Brazilian Journal of Microbiology
https://doi.org/10.1007/s42770-020-00270-9

BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Antifungal compounds with anticancer potential from *Trichoderma* sp. P8BDA1F1, an endophytic fungus from *Begonia venosa*

Diana F. Grigoletto[1] · Daniela B. B. Trivella[2] · André G. Tempone[3] · André Rodrigues[4,5] · Ana Maria L. Correia[4] · Simone P. Lira[1]

Received: 27 May 2019 / Accepted: 7 April 2020

Abstract

Fungi in the genus *Trichoderma* are notorious producers of secondary metabolites with diverse applications, such as antibacterial, antifungal, and plant growth-promoting properties. Peptaibols are linear peptides produced by such fungi, with more than 440 compounds described to date, including tricholongins, longibrachins, trichobrachins, and trichovirins. Peptaibols are synthesized by non-ribosomal peptide synthetases and they have several biological activities. Our research group isolated four peptaibols (6DP2, 6DP3, 6DP4, and 6DP5) with antifungal activity against the plant pathogen *Colletotrichum gloeosporioides* and the proteasome (a cancer chemotherapy target) from *Trichoderma* sp. P8BDA1F1, an endophytic fungus from *Begonia venosa*. The ethyl acetate extract of this endophyte showed activity of 6.01% and 75% against *C. gloeosporioides* and the proteasome, respectively. The isolated compounds were identified by MS/MS and compared to literature data, suggesting the presence of trilongins BI, BII, BIII, and BIV, which are peptaibols containing 20 amino acid residues. The minimum inhibitory concentration against *C. gloeosporioides* was 40 μM for trilongin BI, 320 μM for trilongin BII, 160 μM for trilongin BIII, and 310 μM for trilongin BIV. BI–BIV trilongins inhibited proteasome ChTL activity, with IC_{50} values of 6.5 ± 2.7; 4.7 ± 1.8; 6.3 ± 2.2; and 2.7 ± 0.5 μM, respectively. The compounds were tested ex vivo against the intracellular amastigotes of *Leishmania* (L.) *infantum* but showed no selectivity. It is the first report of trilongins BI–BIV with antifungal activity against *C. gloeosporioides* and the proteasome target.

Keywords *Colletotrichum gloeosporioides* · Alcatrazes Island · Peptaibiotics · Peptaibols · Proteasome

Introduction

Fungi in the genus *Trichoderma* (teleomorph *Hypocrea*) are widely used in agriculture as mycofungicides and biofertilizers [1, 2] and known for the production of peptaibiotics [2, 3], with more than 1297 sequences described to date in the "Comprehensive Peptaibiotics Database" [4]. Peptaibiotics are a group of peptide antibiotics containing 4 to 21 amino acid residues, with a molecular mass ranging from 500 to 2200 Da [5]. Peptaibols, the largest group of peptaibiotics, are a class of linear peptides that contain 7 to 20 amino acids and characteristically have an acylated N-terminus group, a C-terminal amino acid, and a high content of α-aminoisobutyric acid (Aib)—approximately 40% of Aib in long peptaibols and from 14 to 56% in short peptaibols [6]. Several peptaibols have been isolated from *Trichoderma* species, including tricholongins [7], longibrachins [8], trichobrachins [9, 10], and trichovirins [9]. Among the secondary metabolites produced by *Trichoderma*, peptaibols are

Responsible Editor: Luis Henrique Souza Guimaraes.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42770-020-00270-9) contains supplementary material, which is available to authorized users.

✉ Simone P. Lira
splira@usp.br

[1] Departamento de Ciências Exatas, Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz", CP 9, Piracicaba, SP 13418-900, Brasil

[2] Centro Nacional de Pesquisa em Energia e Materiais, Laboratório Nacional de Biociências, CP 6192, Campinas, SP 13083-970, Brasil

[3] Instituto Adolfo Lutz, Centro de Parasitologia e Micologia, São Paulo, SP 01246-000, Brasil

[4] Departamento de Biologia Geral e Aplicada, Universidade Estadual Paulista (UNESP), Rio Claro, SP 13506-900, Brasil

[5] Centro de Estudos de Insetos Sociais, Universidade Estadual Paulista (UNESP), Rio Claro, SP 13506-900, Brasil

Published online: 24 April 2020

the most studied group due to their biological activities [11–13].

Thus, the objective of this study was to isolate and characterize the bioactive peptaibols produced by the endophytic fungus *Trichoderma* sp. P8BDA1F1 using bioguided isolation for combating the phytopathogen *Colletotrichum gloeosporioides* and the proteasome, a target for cancer chemotherapy.

Material and methods

General procedures

A solid-phase extraction method using C_{18}/Sep-Pak silica gel columns of different sizes (Phenomenex) and analytical grade solvents (Synth) were used. Silica gel_{254} (Macherey-Nagel) was used for thin-layer chromatography (TLC). Spots were detected under UV light (254 and 365 nm).

