		
		653	M-CH <sub>3</sub> OH		
		635	653-H <sub>2</sub> O		
		593	653-CH <sub>3</sub> OH-H <sub>2</sub> O + H		
		309	Choi-Argininal-NH <sub>2</sub> + H		
		266	Choi-Argininal-CH <sub>3</sub> N <sub>2</sub> -H <sub>2</sub> O + H <sup>a</sup>		
		221	(Leu-Choi) fragment		
		169	Choi + H		
		140	Choi-immonium ion		

**Table 1.** Assignment of cyanopeptide fragmentations (cont.)

Cyanobacteria	Collision energy / V	Fragmentation ions / $m/z$	Fragment assignment	Chemical structure	Reference
	30	945	—		Micropeptin structure and ions assignments adapted from Czarnecki <i>et al.</i> <sup>23</sup>
		927	M-H <sub>2</sub> O + H		
		386	(Ahp-Leu-MTyr)-H <sub>2</sub> O + H		
		209	(Ahp-Leu)-H <sub>2</sub> O + H		
		150	MTyr-immonium		
	35	959	—		
		941	M-H <sub>2</sub> O + H		
	40	386	(Ahp-Leu-MTyr)-H <sub>2</sub> O + H		
		209	(Ahp-Leu)-H <sub>2</sub> O + H		
		181	(Ahp-Leu)-H <sub>2</sub> O-CO + H		
<i>S. brasiliense</i> CCIBt 3094	50	973	—		Cyanopeptolin structure and ions assignments adapted from Welker and von Döhren <sup>2</sup>
		386	(Ahp-Leu-MTyr)-H <sub>2</sub> O + H		
		209	(Ahp-Leu)-H <sub>2</sub> O + H		
	60	181	(Ahp-Leu)-H <sub>2</sub> O-CO + H		
		150	MTyr-immonium		
		70	Arg fragment		

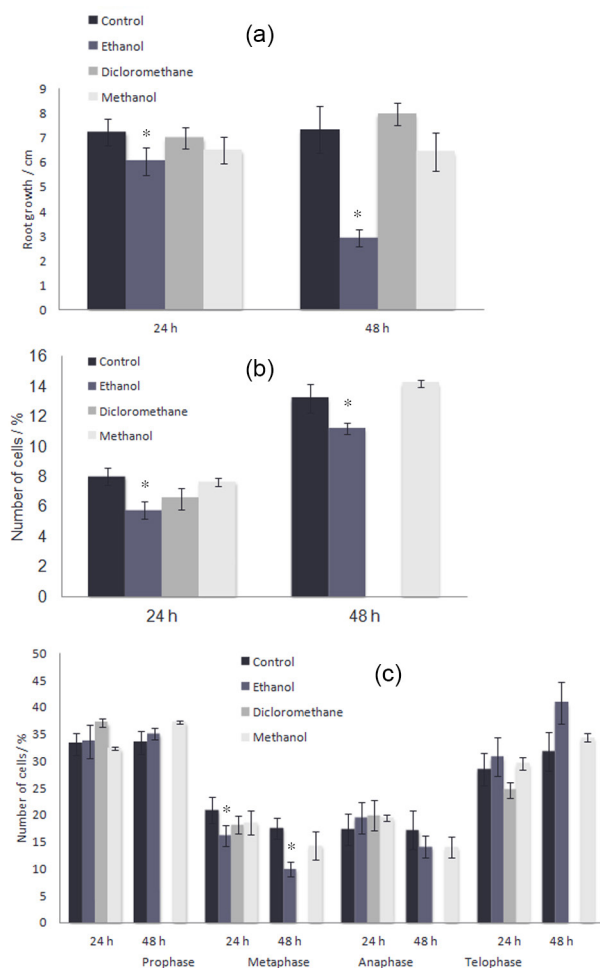
<sup>a</sup>CH<sub>3</sub>N<sub>2</sub>-ureido-group of arginal.**Figure 3.** *Allium cepa* roots treated with extract of *Sphaerocavum brasiliense* CCIBt 3094 for 24 h and 48 h. Legend: C: negative control; SI: solvent; Ex: extract.

