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Research Letter

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RESEARCH LETTER – Biotechnology & Synthetic Biology

Gloeosporiocide, a new antifungal cyclic peptide from *Streptomyces morookaense* AM25 isolated from the Amazon bulk soil

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One sentence summary: A novel antifungal compound produced by an Amazon actinobacteria (*Streptomyces morookaense* AM25) and its *in vitro* activity.

Editor: Stefan Olsson

ABSTRACT

Actinobacteria are known by their ability to produce several antimicrobial compounds of biotechnological interest. Thus, in this study, we isolated and identified by partial 16S RNA sequencing ~100 actinobacteria isolates from guarana (*Paullinia cupana*) bulk soil. Besides, we isolated from the actinobacteria *Streptomyces morookaense* AM25 a novel cyclic peptide, named gloeosporiocide, molecular formula $C_{44}H_{48}N_{11}O_7S_3$ (calculated 938.2901), and characterized by the presence of cyclized cysteins to form three thiazols. The novel compound had activity against the plant pathogen *Colletotrichum gloeosporioides*, assayed by the paper disk diffusion method (42.7% inhibition, 0.1 mg disk^{-1}) and by the microdilution assay (1.25 g L^{-1}). Our results reveal the potential of the actinobacteria from the Amazon rhizospheric soils as biocontrol agents as well as producers of new compounds with antifungal activity. Thus, this work constitutes a step forward in the development of the biotechnology of actinobacteria in the production of compounds of agronomic interest.

Keywords: actinobacteria; anthracnose; *Colletotrichum gloeosporioides*; guarana; *Paullinia cupana*

INTRODUCTION

Actinobacteria are widely distributed in terrestrial and aquatic ecosystems. This group is an important source of secondary metabolites (McIntosh, Donia and Schmidt 2009; Zhao *et al.* 2018). Compared to other bacteria, actinobacteria stand out as a rich source of structurally complex peptides. These classes of compounds exhibit known potential activity against several pathogens (Zhao *et al.* 2018). Biologically active cycle peptides produced by the genus *Streptomyces* include cyclomarin

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A (Renner *et al.* 1999), piperazimycins (Miller *et al.* 2007), ohmyungsamycins A and B (Um *et al.* 2013) and desotamide B (Song *et al.* 2014), among others. The efforts to discover new cyclic peptides may provide the improvement of plant diseases control, which depends massively on conventional biocides (Lee and Kim 2015).

Among hundreds of plant pathogens, the genus *Colletotrichum* was classified as the eighth most important plant pathogen in the world from the economic and scientific point of view (Dean *et al.* 2012). It is one of the causal agents of anthracnose disease along with the genus *Gloeosporium* (Greene 1969). This disease infects several plants of agronomic interest, such as cereals, legumes, vegetables, ornamental plants and various fruits (Dean *et al.* 2012; Oliveira *et al.* 2018). Despite the wide geographic incidence of this plant pathogen, the greatest damage is recorded in tropical and subtropical regions, due to the hot and humid environment (Sharma, Maymon and Freeman 2017). For instance, guarana productivity has been strongly affected by the presence of the anthracnose disease caused by *Colletotrichum gloeosporioides* (Bonatelli *et al.* 2016; Silva *et al.* 2016).

Anthracnose in guarana is characterized by the appearance of rounded necrotic orange-colored lesions on leaf surfaces (Silva *et al.* 2006). In general, plants can be infected in a highly aggressive way at any stage of development (Jeffries *et al.* 1990) and the main symptoms observed are necrotic lesions on leaves, stems, flowers and fruits (Ojola *et al.* 2018).

Due to the importance and the economic losses of the anthracnose to agriculture, the control of this disease is done with intensive use of fungicides (He *et al.* 2019). However, the excessive use of certain agrochemicals leads to the selection of resistant plant pathogens and the increased severity of some diseases (Feng, Zhang and Zhang 2019), in addition to increasing environmental contamination (Carneiro, Ribeiro and Bastos 2019). Therefore, it is necessary to search for new antifungal compounds against *Colletotrichum* spp., mainly in negligence tropical crops.

Thus, the current research aimed to isolate and identify actinobacteria from guarana (*Paullinia cupana*) bulk soil in Amazon with activity against *C. gloeosporioides* in order to discover a new antifungal compound.

MATERIALS AND METHODS

General procedures

Optical rotations were measured on a Polarimeter (Polartronic H Schimidt + Haensch, Berlin, Germany) at 20°C in a 1dm tube . The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data were recorded on a Bruker 14.1 Tesla, AVANCE III model (Billerica, USA) spectrometer (operating at 600.13 MHz for hydrogen frequency) with cryoprobe. The chemical shifts were given on a δ (ppm) scale and referenced to the tetramethylsilane (TMS). Correlated spectroscopy (H, H-COSY), Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments were analyzed using the TopSpin 3.2 version. NMR spectra were obtained in d_6-DMSO and kept the temperature at 298 K during all the experiments. The high-resolution mass spectrum (UPLC-EM) was acquired under the following conditions: the mass spectrometer used was Waters® XEVO G2-XS QTOF (Milford, EUA) in positive and negative mode, with ZSpray detector (ESI/APCI/ESCi®), detection range between 100 to 1200 Da and ionization energy of 30 eV. In high-performance liquid chromatography (HPLC) the Agilent® HP 1100 (UV/Vis series, Waldbronn, Germany) was used with a quaternary pump coupled to an ultraviolet detector MWD (Multiple Wavelength Detector), C_{18} column (Phenomenex® Kinetex, 5 μm, 250 × 4.6 mm, Torrance, EUA). The mobile phase H_2O:acetonitrile (50:50, v/v), with flow 1 mL min^{-1} and wavelength (λ) of 230 nm. The solvents used were HPLC grade. Solid phase extraction was performed using prepacked Solid Phase Extraction (SPE) (Phenomenex Strata®, Torrance, EUA) columns of different size and stationary phase of silica gel Alugram® TLC sheets of silica-gel_{254} (Macherey-Nagel, Düren, Germany) were used in thin layer chromatography and inspected in ultraviolet light (254 and 365 nm).