High-performance liquid chromatography (HPLC) was performed using an Agilent 1100 Series UV/Vis with a quaternary pump, coupled to a UV detector MWD (multiple wavelength detector), using a reversed-phase column C_{18} (250 × 4.60 mm, 5 μm; Phenomenex Kinetex), with a mobile phase H_2O/ACN (60:40, v/v), flow rate of 1 mL min^{-1}, and $\lambda = 220$ nm. HPLC-grade solvents were used ("Baker Analyzed" from J. T. Baker, Phillipsburg, N.J.).

High-resolution mass spectrometry (UPLC-MS and MS/MS) was performed using a Waters Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometer. The mass spectrometer was operated in electrospray positive ion mode under the following conditions: mass range, 100–2200 Da in the full-scan mode; capillary voltage, + 1.2 kV; collision energy, 60 V; heated capillary temperature, 120 °C; cone gas rate, 50 units; desolvation gas flow rate (Lh^{-1}), 750; column, Acquity UPLC BEH C_{18} (2.1 × 100 mm, 1.7 μm); and mobile phase, ultrapure H_2O (A) and ACN (B) gradient, with an initial ratio of 98A:2B, reaching 100% B after 9 min and finishing after 10 min. The flow rate was 0.5 mL min^{-1}, and the temperature was 4 °C. The *m/z* data was acquired in the centroid mode. Data acquisition was performed using the software MassLynx.

Optical absorbance readings were recorded at 620 nm using the TECAN reader—model SUNRISE, operated by Magellan v.7.1 software.

Biological material

The endophytic fungus was isolated from leaves of *Begonia venosa* collected on Alcatrazes Island, São Paulo, Brazil (24° 06′ 027″ S and 45° 41′ 640″ W), in September 2013.

Fig. 1 Phylogenetic tree inferred under the neighbor-joining algorithm of *Trichoderma* species of the Longibrachiatum clade and the isolate P8BDA1F1 (obtained from *B. venosa*). The tree is based on an alignment of 592 pb of the elongation factor 1-alpha gene. *Trichoderma* species are followed by culture collection number and GenBank accessions in parentheses. Number on branches are bootstrap support values under 10,000 pseudoreplicates

Botanical identification

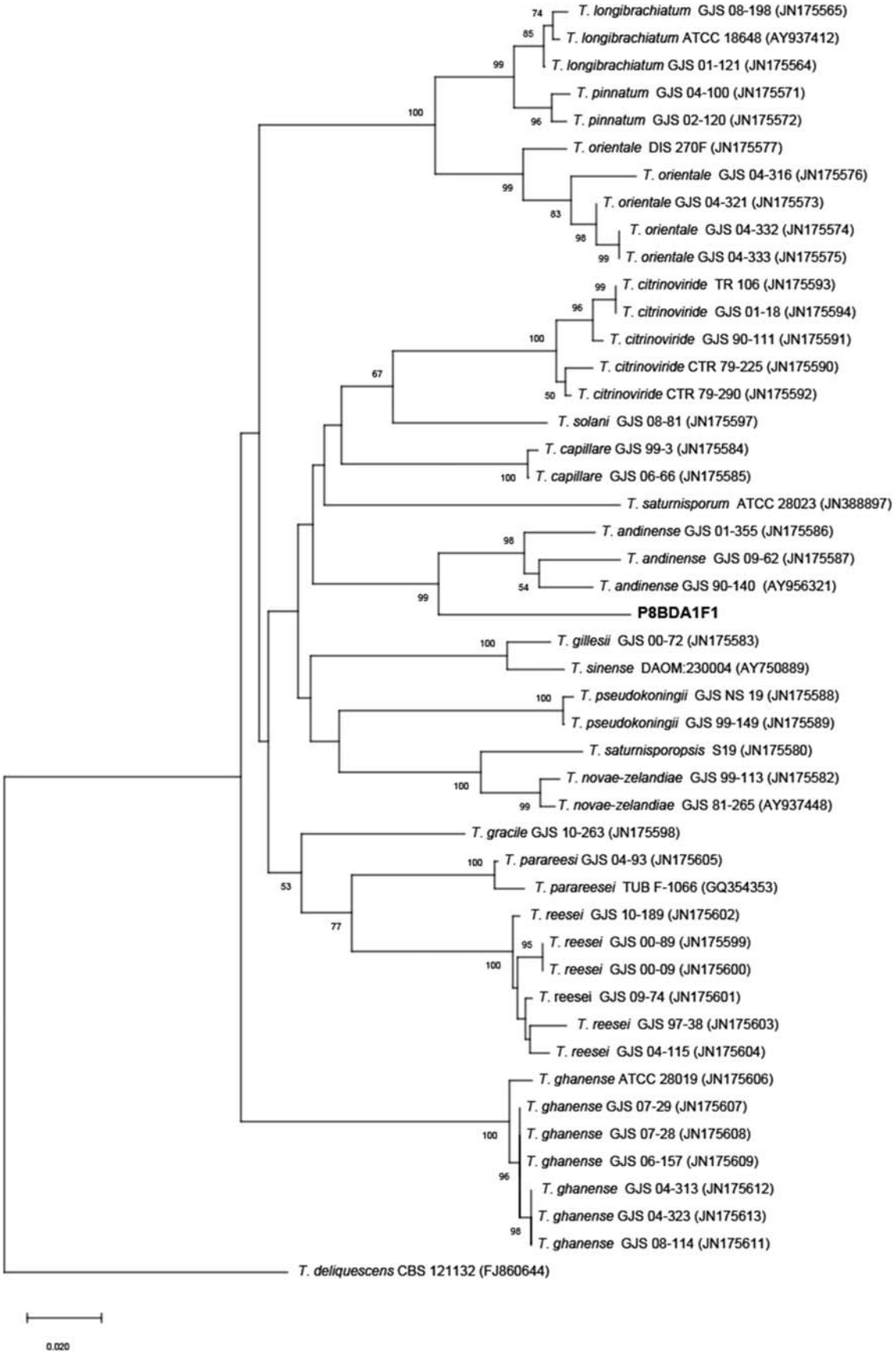
The plant was identified based on the botanical and morphological characteristics of the specimens by Dr. Marco Antônio de Assis (Department of Botany, UNESP - Rio Claro-SP, Brazil) and Dr. Eliane Jacques (Department of Botany, UFRJ - Rio de Janeiro-RJ, Brazil), and one specimen was deposited in the Herbarium Rioclarense (HRCB), under the code HRCB 64227.