peptides inhibit serine proteases<sup>26</sup> making them an important molecule for the development of drugs against various human diseases. Aeruginosins have been tested against several proteases, such as thrombin, trypsin and plasmin. Processes of trypsin inhibition are considered of interest for the treatment of pancreatic disorders such as pancreatitis.<sup>27</sup> Cyanopeptolins are known as trypsin, chymotrypsin, elastase, plasmin, kallikrein and factor XI inhibitors<sup>28</sup> and can be used to treat asthma and viral infections.<sup>29</sup> In addition, some cyanopeptolin variants are known as specific elastase inhibitors being critical for the treatment of emphysema,

which is mediated by the action of excessive elastase.<sup>30</sup> The high level of trypsin inhibition observed for the extracts of *S. brasiliense* CCIBt 3094, CCIBt 3096, and CCIBt 3316 demonstrates a great potential for bioprospection of these strains. *Sphaerocavum brasiliense* CCIBt 3179 presented no inhibitory activity at the tested concentration. MS analysis of the extract of this strain presented no protease inhibitor production, which suggests that despite the positive results in molecular analysis for aeruginosin and cyanopeptolin synthetase genes, they were somehow not expressed.

Genotoxicity studies are important for testing new drugs,<sup>31–33</sup> and they must be performed in the early stages in order to predict potential genotoxic and/or carcinogenic activity and to assist in obtaining new chemical structures less toxic.<sup>31,34</sup> Plant bioassays were validated for testing complex mixtures for *in situ* monitoring and laboratory testing of the genotoxicity of environmental pollutants.<sup>35</sup> Herein, the *Sphaerocavum* ethanolic and dichloromethane extracts showed a potential cytotoxic effect in the *Allium* test. This fact could be linked to the several biocompounds that are produced by cyanobacteria. However, they did not present genotoxic effects.

Aeruginosins and cyanopeptolins are synthesized by the non-ribosomal pathway and can be found as non- mono-



**Figure 4.** Toxicity and cytotoxicity of *S. brasiliense* extracts in *A. cepa* system. Root growth in (a), mitotic index in (b) and mitotic index phase of meristematic cells treated for 24 and 48 h with ethanolic, dichloromethane and methanolic extracts in (c).

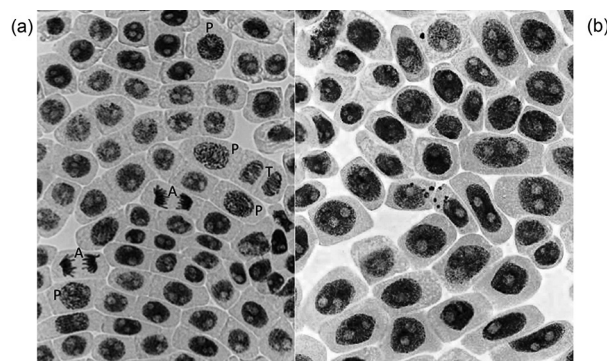
or di-chlorinated molecules.<sup>15</sup> The presence of chlorine in aeruginosin and cyanopeptolin reveals the potential for inhibition activities.<sup>36</sup> In this study cyanopeptides produced by the genus *Sphaerocavum* were found as chlorinated molecules which make them attractive drug targets.

## Conclusions

Protease inhibitors are important biomolecules in many biological processes and are related to several human

**Table 2.** Cyanobacterial extracts with inhibitory trypsin activity

Sample	Extract concentration / $\mu\text{g}$	Trypsin activity / ( $\text{mU mL}^{-1}$ )	Inhibition / %
Negative control ( <i>C. raciborskii</i> 339)	44.6	174.59	0
<i>S. brasiliense</i> CCIBt3094	42.8	25.66	76
<i>S. brasiliense</i> CCIBt3316	53.5	47.09	56
<i>S. brasiliense</i> CCIBt3096	27.0	23.90	77
<i>S. brasiliense</i> CCIBt3179	27.2	129.80	0



**Figure 5.** *Allium cepa* test for potential cytotoxic, genotoxic and mutagenic effects caused by *S. brasiliense* extracts. Negative control in (a), and dichloromethane extract after 48 h treatment in (b). P = prophase, A = anaphase, T = telophase.

diseases such as thrombosis or cancer. Therefore, efforts have been made to characterize these protease inhibitors by identifying their structure, biosynthesis, physiology, bioactivity, toxicology and factors that regulate their production. New peptides, as well as new congeners of such biomolecules are of great interest. This study presented information on the chemical characterization, molecular genetics and biological activity of the peptide protease inhibitors aeruginosin and cyanopeptolin produced by *Sphaerocavum* genus. The report of metabolites with pharmacological potential produced by *Sphaerocavum* shows the great potential of these cyanobacteria in bioprospecting studies. Also, no microcystin was observed for the evaluated strains, making these strains very promising for drug discovery.

## Supplementary Information

Supplementary information (SYBR Green-stained agarose gel of polymerase chain reaction (PCR) products obtained from CCIBt 3094, 3096, 3179 and 3316 aeruginosin specific primers) is available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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