Isolation of the actinobacteria

Bulk soil was collected at the Experimental Farm of the Federal University of Amazonas, located in Manaus-AM, Brazil, in November 2010. For the isolation, bulk soils were sampled from adult guarana plants (02° 39 '41.4"S and 60° 07 '57.5"W), all belonging to the same clone (series 800), grown on the same soil type and under the same climatic conditions. For the isolation of the actinobacteria, 10 grams of soil from each sample were then weighed into Erlenmeyer flasks containing 90 mL of phosphate-buffered saline (PBS) solution (0.8% NaCl; 0.02% KCl; 0.14% Na_2HPO_4 and 0.024% KH_2PO_4; pH adjusted to 7.4). Samples were packed in a shaker (150 rpm) for 1 h at room temperature. Then, 100 μL were distributed in 1.5 mL microtubes containing 900 μL of sterile saline solution (0.85 M NaCl) to perform the serial dilution, and performed up to 10^{-6}. Fifty microliters of each dilution were scattered with a Drigalski loop in Petri dishes containing ACA culture medium (1% starch; 0.03% casein; 0.2% KNO_3; 0.2% NaCl; 2% K_2HPO_4; 0.005% $MgSO_4$; 0.001% $FeSO_4$ and 1.5% agar) and benomyl fungicide (50 μg mL^{-1}). Five replicates were performed for each dilution. Plates were incubated at 28°C for a period of 7 to 10 days. Purification of the colonies was performed by cross-streaking in Petri dishes containing the ACA medium and isolated colonies were selected. Isolated actinobacteria were preserved in 60% glycerol using the cryopreservation technique. The *actinobacterium* (AM25 strain) is deposited under the code CMAA 1683 in the Collection of Microorganisms of Agricultural and Environmental Importance—CMAA, EMBRAPA Environment (São Paulo- Brazil) registered in World Data Center for Microorganisms—WDCM under number WDCM 1149.

Evaluation of antagonistic bacteria

Actinobacteria were evaluated for activity toward the plant pathogen *C. gloeosporioides* L1 (Bonatelli *et al.* 2016; Silva *et al.* 2016) by a dual-culture *in vitro* assay onto Potato Dextrose Agar (PDA) plates. Each Petri plate was inoculated on the borders with a PDA plugs (2 cm in diameter) from actinobacteria and L1, respectively. The diameter of the inhibition areas was measured after 7 days of incubation at 28°C according to Quecine *et al.* (2016). All the strains were tested in four independent replicates. Scott-Knott tests were performed in Statistical Analysis Software (SAS) Program for Windows (SAS Institute Inc., Cary, NC) and the significance level adopted in all tests was 5%.

The actinobacterium (AM25 strain) showing high antagonistic activity toward *C. gloeosporioides* had its active compound isolated and molecularly identified. Moreover, the selected strain was evaluated against other plant pathogens (*Colletotrichum sublineolum, Fusarium verticillioides, Fusarium oxysporum, Rhizoctonia solani* and *Ceratocystis paradoxa*) obtained from culture collection 'Laboratory of Genetics of Microorganisms', Department of