Fungal identification

The pure culture of the isolate P8BDA1F1 was grown on malt agar 2% (2% malt extract and 1.5% agar) at 28 °C for 7 days. Fresh mycelia were obtained and used for DNA extraction according to the protocol described by Montoya et al. [14]. The *tef*1 gene encoding the elongation factor 1-alpha was amplified using primers described by Atanasova et al. [15]. The amplicon was used in cycle sequencing reactions using BigDye Terminator® v.3.1 Kit (Thermo Fisher Scientific). Bidirectional sequences were generated in ABI 3530 (Thermo Fisher Scientific) using the same primers used for amplification. The *tef*1 sequence was deposited in GenBank under the accession MK070908, then compared with homologous sequences deposited in the database. Sequences of closely related species from two taxonomical studies [16, 17] were retrieved from the database and used for phylogenetic inference. Sequences were aligned in MAFFT [18] and then the phylogenetic tree was built using the neighbor-joining algorithm and Kimura 2-parameters as the nucleotide substitution model in MEGA v. 6.0 [19]. Branch support was assessed using 1000 bootstrap pseudo-replicates. A sequence of *Trichoderma deliquescens* (FJ860644) was used as the external group.

Isolation of the active compounds

The endophytic fungus P8BDA1F1 was grown on a 2% liquid malt medium (9 L, 60 × 150 mL culture medium in 250-mL Erlenmeyer flasks) at 28 °C under agitation (150 rpm) for 5 days. The mycelium was separated from the liquid medium using vacuum filtration, and the liquid medium was extracted with EtOAc to yield the EtOAc extract (1193 mg). The EtOAc extract was subjected to Sep-Pak silica gel column chromatography (10 g) using a gradient of hexane/CH_2Cl_2/MeOH as eluent, and six final fractions were obtained (1–6). Fraction 6 (344 mg) was subjected to further fractionation, this time on a Sep-Pak C_{18} column (10 g) using a gradient of H_2O-MeOH as