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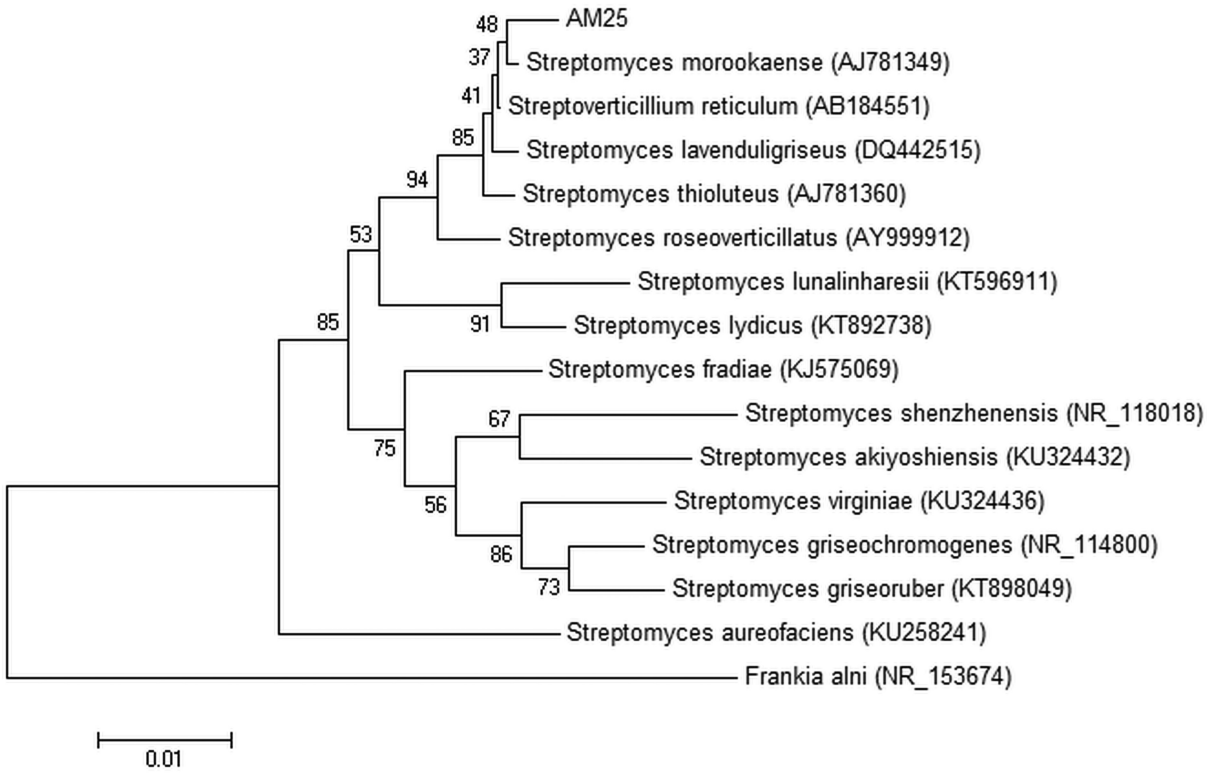

Figure 1. Neighbor-joining similarity tree of partial 16S rDNA gene sequences of representative sequences of species belonging to the genus *Streptomyces*, obtained from Genbank nucleotide sequence database and AM25 (this study). The dendrogram was constructed with MEGA version 6.0 program. *Frankia alni* was used as an outgroup organism. The bars indicate the number of substitutions per site and the robustness of the tree was tested by bootstrapping with 1000 replicates of data.

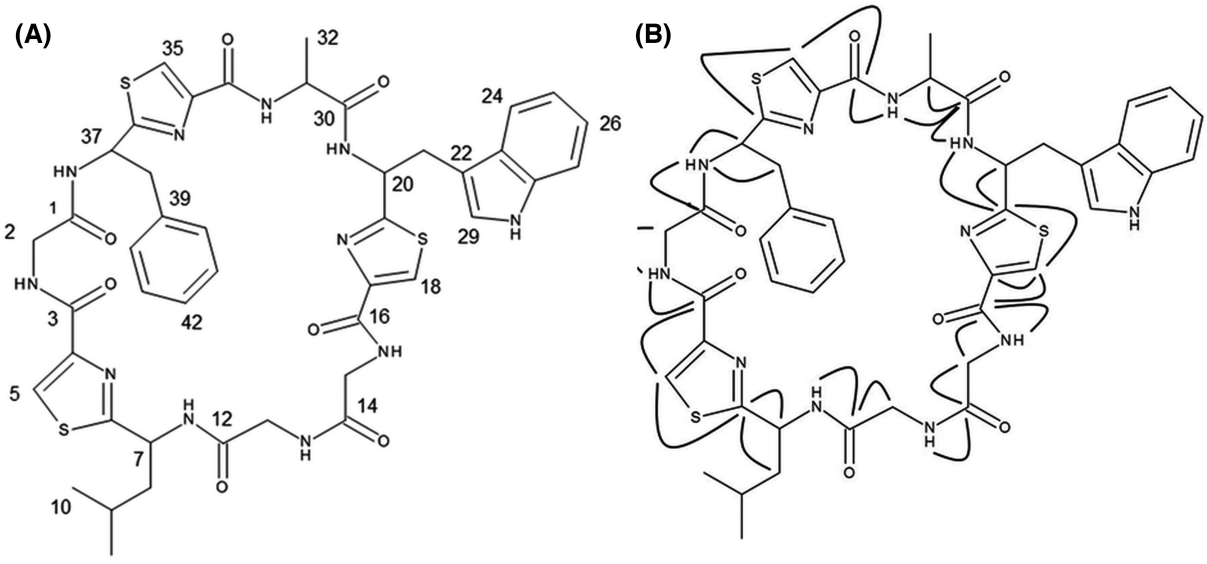

Figure 2. Structure of the antibiotic gloeosporiocide A4C5C2 with numbering of the carbons **(A)** as well as the long-range couplings indicative for structure elucidation, derived from HMBC NMR **(B)**.

Genetics, ESALQ/USP, Piracicaba, SP, Brazil. The phytopathogens were maintained in PDA at room temperature.

The AM25 antagonistic ability against other phytopathogens was assessed using a rating scale (Badalyan, Innocenti and Garibyan 2002) for three main types of interactions: types A and B were classified as deadlock or mutual inhibition, in which neither organism was able to overgrow the other at mycelial contact (A) or at distance (B), and type C was characterized by the overgrowth between the fungi (replacement), without initial deadlock.

Molecular identification

The pure cultures of actinobacteria were grown in liquid TSB medium and incubated at 28°C under constant stirring at 150

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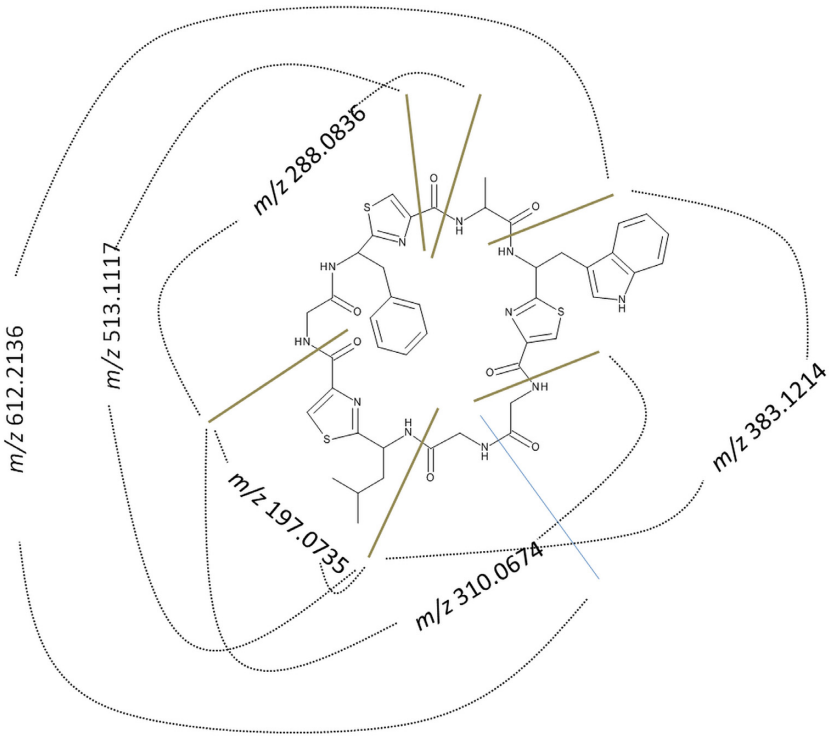

Figure 3. Indicative fragments observed in HR-MS/MS of gloeosporiocide A4C5C3.

rpm for 7 days. After growth, the medium was centrifuged and subjected to DNA extraction according to Khoodoo, Issack and Jaufeerall-Fakim (2002). The amplification reaction for the bacterial 16S rDNA gene was performed using the universal primers for the bacterial domain R1387 and PO27F (Heuer *et al.* 1997).

The actinobacteria had the 16S rDNA partially sequenced, and was performed by the Animal Biotechnology Laboratory, in the Department of Animal Science—ESALQ-USP. The 16S rDNA partial sequences obtained in the sequencing were visualized and edited using the MEGA 6 software (Tamura *et al.* 2013) and analyzed in the database NCBI web server (www.ncbi.nlm.nih.gov), employing the BLASTN tool (BLAST—Basic Local Alignment Search Tool—program (http://www.ncbi.nim.nih.gov/blast/) (Altschul *et al.* 1990). The AM25 strain assayed for characterization of antifungal biomolecule were completely 16S rDNA-sequenced using the 16S rDNA internal primers proposed by Thompson *et al.* (2001).

All nucleotide sequences of the actinobacteria strains were deposited in the GenBank Database under the accession number (MK483220- MK483255, MK038762).

Isolation of the active compound

AM25 strain was cultured in PD culture medium (39 g L^{-1} PD-Acumedia, Neogen, Indaiatuba, Brazil) at 28°C under constant shaking (150 rpm) for 7 days (15 L, 100 × 150 mL in 250 mL Erlenmeyer flasks). After AM25 growth, the metabolic extract was subjected to chromatographic separation in C_{18} Sep-Pak column. Evaporation of the solvent from the extract under vacuum gave the methanolic fraction A4 (403.2 mg). This fraction was separated on a silica gel Sep-Pak column (10 g), with a mobile phase in gradient of dichloromethane, ethyl acetate and methanol. From this separation, five fractions were obtained (A4A-A4E). The bioactive fraction A4C (247.6 mg) was subjected to a new fractionation by chromatography on silica gel column (10 g), with a mobile phase in gradient of dichloromethane, ethyl acetate and methanol. From this chromatographic separation six fractions (A4C1-A4C6) were obtained. The active fraction A4C5 (146.5 mg) was fractionated in a C_{18} column (10 g), with mobile phase in gradient of water and methanol. From this separation five fractions were obtained (A4C5A-A4C5E). The active fraction A4C5C (21.5 mg) was purified on HPLC with C_{18} column and mobile phase in gradient of H_2O: Acetonitrile (50:50) over a period of 20 minutes, yielding three fractions (A4C5C1-A4C5C3), being the active fraction code A4C5C2 (retention time: 4.22 min), 1.7 mg.

Antifungal assays with AM25 compounds

Paper disk diffusion method

In this inhibitory activity assay, a 5 mm diameter disk of L1 was inoculated into one of the margins of a Petri dish containing PDA medium, at a distance of 1.5 cm from the border. At the opposite edge, it was deposited on a 6 mm disk of sterile filter paper in which the fractions to be evaluated were applied. The control plates contained only the plant pathogen, under the same conditions described above. Treatments and controls were maintained in BOD at 28°C for a period of 7 days. After, the plates were photographed and the % inhibition of each fraction tested was evaluated using Image Processing and Analysis in Java (Image J) program.