T. longibrachiatum GJS 08-198 (JN175565)
T. longibrachiatum ATCC 18648 (AY937412)
T. longibrachiatum GJS 01-121 (JN175564)
T. pinnatum GJS 04-100 (JN175571)
T. pinnatum GJS 02-120 (JN175572)
T. orientale DIS 270F (JN175577)
T. orientale GJS 04-316 (JN175576)
T. orientale GJS 04-321 (JN175573)
T. orientale GJS 04-332 (JN175574)
T. orientale GJS 04-333 (JN175575)
T. citrinoviride TR 106 (JN175593)
T. citrinoviride GJS 01-18 (JN175594)
T. citrinoviride GJS 90-111 (JN175591)
T. citrinoviride CTR 79-225 (JN175590)
T. citrinoviride CTR 79-290 (JN175592)
T. solani GJS 08-81 (JN175597)
T. capillare GJS 99-3 (JN175584)
T. capillare GJS 06-66 (JN175585)
T. saturnisporum ATCC 28023 (JN388897)
T. andinense GJS 01-355 (JN175586)
T. andinense GJS 09-62 (JN175587)
T. andinense GJS 90-140 (AY956321)
P8BDA1F1
T. gillesii GJS 00-72 (JN175583)
T. sinense DAOM:230004 (AY750889)
T. pseudokoningii GJS NS 19 (JN175588)
T. pseudokoningii GJS 99-149 (JN175589)
T. saturnisporopsis S19 (JN175580)
T. novae-zelandiae GJS 99-113 (JN175582)
T. novae-zelandiae GJS 81-265 (AY937448)
T. gracile GJS 10-263 (JN175598)
T. parareesi GJS 04-93 (JN175605)
T. parareesei TUB F-1066 (GQ354353)
T. reesei GJS 10-189 (JN175602)
T. reesei GJS 00-89 (JN175599)
T. reesei GJS 00-09 (JN175600)
T. reesei GJS 09-74 (JN175601)
T. reesei GJS 97-38 (JN175603)
T. reesei GJS 04-115 (JN175604)
T. ghanense ATCC 28019 (JN175606)
T. ghanense GJS 07-29 (JN175607)
T. ghanense GJS 07-28 (JN175608)
T. ghanense GJS 06-157 (JN175609)
T. ghanense GJS 04-313 (JN175612)
T. ghanense GJS 04-323 (JN175613)
T. ghanense GJS 08-114 (JN175611)
T. deliquescens CBS 121132 (FJ860644)
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eluent, resulting in five fractions (6A–6E). Fraction 6D (66 mg) was purified on HPLC (on reverse phase C_{18} column) using an elution gradient of A (ultrapure H_2O + 0.1% formic acid) + B (ACN), starting with a ratio of 60A:40B and ending with 100% B in 15 min. It resulted in seven fractions, with four pure compounds: 6DP2 (7.2 mg), 6DP3 (6.1 mg), 6DP4 (7.1 mg), and 6DP5 (6.0 mg).

Test protocols

Antifungal assays

Disk diffusion test The disk diffusion method was performed according to Foster and Woodruff [20]. Each fraction was applied to 6-mm-diameter sterile paper disks (with 2 mg for EtOAc extract, 1 and 0.5 mg for fractions, and 0.2 mg for pure compounds) and placed at the edge of the Petri dish containing 2% malt medium. An inoculum of the pathogen (a 7-mm-diameter disk removed from a 5-day plate) was added to the opposite edge of the Petri dish. Inhibition of mycelial growth around disks was measured after incubation for 5 days at 28 °C in a 2% malt medium. Data were expressed as percentage inhibition with respect to the control using the ImageJ program (Image Processing and Analysis in Java). The fungus *C. gloeosporioides* used in this study was deposited in The Microbial Resource Center of UNESP (WDCM 1043) under the code CRM 1352.

Microdilution assays The microdilution assay was performed in 96-well microplates according to the guidelines of the National Committee of Clinical and Laboratory Standards [21] with modifications. For the assays, the compounds were diluted in DMSO and 2% malt medium to final concentrations of 2500; 1250; 625; 312.5; 156; 78.1; 39; 19.5; 7.7; 4.8; and 2.4 mg mL^{-1} per well. An inoculum suspension of *C. gloeosporioides* (10^5 conidia mL^{-1}, 60 μL) was added to each well, totaling a final volume in the well of 100 μL. Wells containing medium, inoculum, and DMSO were evaluated as a negative control, but without the compounds. The active ingredients captan and difenoconazole from commercial fungicides were used as positive controls. Microplates were incubated in B.O.D. at 28 °C and analyzed in a microplate reader (Tecan Sunrise™, Switzerland, operated by Magellan v 7.1 software) at 620 nm at 12-h intervals for 120 h.