Microdilution assays

The pure compound was evaluated for activity against spores *C. gloeosporioides* by the microdilution assay in multiwell plates according to the Clinical and Laboratory Standards Institute (2017), with modifications. To obtain the spore solution, a Petri dish containing the plant pathogen *C. gloeosporioides* was used and 0.85 M NaCl saline solution was added. The suspension was

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Table 1. Molecular identification of actinobacteria and their ability to inhibit *C. gloeosporioides* (L1) growth.

Strain	Identification	*C. gloeosporioides* growth inhibition[a]
AM01	*Streptomyces* sp.	44.6[a]
AM03	*Streptomyces* sp.	44.6[a]
AM04	*Streptomyces* sp.	39.5[a]
AM07	*Streptomyces* sp.	13.9[d]
AM09	*Streptomyces* sp.	9.3[d]
AM10	*Streptomyces* sp.	13.2[d]
AM17	*Streptomyces* sp.	0.0[e]
AM18	*Streptomyces* sp.	40.1[a]
AM19	*Streptomyces* sp.	6.8[e]
AM20	*Streptomyces* sp.	16.1[d]
AM21	*Streptomyces* sp.	9.3[d]
AM22	*Streptomyces* sp.	18.5[c]
AM23	*Streptomyces* sp.	29.4[b]
AM25	*Streptomyces* sp.	43.4[a]
AM28	*Kitasatospora* sp.	35.2[b]
AM40	*Streptomyces* sp.	22.2[c]
AM41	*Streptomyces* sp.	16.7[c]
AM46	*Streptomyces* sp.	31.5[b]
AM49	*Streptomyces* sp.	21.0[c]
AM50	*Streptomyces* sp.	3.7[e]
AM52	*Streptomyces* sp.	25.3[c]
AM53	*Streptomyces* sp.	11.1[d]
AM54	*Streptomyces* sp.	32.1[b]
AM57	*Streptomyces* sp.	17.9[d]
AM62	*Streptomyces* sp.	12.9[d]
AM63	*Streptomyces* sp.	30.2[b]
AM64	*Streptomyces* sp.	25.9[c]
AM67	*Streptomyces* sp.	14.8[d]
AM69	*Streptomyces* sp.	0.0[e]
AM74	*Streptomyces* sp.	24.7[c]
AM75	*Streptomyces* sp.	13.0[d]
AM80	*Streptomyces* sp.	4.3[e]
AM83	*Streptomyces* sp.	3.70[e]
AM88	*Streptomyces* sp.	22.2[c]
AM90	*Streptomyces* sp.	19.1[c]
AM95	*Streptomyces* sp.	11.1[d]
AM99	*Streptomyces* sp.	28.4[b]

[a]Means of fungal growth inhibition represent four replicate plates. Values with the same letter are not significantly ($P > 0.05$) different according to the Scott-Knott test.

filtered on glass wool and spore determination was performed by Neubauer chamber. In a 96-well plate 40 μL of sterile ultrapure water were added. Then, in the wells of column 1 0.5 mg of the pure compounds diluted in 40 μL ultrapure water was applied and serial dilution was performed up to column 12. The pure compound was evaluated in decreasing concentrations ranging from 2.50; 1.25; 0.62; 0.31; 0.16; 0.08; 0.04; 0.02; 0.009; 0.004 and 0.002 g L^{-1}. After dilution, 60 μL of the 2% malt medium was inoculated into each well along with the spore suspension (2.5×10^5 mL^{-1} cells). For this assay, a growth control row containing 60 μL of medium with spore solution and 40 μL of water was included, but without the compound. As a positive control, the commercial fungicides Captan SC® (Beer-Sheva, Israel) and Dithane NT® (Jacareí, Brazil) diluted in sterile ultrapure water were used at the same concentration (active principle) of the pure compound. The microplates were covered with clear plastic film and incubated in BOD at 28°C for 120 h. The readings were done every 12 h on the TECAN microplate reader, model SUNRISE (Männedorf, Germany), operated by Magellan v 7.1 software, at the wavelength of 620 nm.

RESULTS

Antagonistic activity and identification of actinobacteria

Among ~100 isolates, 37 were characterized to *C. gloeosporioides* growth inhibition and molecularly identified by partial 16S RNA sequencing. All strains, except AM28, were identified as *Streptomyces*. AM28 was identified as *Kitasatospora*. Approximately 16% of strains did not show significant fungal inhibition ability. Five strains induced largely the L1 growth inhibition (AM1, AM3, AM4, AM18 and AM25) (Table 1).

AM25, one of the highest L1 growth inhibition, was identified by total 16S rRNA. According to the dendrogram, AM25 was identified as *Streptomyces morookaense* (Fig. 1).

This strain was able to control the growth of *C. sublineolum*, *F. verticillioides* and *F. oxysporum* without contact.