Proteasome inhibition bioassay

Inhibition assays of the purified 20S proteasome core particle from *Saccharomyces cerevisiae* were conducted as previously reported [22], with modifications according to the assay format. Initially, fungal extracts and fractions were screened in a 384-well plate format. This assay was carried out using 0.25 μg mL^{-1} of proteasome prepared in Tris 25 mM pH 7.5, SDS 0.03%. Chemical samples (1 μL at 1 mg mL^{-1}) were added to wells using a Janus MDT liquid handler (Perkin Elmer) connected to the cell:explorer system (Perkin Elmer) at the Brazilian Biosciences National Laboratory (LNBio, CNPEM). Plates were incubated at room temperature for 1 h before the addition of 5 μL of the fluorogenic substrate suc-LLVY-AMC (R&D Biosciences) at a concentration of 250 μM. Each plate was immediately inserted into the ClarioStar microplate reader (BMG labtech) and the fluorescence ($\lambda_{excitation}$ = 380 nm, $\lambda_{emission}$ = 460 nm) was recorded at time zero (to correct for intrinsic fluorescence) and after 1 h of enzyme reaction. Positive (n = 32) and negative (n = 32) controls were carried out in the same plate to access enzyme activity (DMSO controls = 100% ChTL activity) and inhibition (assay carried out in the absence of the enzyme = 0% of enzyme activity). Controls were used to normalize the data and to access the analytical quality of assays (Z' parameter) [23]. One nanomolar of the proteasome was incubated with different inhibitor concentrations in Tris 100 mM pH 7.5, SDS 0.03% for 30 min at room temperature in 96-well plates, in a final reaction volume of 50 μL. Ten microliters of the fluorogenic substrate at 250 μM was added, resulting in a final substrate concentration of 50 μM. The fluorescence ($\lambda_{excitation}$ = 380 nm, $\lambda_{emission}$ = 460 nm) was recorded at time zero and after 1 h of enzyme reaction using an EnSpire plate reader (Perkin Elmer). Fluorescence was normalized to positive and negative controls. The percentage of remaining enzyme activity was plotted. For dose-response curves, data was plotted as a function of inhibitor concentration and fitted using the normalized logistic 4-parameter equation in GraphPad software version 7 (GraphPad Prism, San Diego).

In vitro assay against *Leishmania* (L.) *infantum*

Isolation of amastigote forms of *L. infantum*

The animals (golden hamsters and BALB/c mice) were obtained from the Adolfo Lutz Institute, São Paulo, Brazil. They were kept under a controlled environment in sterile cages and were given food and water ad libitum. The Ethics Committee on Animal Research approved the project (number CEUA IAL/Pasteur 02/2011), according to the "Guide to the Care and Use of Laboratory Animals" of the National Academy of Sciences. Golden hamsters (*Mesocricetus auratus*) served as hosts for *L. infantum* (MHOM/BR/1972/LD) until approximately 60–70 days after infection. Amastigotes were obtained from the spleens of previously infected hamsters and purified by differential centrifugation. Macrophages were collected from the peritoneal cavity of BALB/c mice by washing them with RPMI-1640 medium supplemented with 10% fetal calf serum and maintained at 37 °C in a humidified incubator containing 5% CO_2 [24].

Determination of 50% inhibitory concentration

Macrophages were collected from the peritoneal cavity of BALB/c mice and incubated at 1×10^5 well^{-1} for 24 h on 16-well plates (NUNC-Thermo, USA). Amastigote forms were prepared in a ratio of 1:10 (macrophages/amastigotes) and incubated in RPMI-1640 medium for 24 h at 37 °C in a humidified incubator containing 5% CO_2. The compounds were incubated with infected macrophages for 72 h. Miltefosine was used as the standard drug. Subsequently, the cells were fixed with MeOH, stained with Giemsa, and observed using a light microscope. The number of intracellular parasites was determined after counting 400 macrophages. The compounds were tested at the maximum concentration of 50 μM and were reported as not active (NA) when the 50% inhibitory concentration (IC_{50}) value was above this concentration [25].

Results and discussion

Botanical and fungal identification

Plant morphological characteristics and identification keys were used to identify the specimen as belonging to *Begonia venosa* Skan ex Hook. This is a critically endangered plant species native to Brazil and is specifically found in the islands of the north coast of São Paulo state [26].

The fungal isolate P8BDA1F1 was identified as belonging to the genus *Trichoderma* using colony morphological characters. Phylogenetic analysis showed that isolate P8BDA1F1 clusters in the Longibrachiatum clade, sensu Samuels et al. [17]. Within this clade, *T. andinense* is its closest relative with high bootstrap support (99%, Fig. 1). However, the isolate P8BDA1F1 differs from strains of the *T. andinense* in relation to the tef1 gene. For instance, the tef1 sequence of isolate P8BDA1F1 showed 89% identity with the tef1 sequence of *T. andinense* GJS 90–140. The differences accounted for 46 substitutions and 8 gaps in a pairwise alignment of 503 bp.

Because the unique position in the Longibrachiatum clade, the isolate P8BDA1F1 may represent a phylogenetic species not yet described. However, sequencing of additional genes, such as *rpb2* (RNA polymerase 2), is necessary to support this hypothesis.