Identification of AM25 active compound

Structure elucidation of the compound was achieved by combining high-resolution mass spectrometry (HRMS) and 1D- and 2D-^{1}H- and ^{13}C-NMR experiments (Figures S1–S6, Supporting Information) (Abraham 1986; Hanssen and Abraham 1988). Under electrospray ionization and positive ion recording A4C5C2 (gloeosporiocide) showed in the HRMS (ESI- (+) -HRMS) an $[M + H]^+$-ion of 938.2863 which fits to the sum formula of $C_{44}H_{48}N_{11}O_7S_3$ (calculated 938.2901). A doublet at $\delta_H = 1.31$ of 3H displayed couplings to a methine proton at $\delta_H = 4.46$ and a carbonyl group at $\delta_C = 172.0$. These resonances were in accordance with an alanyl moiety (Fig. 2A). The methine proton at $\delta_H = 4.46$ had couplings to an amide proton at $\delta_H = 7.97$ and a carbonyl at $\delta_C = 159.9$. The alanyl carbonyl ($\delta_C = 172.0$) was connected to a methine proton at $\delta_H = 5.36$ which belonged to a spin system typical for a tryptophanyl residue. The tryptophanyl methine proton also coupled with a carbon at $\delta_C = 173.4$ (Fig. 2B). This carbon turned out to belong to a 2,4-disubstituted thiazole spin system. Its 5-H at $\delta_H = 8.17$ displayed in the HMBC NMR a long-range cross peak to another carbonyl carbon at $\delta_C = 161.4$. Further analyses demonstrated that this carbonyl was connected with two glycyl moieties. The carbonyl of the second glycine coupled to an amide proton at $\delta_H = 8.40$ belonging to a leucyl derivative. Further investigations revealed that the leucyl carbonyl has been derivatized in A4C5C2 and was part of a thiazole moiety. These thiazoles are caused by post-translational modifications of cysteine with the adjacent amino acids (Walsh, Acker and Bowers 2010).The 5-H of this thiazole had long-range couplings within this aromat but also to a carbonyl group at $\delta_C = 160.8$. This carbonyl coupled to an amide proton at $\delta_H = 8.26$ which was part of another glycyl residue. The carbonyl of this amino acid at $\delta_C = 169.2$ was attached to an amide ($\delta_H = 8.85$, d) which was part of a spin system typical for phenylalanine. The α-proton of this amino acid displayed again the typical couplings observed for a thiazolyl moiety. In Table 2, we find the ^{1}H and ^{13}C NMR (Figures S1 and S5, Supporting Information) values of the isolated compound gloeosporiocide.

After the detailed analysis of the NMR spectra, antibiotic gloeosporiocide A4C5C2 turned out to be a derivatized peptide. The molecular formula determined form HRMS required a cyclic peptide. In this cyclic peptide, all cysteins were cyclized to form three thiazols. Although the three 5-Hs of the thiazols could be

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Table 2. ^{1}H and ^{13}C NMR data of the compound gloeosporiocide.

N°	Amino acid	δ_H (mult*, J Hz) d_6-DMSO; 600 MHz	δ_C d_6-DMSO; 150 MHz
1	Pro-I	—	169.2
2		3.72, 1H, dd (16; 6) 4.00, 1H, dd, (16; 7)	42.3
2-NH		8.26, 1H, dd (6; 6)	—
3	Thiazole-I	—	160.8
4		—	148.7
5		8.16 1H, s (br)	124.5
6		—	173.2
7	Leu	5.17, 1H, ddd (10; 9; 5)	49.1
7-NH		8.40, 1H, d (8.5)	—
8		1.69, 1H, ddd (13; 9; 5) 1.83, 1H, ddd (13; 10; 5)	43.3
9		1.64, 1H, m	24.7
10		0.87, 3H, d (6.5)	21.7
11		0.88, 3H, d (6.5)	23.3
12	Pro-II	—	168.8
13		3.74, 2H, d (5)	42.2
13-NH		8.11, 1H, d (5.8)	—
14	Pro-III	—	169.5
15		3.92, 1H, dd (16; 6) 3.72, 1H, dd (16; 6)	42.9
15-NH		8.55, 1H, t (5.7)	—
16	Thiazole-II	—	161.4
17		—	148.7
18		8.17, 1H, s (br)	124.5
19		—	173.4
20	Try	5.36, 1H, ddd (7; 7; 7)	52.9
20-NH		8.88, 1H, d (7)	—
21		3.34, 1H, m	30.8
22		—	109.9
23		—	127.3
24		7.52, 1H, d (7.5)	118.5
25		6.96, 1H, dd (7; 7)	118.9
26		7.05, 1H, dd (7; 7)	121.3
27		7.31, 1H, d (8)	111.7
28		—	136.4
28-NH		10.83, 1H, s (br)	—
29		7.16, 1H, d (2)	124.3
30	Ala	—	172.0
31		4.44, 1H, dq (7; 7)	48.2
31-NH		7.96, 1H, d (6.5)	—
32		1.30, 3H, d (7)	18.9
33	Thiazole-III	—	159.9
34		—	148.7
35		8.17, 1H, s (br)	124.5
36		—	173.2
37	Phe	5.34, 1H, m	53.1
37-NH		8.85, 1H, d (7)	—
38		3.16, 1H, dd (14; 9) 3.26, 1H, dd (14; 5)	40.1
39		—	137.5
40, 41, 43, 44		7.24, 4H, m	129.2
42		7.20, 1H, m	126.9

***mult:** s: singlet, d: doublet, dd: doublet of doublets, ddd: doublet of doublet of doublet, t: triplet, m: multiplet; br: broad

resolved in the 600 MHz-NMR spectrum, they were too close together for an unambiguous connection of the three partial structures using 2D-NMR spectra.

To determine the sequence of these partial structures in the molecule, HRMS (Figures S3 and S4, Supporting Information) was applied (Yakimov *et al.* 1999). While direct sequencing of linear peptides usually does not give substantial problems, sequencing of cyclic peptides does not lead to their structures. An alternative is the analysis of fragments of these cyclic peptides using multistage mass spectrometry (Eckart 1994; Siegel *et al.* 1994).

A fragment at m/z 197.0735 could be identified as leucyl-thiazole moiety, m/z 288.0836 as Gly-Phe-thiazolyl and m/z 383.1214 as Gly-Gly-Thia-Trp. The latter connected two partial structures deduced from the NMR data and fragment m/z 513.117 fitting to Leu-Thia-Gly-Phe-Thia gave the remaining

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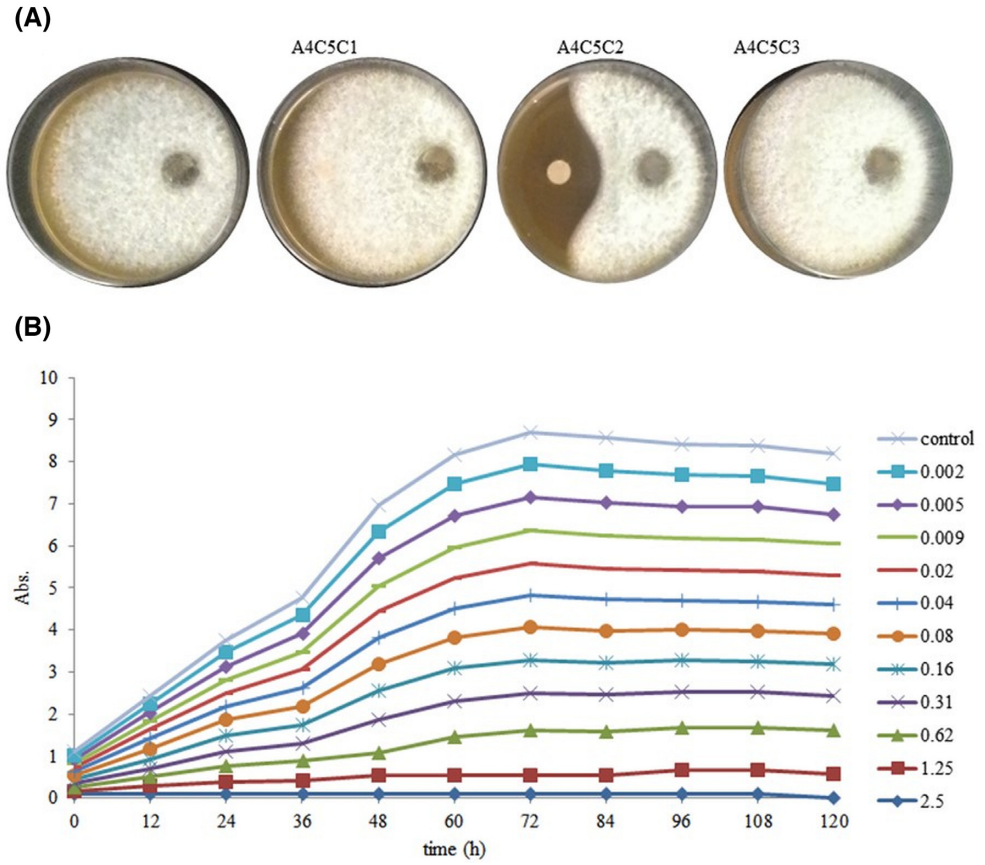

Figure 4. Bioassay performed with the three fractions obtained after HPLC separation (A4C5C1- A4C5C3) against the plant pathogen *C. gloeosporioides*. Control (a) contained only the plant pathogen. Fungal growth was measured after 7 days of incubation at 28°C in PDA **(A)**. Determination of the MIC of the isolated compound A4C5C2 by the microdilution technique **(B)**.

connections. This was corroborated by the fragment Gly-Leu-Thia-Gly-Phe-Thia-Ala (m/z 612.2136) (Fig. 3).

Gloeosporiocide A4C5C2 has [α] D^{20}–48.1 (c 0.001, MeOH). As hydrolysis of the peptide to obtain the individual amino acids for the assessment of their absolute configuration would lead to racemization at the chiral centers adjacent to thiazoles (Boden and Pattenden 2000), ozonolysis followed by acidic hydrolysis to the free amino acid and derivatization with optically pure reagents to determine the chirality of the amino acids were required (Perez and Faulkner 2003). Due to scarcity of isolated A4C5C3, this analysis could not be performed and the absolute configuration of the metabolite remained unsolved.

The compound A4C5C2 was identified as a new cyclic peptide, named gloeosporiocide, molecular formula $C_{44}H_{48}N_{11}O_7S_3$ (calculated 938.2901), characterized by the presence of cyclized cysteins to form three thiazols.

Bioguided isolation of the active compound and its MIC

In a paired culture assay, the AM25 inhibited in 43.17% the mycelial growth of *C. gloeosporioides*. Disk diffusion test demonstrated that the methanolic fraction (A4) inhibited 51.39%. The methanol fraction (A4) was subjected to chromatographic separation, resulting in five fractions (A4A-A4E). The fractions A4C (53.68%) and A4E (20.61%) presented the highest percentage of inhibition to the mycelial growth of *C. gloeosporioides* (1 mg disk^{-1}). As the chemical profile of these fractions was similar, the A4C fraction was selected due to the amount of mass in mg.