Isolation and identification of the compounds

After incubation in a liquid medium, filtration and partition using ethyl acetate, the EtOAc extract (1193 mg) was obtained. This extract was submitted to inhibition assays against *C. gloeosporioides* and proteasome chymotrypsin-like (ChTL) subunit, showing 6.01% and 75% activity, respectively. This extract was fractionated by chromatography on a silica gel Sep-Pak column, yielding six fractions. The sixth fraction (344 mg) showed 30.66% activity against *C. gloeosporioides*

Table 1 The $[M+Na]^+$ ions and the diagnostic fragment mass ions of b_{13} and y_7 series ions observed by MS/MS of the isolated compounds 6DP2–6DP5/trilongins BI–BIV

Diagnostic ions	Trilongin BI/6DP2	Trilongin BII/6DP3	Trilongin BIII/6DP4	Trilongin BIV/6DP5
$[M+Na]^+$	1958/1959	1972/1973	1972/1973	1986/1987
b_{13}	1163	1163	1177	1177
b_{12}	1078	1078	1092	1092
b_{11}	965	965	979	979
b_{10}	908	908	922	922
b_9	823	823	837	837
b_8	724	724	738	738
b_7	639	639	653	653
b_6	511	511	525	525
b_5	440	440	440	440
b_4	355	355	355	355
b_3	284	284	284	284
b_2	199	199	199*	199*
b_1	128*	128*	128*	128*
y_7	774	788/789	774	788/789
y_6	623	637	623	637
y_5	495	509	495	509
y_4	367	381	367	381
y_3	282	282	282	282
y_2	197	197	197	197

*These fragments were not observed for the isolated compounds

and was resubmitted to chromatography on a Sep-Pak C_{18} column, obtaining five fractions. The fourth fraction (63 mg) showed 12.44% activity against *C. gloeosporioides* and was purified on HPLC using a C_{18} column, where four fractions were obtained: 6DP2, 6DP3, 6DP4, and 6DP5. The obtained compounds were then fragmented by MS/MS (Supplementary Material, Fig S1-S8) and compared to compounds found in literature and in the Dictionary of Natural Products database [27].

The four compounds isolated from *Trichoderma* sp. were identified by comparison of MS/MS fragmentation pattern [28]. Table 1 shows the diagnostic fragment ions from b- and y-series.

Therefore, by comparing the diagnostic ions obtained, it was possible to identify the compounds as trilongins BI, BII, BIII, and BIV, of the class of peptaibols.

The isolated compounds trilongin BI (1936 Da, fragments *m/z* 1163 and *m/z* 774), trilongin BII (1950 Da, fragments *m/z* 1163 and *m/z* 788), trilongin BIII (1950 Da, fragments *m/z* 1177 and *m/z* 774), and trilongin BIV (1964 Da, fragments *m/z* 1177 and *m/z* 788) are 20-residue peptaibols containing an α-aminoisobutyric acid acetylated at the N-terminus and an amino alcohol C-terminal.

The compounds trilongins BI–BIV are isomeric, or very similar, to other peptaibols already described in the literature [3].

Differences in the chemical structures and conformation of peptaibols contribute to a variety of biological activities, which is also attributed to the formation of voltage-dependent ion channels in the lipid membranes, most likely due to their amphipathic nature [11].

The study conducted by Marik et al. [29] examined the structural diversity and bioactivity of peptaibols produced by 17 species belonging to the genus *Trichoderma*, Longibrachiatum clade. HPLC-ESI-MS investigations revealed several new and recurring 20-residue peptaibols. Furthermore, bioactivity tests showed that peptaibols isolated in the study may have plant protection applications.

BI–BIV trilongins are thermostable and mitochondriotoxic compounds for mammalian cells capable of forming voltage-dependent Na^+/K^+ permeable channels in biomembranes [28].

Evaluation of the active compounds

The four isolated trilongins showed fungistatic activity against *C. gloeosporioides* (from guarana plant) in the disk diffusion test. The percentages of mycelial growth inhibition were 41.43% for trilongin B1; 44.46% for trilongin BII; 44.97% for trilongin BIII; and 39.07% for trilongin BIV (200 μg of compound per disk). The pure compounds were then tested against spores of *C. gloeosporioides* using the microdilution assay. The compounds were incubated for 120 h with readings every 12 h. The graphs are shown in Fig. 2.

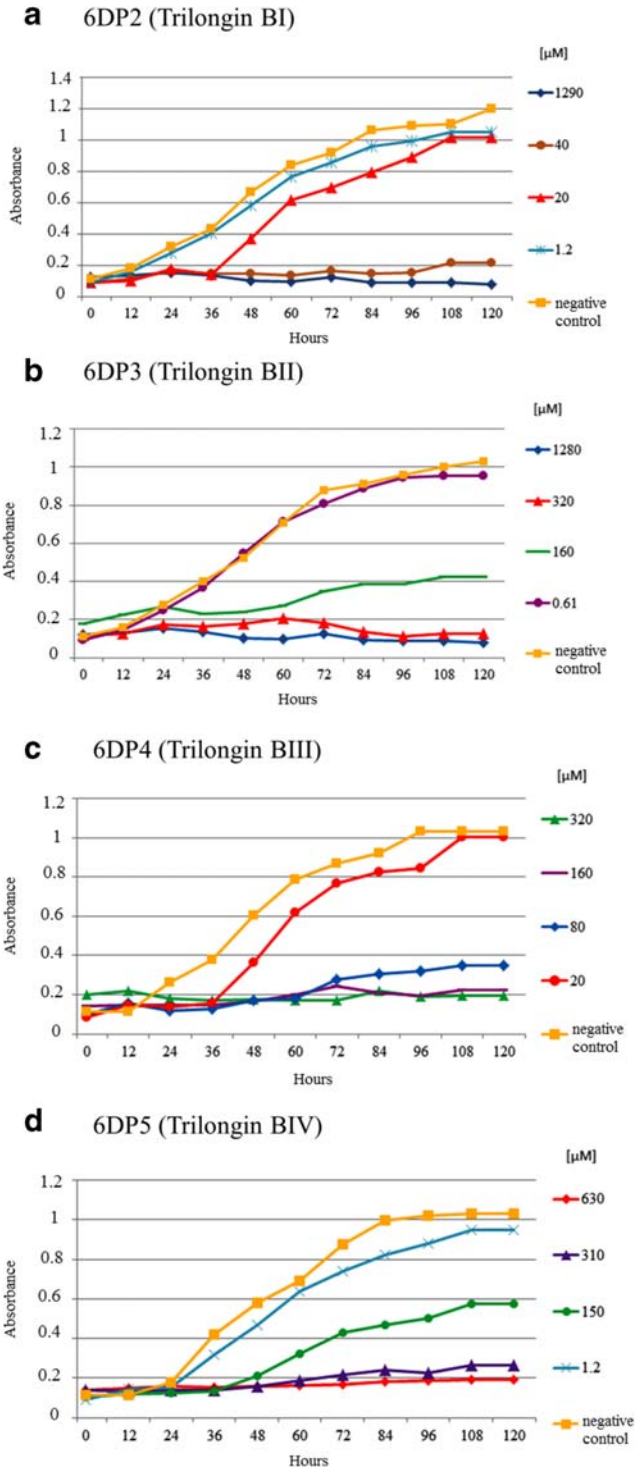

Fig. 2 Data obtained in microdilution assay of the isolated compounds trilongins BI (**a**), BII (**b**), BIII (**c**), and BIV (**d**) against spores of *C. gloeosporioides*

The negative control was comprised of 2% malt medium, spores of *C. gloeosporioides*, and DMSO in the same concentration used to dilute the mixture of compounds tested. The minimum inhibitory concentration (MIC) of the trilongin mixture was 312 μg mL^{-1}. For trilongins BI, BII, BIII, and BIV

Table 2 Minimum inhibitory concentration (MIC) of the compounds trilongins BI, BII, BIII, and BIV against spores of *C. gloeosporioides*

Isolated compounds	MIC (μg mL^{-1})	MIC (μmol mL^{-1})
6DP2	78.1	40
6DP3	625	320
6DP4	312	160
6DP5	625	310
4 peaks	312	–

(Fig. 2a–d), the MIC against *C. gloeosporioides* was 40, 320, 160, and 310 μM, respectively, as shown in Table 2.

This result demonstrates that these compounds do not act synergistically against spores of *C. gloeosporioides*.

The difference in bioactivity due to structure is discussed in the study by Marik et al. [29]. The authors point out that different residues, such as Aib at position R17, as in the case of the compounds paracelsin B and brevicelsin I, result in a highly dynamic folding process, while Val at the same position (in paracelsin H and brevicelsin IV) led to less energy stable conformations. In the present study, 6DP2 and 6DP4 have 774 y-ion, while 6DP3 and 6DP5 have 788 y-ion. Thus, the results on bioactivity differences can be explained by this conformational structure dynamics caused by the different residues.

The isolated BI–BIV trilongins were tested against intracellular amastigotes of *L. infantum*, at different concentrations. The compounds show no activity against amastigotes at concentrations not toxic to macrophages, with no selectivity (activity against the parasite at safe concentrations for host cells).

The EtOAc extract from *Trichoderma* sp. was active against the proteasome in a high-throughput screening assay for proteasomal inhibitors using a library of fungal extracts. This sample showed 75% inhibition against the chymotrypsin-like subunit (ChTL) of the proteasome, with a *Z* factor of 0.7. The trilongins BI–BIV were confirmed as proteasome inhibitors in an initial assay performed at 100 μM (Fig. 3a). Then, dose-response analyses were performed for the trilongins (Fig. 3b).

The dose-response analyses generated curves, which provided the following IC_{50} values: trilongin BI, 6.5 ± 2.7; trilongin BII, 4.7 ± 1.8; trilongin BIII, 6.3 ± 2.2; and trilongin BIV, 2.7 ± 0.5 μM for the ChTL subunit of the proteasome.