From the chromatographic separation of the A4C fraction, six fractions were obtained. The A4C5 fraction inhibited in 47.97% the mycelial growth of *C. gloeosporioides* (0.5 mg disk^{-1}). From the separation of the A4C5, five fractions were obtained. The A4C5C fraction inhibited in 53.82% the mycelial growth of the plant pathogen (0.3 mg disk^{-1}) and was separated in three fractions on HPLC (A4C5C1- A4C5C3). The active fraction A4C5C2 presented 42.68% inhibition in the mycelial growth of *C. gloeosporioides* (0.1 mg disk^{-1}) (Fig. 4).

The isolated compound showed spore inhibition at all evaluated concentrations. We verified that all growth was lower than the positive control. From the concentration of 1.25, it was noted that there was no growth and the reading remained constant. Due to the lack of mass of the compound, intermediate concentrations were not evaluated. The Score® (São Paulo, Brazil) fungicide presented MIC 0.02 mg mL^{-1} and the fungicides Captan SC and Dithane NT were not able to inhibit the germination of *C. gloeosporioides* spores at the concentrations evaluated (not shown).

DISCUSSION

Actinobacteria are known for their ability to produce compounds of different chemical properties. Once isolated they could be used in the development of new agricultural pesticides (Ranjan and Jadeja 2017), because despite all the technological development initiated in the 1940s, weeds, insects and plant pathogens remain a problem for agriculture. In recent years, the

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search for actinobacteria from few exploited habitats or areas of high biodiversity has increased as the discovery of new antimicrobial compounds is becoming increasingly scarce (Ranjan and Jadeja 2017).

In order to isolate new bioactive secondary metabolites of actinobacteria, researchers have studied actinobacteria from areas of high biodiversity (Gebreyohannes *et al.* 2013; Álvarez-Pérez *et al.* 2017; Mohamed *et al.* 2017; Elbendary *et al.* 2018). Other important source of new actinobacteria with unexplored molecules should be explored, for instance, the 'hotspot' biodiversity from Amazonia that is hosting a microbial diversity poorly understood (Kim *et al.* 2007; Huang *et al.* 2015; Batista *et al.* 2018).

Most of our isolated actinobacteria from bulk soil from guarana, a typical Amazonian crop, were identified as *Streptomyces* sp. *Streptomyces* spp. are capable of producing mycelium (Pettis 2018) and comprehends >615 species described (Labeda *et al.* 2012). This genus is found mainly in soils (Chater 2006; Letek *et al.* 2012). This genus has been described as controlling *Botrytis, Pythium, Aphanomyces, Phytophthora, Rhizoctonia*, *Fusarium* (Shih *et al.* 2003; Minuto *et al.* 2006; Ge *et al.* 2015) as well as *Colletotrichum* spp., causal agent of antrachnose (Palaniyandi, Yang and Suh 2013; Palaniyandi, Yang and Suh 2016; Jeon *et al.* 2016). Among the *Streptomyces* isolated from Amazonian soil, just 5% were not able to inhibit the *C. gloeosporioides* L1 growth. AM25 strain demonstrated a high ability to control L1 as well as other *C. sublineolum*, *F. oxysporum and F. verticillioides*. This strain has been identified as *S. morookaense*, previously described as an important producer of antibiotic (Feng *et al.* 2007).

Streptomyces is responsible for the synthesis of almost 40% of all known bioactive compounds (Bérdy 2005; Goodfellow and Fiedler 2010; Silva *et al.* 2013).

Cyclic peptides have been of interest to researchers because of their wide spectrum of pharmacological activity and because of interesting chemical structures. An example is the class of marine-derived cyclic peptide that exhibits antiviral and immunomodulating activities, being rich in proline and usually containing seven or eight amino acids (Randazzo *et al.* 1998). The cyclic peptides axinellins A and B (Figure S7A, Supporting Information) were isolated from the marine sponge *Axinella carteri* and showed in vitro antitumor activity against bronchopulmonary carcinoma and non-small cell lung cancer (NSCLC-N6) with IC_{50} of 3.0 and 7.3 μg mL^{-1}, respectively. In the literature, other cyclic peptides presenting antifungal activity have been reported, as the compounds glomecidin (Kunihiro and Kaneda 2003), nocardiamides A and B (Wu *et al.* 2013), ambobactin (Wei, Zhang and Ji 2015) (Figure S7A, Supporting Information), laxaphycins (Frankmölle *et al.* 1992), iturin A (Han *et al.* 2015) (Figure S7B, Supporting Information) among others.

One of the advantages of using cyclic peptides as fungicides is that these compounds bind stably with target sites and possess more rigidity over the linear form, what makes them resistant to hydrolysis by proteases. Moreover, these compounds are degraded under field conditions (Lee and Kim 2015).

In the present work, we reported the isolation and molecular identification of actinobacteria from Amazonian bulk soil demonstrating their ability to inhibit the growth of *C. gloeosporioides* L1. One strain, *S. morookaense* AM25, also showed the potential to control other phytopathogens. AM25 had the structure elucidation of its antifungal molecule, a new cyclic peptide, gloeosporiocide, which showed to be active against (mycelium and spores) the plant pathogen *C. gloeosporioides*, which causes anthracnose disease in guarana plants.

Our data clearly confirmed the potential of the genus *Streptomyces* as a good producer of new metabolites and emphasize the necessity of continued efforts to discover new compounds with biological activity of unexplored places, such as Amazon.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

Compliance with ethical standards

Ethical statement approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of interests. None declared.

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