Even though IC_{50} values are high (~ 1 μM) compared to available proteasome inhibitors (~ 1 nM), a new class of proteasome inhibitors stands out.

Thus, the isolated compounds proved to be efficient in inhibiting both the proteasome (IC_{50} between 2.7 and 6.5 μM) and the phytopathogen *C. gloeosporioides* (MIC between 40 and 320 μM).

In our study, the trilongins showed no selectivity against intracellular amastigotes of *L. infantum*. This lack of efficacy against intracellular parasites can be attributed to the possible lack of macrophage receptors responsible for the internalization of compounds, but also intrinsic biochemical resistance of the parasite against the compounds.

The trilongins isolated in our study were active against the proteasome target. The proteasome is responsible for 80% of cellular proteolysis and interferes with several cellular processes, such as cycle control, differentiation, immune response, amino acid recycling, and apoptosis [30, 31]. Moreover, the proteasome has also been identified as a strategic target for malaria [32] and human trypanosomiasis [33].

Conclusion

Our results indicate that trilongins BI–BIV isolated from *Trichoderma* sp. may be effective in inhibiting the activity of *C. gloeosporioides* and the proteasome target, confirming these compounds as candidates for drugs and agrochemicals. In the best of our knowledge, this is the first report of

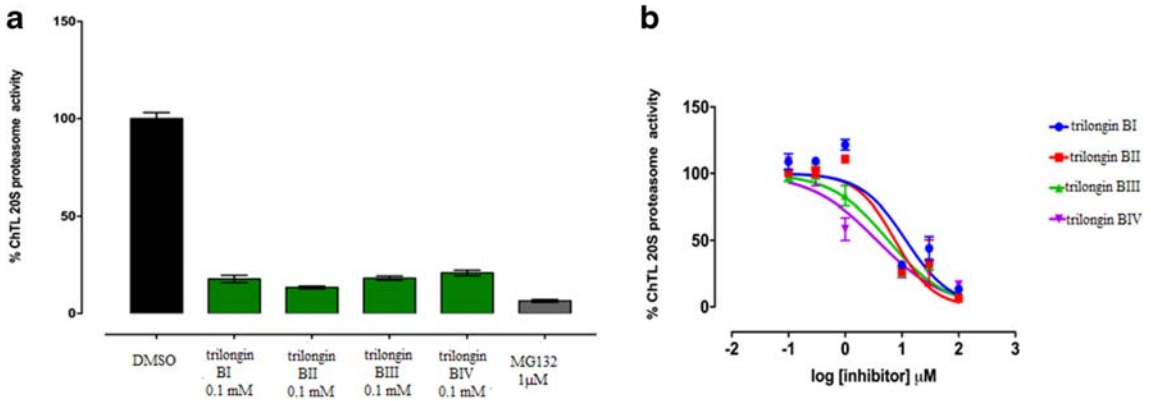

Fig. 3 Proteasome ChTL subunit inhibition by trilongins isolated from *Trichoderma* sp. **a** Isolated compounds assayed at a single concentration of 100 μM. Internal enzyme activity positive (DMSO) and negative (MG132 at 1 μM) controls are shown for reference. **b** Concentration-response curves of isolated trilongins BI–BIV (6DP2–6DP5). Enzyme activity data was normalized by DMSO controls (100% ChTL activity) and sample blanks (time zero of enzyme reaction in each well). Data show average ± SEM of triplicates

trilongins BI–BIV possessing antifungal activity against *C. gloeosporioides* and the proteasome target, showing great potential in the study of a new class of inhibitors.

Acknowledgments We thank AZN Fernandes for enzyme preparation and LQPN, LBE, LPP, and LEC for facilities (LNBio, CNPEM). We thank Dr. Roberto G. de S. Berlinck for the equipment and support, Dr. Marco A. de Assis and Dr. Eliane Jacques for plant identification, Marta L.Lima by performing the ex vivo assay against the intracellular amastigotes of *L. infantum*, and Instituto Chico Mendes and Ibama for helping with plant collections in the Island. SISBio authorization under an accession number (37256-4) and at Sisgen by the code A046A6C.

Funding information Financial support was provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grants 2014/15760-3 and 2014/10753-9) and BIOTA/BIO*prospec*TA FAPESP (grant 2013/50228-8). Scholarships to Diana F. Grigoletto (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior 88881.133610/2016-01 and Conselho Nacional de Desenvolvimento Científico e Tecnológico 142079/2016-2) and Ana M. Correia (FAPESP: 2014/12021-5) are also gratefully recognized.

Compliance with ethical standards

The Ethics Committee on Animal Research approved the project (number CEUA IAL/Pasteur 02/2011), according to the "Guide to the Care and Use of Laboratory Animals" of the National Academy of Sciences.

Conflict of interest The authors declare that they have no conflict of interest.

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